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Reptilian MPR 300 is also the IGF-IIR: Cloning, sequencing and functional characterization of the IGF-II binding domain

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ABSTRACT

The mammalian cation-independent mannose 6-phosphate/insulin-like growth factor (IGF)-II receptor binds IGF-II with high affinity. Ligands transported by the MPR 300/IGF-IIR include IGF-II and mannose 6-phosphate-modified proteins. By targeting IGF-II to lysosomal degradation, it plays a key role in the maintenance of correct IGF-II levels in the circulation and in target tissues. Although, from our studies we found homologous receptor in *calotes* but its functional significance was not known. We present here the first report on the *calotes* MPR 300/IGF-IIR binds IGF-II with K_d of 12.02 nM; these findings provide new and strong evidence that MPR 300/IGF-IIR in *Calotes versicolor* binds IGFII with high affinity.

1. Introduction

In mammals it is well established that two homologous but distinct transmembrane glycoproteins designated as the cation independent receptor (CIMPR or MPR 300) and cation dependent receptor (CDMPR or MPR 46) mediate the transport of lysosomal enzymes. The MPR 300 protein in addition to having two mannose 6-phosphate binding domains it has a distinct IGF-II binding domain and is therefore referred to as the mannose 6-phosphate/IGF-II receptor. Mammalian MPR 300/IGF-IIR has been found to bind a variety of ligands and hence is a multifunctional protein [1]. This receptor, along with the smaller cation-dependent mannose 6-phosphate receptor (CDMPR), is responsible for the intracellular targeting of lysosomal proteins to the lysosomes [2]. The extracytoplasmic region of the MPR 300/IGF-IIR contains 15 repetitive domains that are homologous to each other and to the single domain structure of MPR 46. Domains 3 and 9 of human MPR 300/IGF-IIR (as well as other known vertebrates MPR 300/IGF-IIR's) bind mannose 6-phosphate containing ligands while domain 11 has a high affinity binding site for human IGF-II, and is responsible for the internalization and clearance of IGF-II with the involvement of domain 13 [3]. Growth factors such as the IGF-I and -II are conserved throughout the vertebrates and play an essential role in biological processes, such as growth, development, and metabolism [4–6]. The MPR 300/IGF-IIR mediates endocytosis of insulin-like growth factor II, resulting in growth factor degradation in lysosomes [7,8]. This degradation is an important regulator of growth factor activity *in vivo*, as shown by the phenotype of receptor deficient mice [9]. A critical isoleucine residue located at position 1572 in the 11th domain of the human MPR 300/IGF-IIR protein has been suggested to be important for IGF-II binding. This isoleucine has been shown to be conserved also among several other mammalian species which are known to bind human IGF-II (hIGF-II). However in an earlier study it was shown that purified MPR 300/IGF-IIR protein from chicken liver and *xenopus*, showed weak binding to IGF-II [10]. Subsequent cloning and characterization of the chicken MPR 300/IGF-IIR revealed that the mannose 6-phosphate binding regions of the receptor are same as in the mammals, but in the IGF-II binding domain the isoleucine located at 1552 position is replaced by leucine and the weak binding of the chicken protein to IGF-II has been attributed to this change [11]. Therefore it was proposed that the acquisition of an IGF-II binding site by the MPR 300/IGF-IIR is a late event in evolution. In a separate study it was shown that the early vertebrate fish MPR 300/IGF-IIR can bind IGF-II with high affinity [12]. Other studies suggest that in addition to isoleucine, other parts of the IGF-II binding domain may also play an important role in IGF-II binding and this property appears to have occurred after the divergence of marsupial and placental mammals from their common ancestor with egg-laying mammals, and it has been suggested that this acquisition was a major factor in driving the evolution of an imprinted MPR 300/IGF-IIR in some mammals. After the work of Mendez et al. [12], we have also

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found that the chicken MPR 300/IGF-IIR that falls above the fish in the vertebrate linage also shows IGF-II binding [13]. Towards understanding the evolution of the MPR proteins, research work in our laboratory has been focused on the identification, purification, biochemical characterization, cloning and sequencing of the MPRs from non-mammalian vertebrates. Although the reptilian MPR proteins (MPR 300/IGF-IIR and CD MPR) have been purified from *calotes* liver tissue and were shown to exhibit similar molecular masses as the mammalian (goat) receptors, so far there has been no detailed studies on the structural and functional characterization of the reptilian receptors. We identified mammalian homologues of the receptors from reptiles, amphibians and fish. Extensive characterization of the early vertebrate zebra fish MPR 46 revealed that the receptor is conserved from fish to mammals [14]. Among the invertebrates we identified the mammalian homologues of the receptors in echinodermates and molluscs [15]. Studies carried out by other workers on zebra fish MPR's also revealed that the both receptors are conserved in the vertebrates and the MPR 300/IGF-IIR also exhibits repetitive cassette structures like the mammalian protein and the domains 3 and 9 responsible for mannose 6-phosphate ligand binding are highly conserved [16]. These studies thus clearly demonstrate the conserved mannose 6-phosphate binding pattern observed among the different vertebrate MPR 300/IGF-IIR proteins. However, no such conclusive evidence has been shown so far for the IGF-II binding domains between different non-mammalian vertebrate MPR 300/IGF-IIR's. Therefore, it is important to gain new insights into the IGF-II binding domain of the non-mammalian vertebrate MPR 300/IGF-IIR's to establish the structure and functional significance of its domain structures. The objectives of the present study were therefore, (i) to localize the MPR 300/IGF-IIR protein in the *calotes* liver tissue, (ii) to analyze the interaction between the human IGF-II and purified reptilian *calotes* MPR 300/IGF-IIR protein (heterologous system) and (iii) to clone the 11th domain of the *calotes* MPR 300/IGF-IIR using an RT-PCR approach and make a structural comparison with the available IGF-II domain sequences from other vertebrates.

2. Materials and methods

O-Phosphonomannan was a generous gift from Dr. M.E. Slodki, USDA, IL, and USA. Garden lizards liver was collected from the local animal supplier and from some animals; the testis and other tissues used in the study were separated. Human IGF-II, insulin, mannose 6-phosphate, DSS (disuccinamidyl suberate) were purchased from Sigma. Molecular biology reagents and TA cloning vector were purchased from Invitrogen. Both the liver and testis were kept frozen at −80 °C. Affinity purified antibody to the goat MPR 300 protein was obtained as described earlier [17].

2.1. Tissue localization of MPR 300/IGF-IIR receptor

Calotes were anaesthetized with chloroform and received a retrograde perfusion through the abdominal aorta with phosphate buffered saline for 1 min immediately followed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. After 5 min of perfusion, portions of two liver lobes were excised and were cut into 1 mm thin slices that were immersion-fixed in the same fixative for an additional hour. For immunofluorescence semithin (20 μm) cryo-sections from prefixed livers were prepared on microtone. For double immunofluroscence staining, liver sections (20 μm) were incubated overnight with a combination of anti-M6P/IGF-II receptor (1:300) and anti-Lamp1 (1:200). After incubation with the primary antiserum, sections were rinsed with PBS, exposed to FITC- or TRITC-conjugated secondary antibodies (1:200) for 1 h at room temperature, washed thoroughly with PBS and then cover-slipped with Vectashield mounting medium. Immunoassayed sections were examined under a Leica fluorescence microscope and the photomicrographs were taken with a Nikon 200 digital camera.

2.2. Quantification of MPR 300/IGF-IIR in different tissues of reptilian

Quantification of MPR 300/IGF-IIR in membrane extract of different tissues was done by ELISA as described in [16].

2.3. Purification of MPR 300/IGF-IIR on PM-affinity chromatography

Membrane proteins extraction, purification of MPR 300/IGF-IIR on sepharose-divinyl sulfone-phosphomannan affinity matrix, analyzing the purity on SDS–PAGE and western blot was done as described in [17].

2.4. Protein estimation

Protein estimation was done using bicinchoninic acid reagent following manufacturer's instructions (Sigma, USA).

2.5. Ligand blot assay and cross-linking experiment

Ligand blot analysis between MPR 300/IGF-IIR and biotinylated IGF-II was done as described [12]. Cross-linking experiment was done as described in [13].

2.6. ELISA binding assay to determine dissociation constants (K_d) for the binding of MPR 300/IGF-IIR to IGF-II

A novel ELISA based immunoassay was used to measure the binding affinity of MPR 300/IGF-IIR protein to hIGFII [18]. ELISA assays were performed with purified MPR 300/IGF-IIR directly in 96-well polystyrene disposable sterile ELISA plates. Purified MPR 300/IGF-IIR (100 ng), in 100 μl of interaction buffer (20 mM Tris (pH 8.3), 150 mM NaCl, 0.2% Tween 20), was adsorbed to the wells of an ELISA plate by overnight incubation at 4 °C. Subsequently, unbound proteins were removed, and wells were washed three times with interaction buffer. The non-specific sites in the wells were blocked by incubating for 1 h with the blocking buffer (interaction buffer containing 5% bovine serum albumin). Then the blocking buffer was removed, and the wells were washed with interaction buffer with different concentrations of biotinylated hIGF-II (5–60 nM), in 100 μl of interaction buffer containing 0.1% bovine serum albumin, was then added to the wells, and the plate incubated for 1 h at room temperature. Following the interaction period, unbound hIGF-II protein was removed, and the wells were washed with interaction buffer. Next, polyclonal antibody against to the human IGFII was added to the wells, incubated for 1 h, followed by incubation with HRP-conjugated secondary antibody for 1 h and the liquid in the wells were removed. The HRP-conjugated antibody bound to the biotinylated hIGF-II protein-MPR 300/IGF-IIR complex was detected using the chromogenic substrate TMB/H_2O_2. Colour development was stopped after 15 min by adding 1 N HCl, and the absorbance was measured at 450 nM using a Bio-Rad model 450 microplate reader.

2.7. RT-PCR

The cDNA sequences for the putative IGF-II binding region was obtained using total RNA extracted from garden lizard liver, followed by reverse transcription-polymerase chain reaction (RT-PCR). Total *calotes* liver RNA was extracted using the RNeasy Mini Kit, and first-strand cDNA was synthesized using 0.5 μg of oligo