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Mannose-6-phosphate receptors (MPR 300 and 46) from the highly evolved invertebrate *Asterias rubens* (Echinodermate): biochemical and functional characterization of MPR 46 protein

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Abstract Mammalian mannose 6-phosphate receptors (MPR 300 and 46) mediate transport of lysosomal enzymes to lysosomes. Recent studies established that the receptors are conserved throughout vertebrates. Although we purified the mollusc receptors and identified only a lysosomal enzyme receptor protein (LERP) in the *Drosophila melanogaster*, little is known about their structure and functional roles in the invertebrates. In the present study, we purified the putative receptors from the highly evolved invertebrate, starfish, cloned the cDNA for the MPR 46, and expressed it in $mpr^{(-/-)}$ mouse embryonic fibroblast cells. Structural comparison of starfish receptor sequences with other vertebrate receptors gave valuable information on its extensive structural homology with the vertebrate MPR 46 proteins. The expressed protein efficiently sorts lysosomal enzymes within the cells establishing a functional role for this protein. This first report on the invertebrate MPR 46 further confirms the structural and functional conservation of the receptor not only in the vertebrates but also in the invertebrates.

Keywords MPR proteins · Mannose 6-phosphate receptors · Starfish · Goat · Phosphomannan gel · Echinodermata · Invertebrate · Evolution

Introduction

In mammals, lysosomal enzymes are targeted to lysosomes by two mannose 6-phosphate receptor proteins designated as MPR 46 and 300 (apparent molecular masses Mr 46 and Mr 300 kDa respectively). The mammalian receptors have been extensively characterized. Sequence and structural studies of both receptors from mammals have clearly established that the MPR 46 protein is a transmembrane glycoprotein made of a single amino terminal domain, a short transmembrane domain, a cytoplasmic tail and exists as a dimer in membranes. On the other hand the MPR 300 protein consists of a single polypeptide chain with 15 repetitive domains in the amino terminal region, a transmembrane domain and a cytoplasmic tail. Each of the 15 repetitive domains of the MPR 300 are homologous to each other and to the single domain of the MPR 46 protein ([1] and the references therein). MPR 300 has also been shown to be a multifunctional protein which, in addition to binding mannose 6-phosphate containing ligands, also binds human IGF-II, retinoic acid, urokinasetype-1 plasminogen activator receptor [2, 3]. The existence of two homologous proteins with overlapping, but distinct functions has raised the question of at what stage in the phylogenetic tree they began to appear. Towards understanding the evolution of these interesting proteins, our laboratory has contributed significantly to identify these putative receptors among the different non-mammalian vertebrates as well as from the invertebrates [4–6]. cDNA clones for several mammalian and for chicken MPR 300 and partial cDNA clone for chicken MPR 46 have been published [7]. We recently obtained a full-length cDNA clone for both receptors in fish [8, 9]. Comparison of the structural domains of the receptors in the vertebrates suggests that these are structurally related proteins with highly conserved ligand binding domains and exhibit distinct phosphomannan binding ability. However little is known about the lysosomal enzymes, their sorting mechanism and the biogenesis of lysosomes among the non-mammalian vertebrates and

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invertebrates. A comparative study using different model organisms and animal species should help to uncover the individual contributions of the two receptors to normal physiology and to pathological processes in these organisms.

Among the invertebrates, in molluscs (*unio*, a fresh water mussel that belongs to the phylum Mollusca) [6], in addition to the putative receptors we have also isolated and characterized a glycosylated form of α-fucosidase enzyme, which showed specific interaction *in vitro* with the purified MPR 300 protein from mollusc as well as from the goat, suggesting that molluscs might have similar targeting mechanisms for transport of lysosomal enzymes as the mammals [10]. However, in the *Drosophila melanogaster* that falls below the molluscs in the evolutionary tree, only a lysosomal enzyme receptor protein was identified by us that exhibited partial sequence homology to the human receptor, but failed to bind the multivalent phosphomannan gel [11] and no MPR 46 protein could be detected in *Drosophila*. In the arthropoda (prawn) and annelidae (earthworm) we could identify only MPR 300 like polypeptides and no MPR 46 protein could be detected [12]. All these species fall below the molluscs in the evolutionary tree. Although the putative receptors (receptors with phosphomannan binding ability and reacting with the mammalian receptor antibodies) have been identified in the molluscs, no information is available on the lysosomal enzymes and their receptors in the highly evolved invertebrates, the echinodermates. To gain further insight into the appearance of the receptors in these and to obtain new information on the structure and functional significance of the receptors among the invertebrates, the present work was carried out with the following objectives. (1) To check if the highly evolved invertebrates contain the putative MPR proteins, (2) whether they can bind the multivalent phosphomannan gel like the mollusc and vertebrate receptors, (3) analyze their biochemical and immunological properties, (4) obtain the first cDNA clone for the invertebrate MPR 46 gene and (5) to express the cDNA of the starfish MPR 46 protein in $mpr^{(-/-)}$ mouse embryonic fibroblast cells to study its functions.

Materials and methods

Starfish animals were collected from North Sea, Germany. From some animals, the gonads were separated under sterile conditions. Both the animals and gonads were kept frozen at minus 80°C, and were kindly provided by Prof. Dr. Soerge Kelm, University of Bremen, Bremen, Germany. In some experiments, gonads were directly used.

Preparation of the total membrane extracts

The whole animal tissue was used to prepare the acetone powder as described earlier [4]. All operations were carried out at 4°C. Fifty grams of the acetone powder prepared was homogenized with 300 ml of 50 mM imidazole buffer pH 7.0, 150 mM sodium chloride containing 0.1 mM PMSF and the homogenate stirred overnight. The suspension was clarified by centrifugation at 13,583×*g* for 15 min, and the supernatant discarded. The pellet was homogenized with 300 ml of 50 mM sodium acetate buffer pH 5.0, containing 150 mM sodium chloride, and centrifuged as described above. The pellet obtained was finally homogenized with 300 ml of 50 mM imidazole-HCl buffer pH 7.0, containing 5 mM sodium β-glycerophosphate, 150 mM sodium chloride. To this Triton X-100 and sodium deoxycholate were added to a final concentration of 1% and 0.1%, respectively and the suspension stirred overnight. This was then centrifuged at 670×*g*, for 15 min, and the supernatant was recentrifuged at 32,869×*g* for 30 min. The clear membrane extract was used as the source of the receptors. When the gonads were used, membrane proteins were directly extracted from them as described above for the purification of the receptors.

Affinity chromatography on phosphomannan–Sepharose gel

Phosphomannan–Sepharose (PM) gel was prepared as described earlier [4]. To purify MPR 300, EDTA was added to the membrane extract to yield a final concentration of 2 mM, and applied on a PM gel equilibrated with column buffer (50 mM imidazole buffer (pH 7.0), containing 5 mM sodium β-glycerophosphate, 150 mM sodium chloride, 0.05% Triton X-100, 2 mM EDTA (EDTA buffer). To purify MPR 46, $MnCl_2$, $CaCl_2$, and $MgCl_2$, were added to the membrane extract to a final concentration of 10 mM and applied to a separate PM gel equilibrated with column buffer described above except that the buffer had the three divalent metal ions in place of EDTA and no sodium chloride (metal ion buffer). This buffer allowed purification of the mollusc MPR 46 [6]. The membrane extracts were passed through two separate PM gels equilibrated with the EDTA and metal ion buffers for the isolation of the MPR 300 and 46, respectively. After the sample was passed through the gels, they were washed extensively with the respective column buffers, and the bound protein was eluted using 5 mM mannose 6-phosphate in column buffer. Aliquots were further analysed by SDS-PAGE as described below.

Protein estimation

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

Isolation of total RNA from starfish gonads

Total RNA from 20 mg of starfish gonads was isolated with the RNeasy kit (Qiagen) according to the manufactures

instructions. Purity and integrity of the total RNA was analysed by 1% agarose gel electrophoresis under denaturation conditions.

RT-PCR

From the total RNA isolated, cDNA was synthesized by Reverse Transcription (MBI Fermentas, India) following the kit protocol with oligo (dT) as the primer. The cDNA synthesized was used as a template for amplification of MPR 46 gene using the following degenerative primers designed based on the conserved sequences of known MPR 46 proteins from different vertebrates. The primers are; sense primers ('5-GTGCTGGTSAGTGAATCYTAGG-3'), (**'5-ATGCTGAACAGTGTAAGG-3'**) and anti sense primer ('5-CGTTCGGTAGYAARTGRTGATC-3'; **'5-CATCGG TAGCAAGTGATC-3'**). The MPR 46 gene was amplified by PCR with Taq polymerase and 12 pmol of the respective forward and reverse primers with the following conditions (95°CX15'; 94°CX1';55°C X 1'; 72°CX 1'; for 32 cycles).

Cloning of MPR 46

The PCR product was analysed on 1% agarose gel electrophoresis. The single band obtained was excised; gel purified and subjected to TA cloning into pTZ57R vector (MBI Fermentas). The positive clones were selected by blue/white selection and the plasmid DNA was isolated by mini kit (MBI Fermentas). The size of the insert was confirmed by restriction digestion of the plasmid DNA with EcoRI and Hind III enzymes.

Northern blot

Total RNA (15 μg) from starfish gonads was subjected to denaturing 1% agarose gel electrophoresis and transferred to Hybond N membrane (GE). The membrane was cross-linked for 45 s in a UV cross-linker and incubated for 3 h at 42°C in hybridization buffer. cDNA fragment of MPR 46 (819 bp) was radiolabeled with α ^{32}PdCTP (50 μCi). Labeled cDNA fragment was denatured at 98°C for 5 min before dilution with hybridization buffer. Hybridization was carried out at 42°C overnight. Membrane was washed for 10 min at RT and 30 min at 62°C with 20× SSC buffer containing 0.1% SDS followed by exposure to Kodak film overnight at −70°C with an intensifying screen. Alternatively the membrane was exposed to phosphor imaging.

DNA sequencing

DNA sequencing was done at Biosereve sequencing Pvt. Ltd. Sequence comparisons were performed with the CLUSTAL W method available online at http://www.justbio.com.

Construction of expression vectors

The 819 bp full length starfish MPR 46 cDNA that was originally cloned into pTZ57R vector was amplified by PCR using sense (Forward (5'-CG G*AATTC **ATGCTGAA CAGTGTA**-3') and anti-sense (Reverse 5'- CCG C*TCGAG *TCA* **CATCGGTAGCAA**-3') primers and sub cloned into mammalian expression vector pcDNA.6/V5-His A (Invitrogen) as described in [13]. The sequences shown in bold can anneal with the amplified fragment. Asterisk (*) denotes the cleavage site and the stop codon sequence is shown in italics.

Cell culture and transfection

Mouse embryonic fibroblasts [mpr$^{(-/-)}$ MEF] were grown in Dulbecco's minimal essential medium supplemented with Glutamax-I (Gibco/Invitrogen) and 10% fetal calf serum (FCS). Transfection of MEF cells deficient in MPR 46 and MPR 300 [mpr$^{(-/-)}$ MEF] with 20 μg of pcDNA.6/V5-His A containing the starfish MPR 46 gene by calcium precipitation was done as described [13]. Stable cell lines expressing the starfish MPR 46 were selected in Blasticidin S antibiotic containing medium at a final concentration of 15 μg/ml. The selection medium was changed once every 3 days.

Antibodies

Purified MPR 300 from starfish animal was detected by affinity purified antibody to the goat MPR 300 protein that was obtained as described earlier [14]. MPR 46 purified from starfish as well as from expressed MEF cells was detected by goat MPR 46 antiserum, MSC1 antibody (affinity purified human cytoplasmic tail antibody) and 218 peptide antiserum (antiserum raised against peptide 218 in the cytoplasmic tail of human MPR 46). Mouse lysosomal associated membrane protein (LAMP1) was detected using monoclonal anti-mouse LAMP1 antibody. Cathepsin D, arylsulfatase A and β-hexosaminidase were detected using a rabbit antiserum [15].

Preparation of cell lysate and extraction of membrane proteins from the transfected cells

From the mock transfected (vector alone) and the starfish MPR 46 expressing mpr$^{(-/-)}$ MEF cells, the cell lysate and membrane proteins were prepared as described [13].

Purification of starfish MPR 46 protein

The expressed starfish MPR 46 protein from stably transfected cell lines was purified by affinity chromatography on phosphomannan gel (PM gel), as described above in presence of metal ions. The wash fractions were collected and elution

was performed with 5 mM glucose 6-phosphate followed by 5 mM mannose 6-phosphate (M6P) in the column buffer.

SDS-PAGE and western blot analysis

Samples were analysed by SDS-PAGE under non-reducing and reducing conditions as described [16]. Proteins were detected using silver staining method [17]. For the immunological detection of MPR proteins, aliquots of the purified proteins were electrophoresed, and the proteins transferred to PVDF membrane as described [18]. For the detection of MPR 300, 10 μg of affinity purified goat MPR 300 IgG was used as the primary antibody, purified goat and *unio* MPR 300 (invertebrate, mollusc) were used as positive controls. For the detection of MPR 46, goat MPR 46 was used as a positive control. For the detection of MPR 46, antiserum raised against purified goat MPR 46 protein, (1:500 dilution) as well as 10 μg of the MSC1 antibody and 218 tail peptide antiserum described above were used. For analysis of cathepsin D, 50 μg of total cellular protein was subjected to immunoblotting and the blot was probed with antiserum specific for cathepsin D. The blots were developed using the enhanced chemiluminescence light-based immuno detection system [(ECL) Amersham Pharmacia Biosciences].

Immunofluorescence

Immunostaining analysis was performed as described [13], using a confocal laser scanning microscope. Primary antibodies were as described above; fluorescence-conjugated secondary antibodies were from (Cal Biochem, India) respectively.

Metabolic labeling and immunoprecipitation

MEF cells were incubated in methionine-free medium for 1 h and then labeled with 400 μci, of ^{35}S-methionine in the same medium containing 5% dialyzed FCS for 8 h. Arylsulfatase A was immunoprecipitated from the cells and medium as described previously [18] with rabbit antiserum against human recombinant arylsulfatase A. Quantification of band intensities was done with densitometer.

Co-immunoprecipitation

Coimmunoprecipitation was performed for β-hexosaminidase and starfish MPR 46 as described [13], except that β-hexosaminidase antiserum was used in this study in place of cathepsin D antiserum used earlier [13].

Chromatography on MPR 46 and MPR 300 Affinity gel

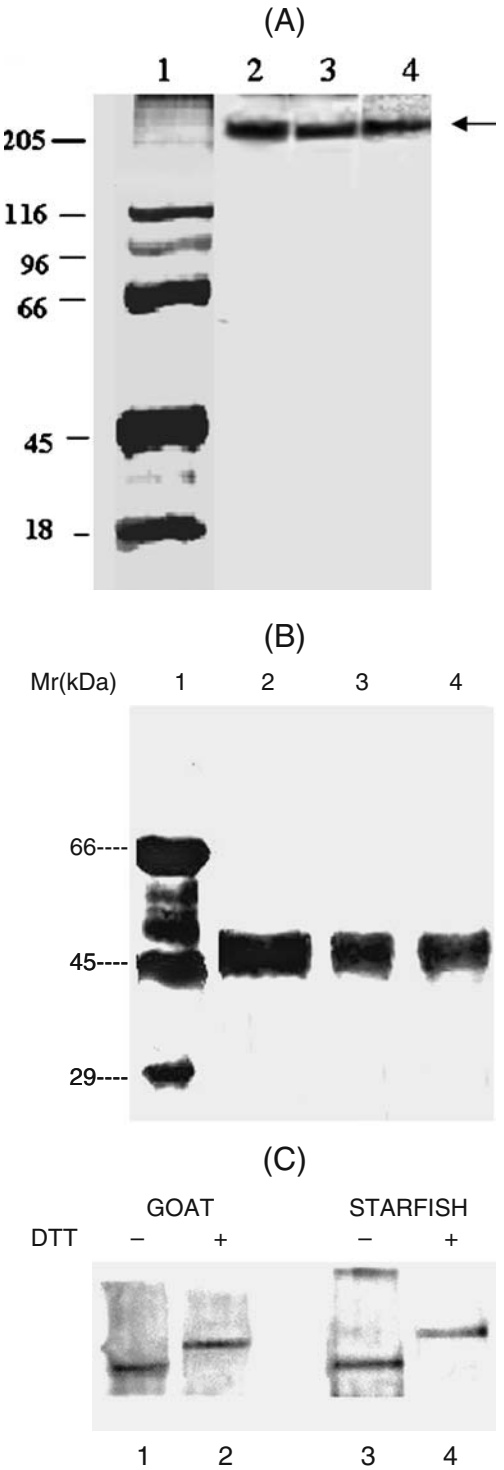
Purified goat MPR 46 and 300 proteins were coupled to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions. The secreted medium obtained after labelling with ^{35}S-methionine was subjected to MPR affinity chromatography as described [19]. The mannose 6-phosphate eluates were precipitated with 10% trichloroacetic acid; pooled and dissolved in 50 μl of 0.4 M Tris-HCl, heated for 5 min at 95°C in 10 mM dithiothreitol, 1% SDS; and analysed by SDS-PAGE (10%) and fluorography.

Fig. 1 SDS-PAGE analysis of M6P elutions from PM column **a** 10% SDS-PAGE analysis of the purified starfish MPR 300 protein. *Lane 1*, Molecular weight marker, *lane 2* goat MPR 300 (positive control), *lanes 3* and *4* represent starfish MPR 300 proteins from whole tissue and gonads respectively. *Arrow* indicates the position of the receptor. **b** 10% SDS-PAGE analysis of the purified goat and starfish MPR 46 protein. *Lane 1*, molecular weight marker, *lane 2* goat MPR 46 (positive control) *lanes 3* and *4* are starfish MPR 46 from whole tissue, and gonads. **c** 7.5% SDS-PAGE analysis of the purified goat (*lanes 1* and *2*) and starfish (*lanes 3* and *4*) MPR 300 proteins. *Lanes 1* and *3* under non-reducing conditions and *lanes 2* and *4* under reducing conditions

Results

Purification and characterization of the star fish MPR proteins

When the total membrane extracts obtained from the starfish were passed through two separate sepharose–phosphomannan gels (1) either in the presence of 2 mM EDTA, or (2) in the presence of divalent metal ions as described under methods, some protein was bound on the gel that could be eluted using 5 mM mannose 6-phosphate. When aliquots of the eluates obtained from 1 and 2 were analysed in 10% SDS-PAGE and the protein bands detected by silver staining the MPR 300 protein band as well as the MPR 46 protein band could be easily detected in both the eluates. The protein bands migrated to the same extent in the gels as the mammalian homologues, suggesting that the proteins isolated in this study represent the MPR proteins in starfish (Fig. 1a,b). Further, when the MPR 300 purified from starfish was analysed by SDS-PAGE under non-reducing and reducing conditions, there was a decreased mobility of the protein band in the gel suggesting that the reduction caused the opening up of the disulfide bridges in the protein. Goat receptor used as a control also gave similar results (Fig. 1c). From 50 g of the whole animal acetone powder 150 μg of the total protein could be quantified in the mannose 6-phosphate eluates. In order to analyze if both the MPR proteins purified from starfish are recognized by the antibodies to the goat receptors, western blot analysis was performed for the purified goat and starfish receptors. From Fig. 2a it is evident that the antibody to the goat MPR 300 protein shows immunoreactivity with purified goat, *unio* as well as the starfish



MPR 300 proteins. From Fig. 2b it is evident that the antiserum to goat MPR 46 shows immuno-reactivity with purified goat and starfish receptors. In addition, the purified starfish receptor also cross-reacted with the MSC1 antibody (Fig. 2c) that has been shown by us to recognize the MPR 46 protein from different vertebrates and molluscs [6]. These data obtained, thus confirm that the receptor proteins among the different vertebrates, molluscs and echinodermates (starfish) are immunologically related.

Molecular cloning and sequencing of starfish MPR 46

We obtained a full length cDNA clone for putative starfish MPR 46 protein. Total RNA isolated from starfish gonads (Fig. 3a) was used for synthesis of cDNA by RT-PCR with oligo (dT) as the primer. In a PCR reaction a 819 bp fragment was amplified by employing the combinations of

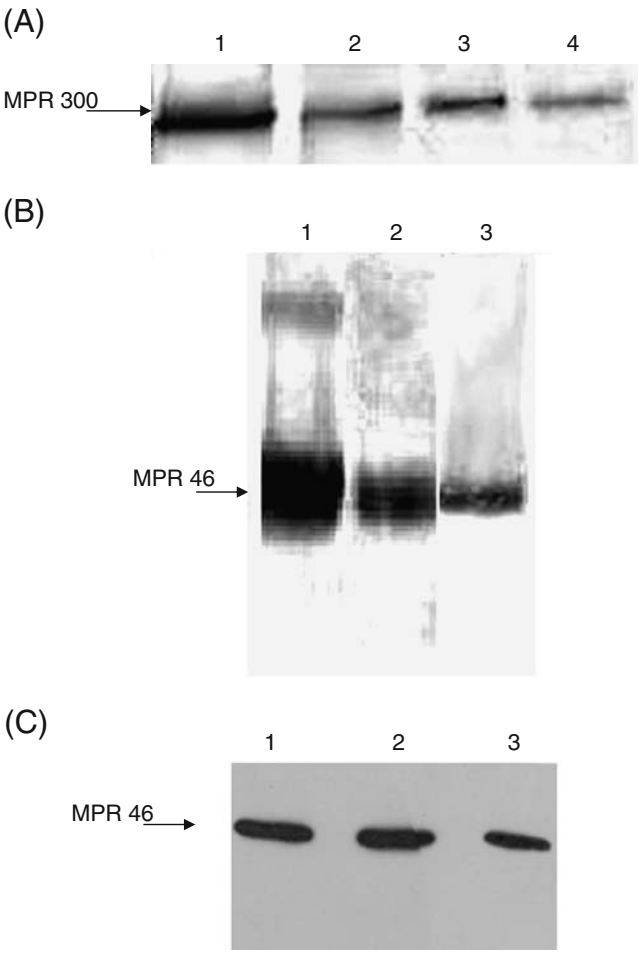


Fig. 2 Western blot analysis of purified MPRs **a** Western blot analysis of starfish MPR 300 protein (10% SDS-PAGE under reducing conditions). *Lanes 1* and *2* represent purified goat and *unio* MPR 300 protein (positive controls), *lanes 3* and *4* are starfish MPR 300 from whole tissue and from gonads, detected using affinity purified goat MPR 300 antibody. The *arrow* indicates the position of the MPR 300 protein. **b** Western blot analysis of goat and starfish MPR 46 proteins (10% SDS-PAGE) detected using goat MPR 46 antiserum. *Arrow* indicates the position of the MPR 46 protein. *Lane 1* goat MPR 46, (additional band seen above represents the dimeric form of the receptor). *Lane 2* and *3* are Starfish MPR 46 from whole tissue and from gonads. **c** Western blot analysis of MPR 46 detected using affinity-purified MSC1 antibody. *Lane 1* goat MPR 46, *lane 2* and *3* are Starfish MPR 46 from whole tissue and from gonads. *Arrow* indicates the position of the receptor

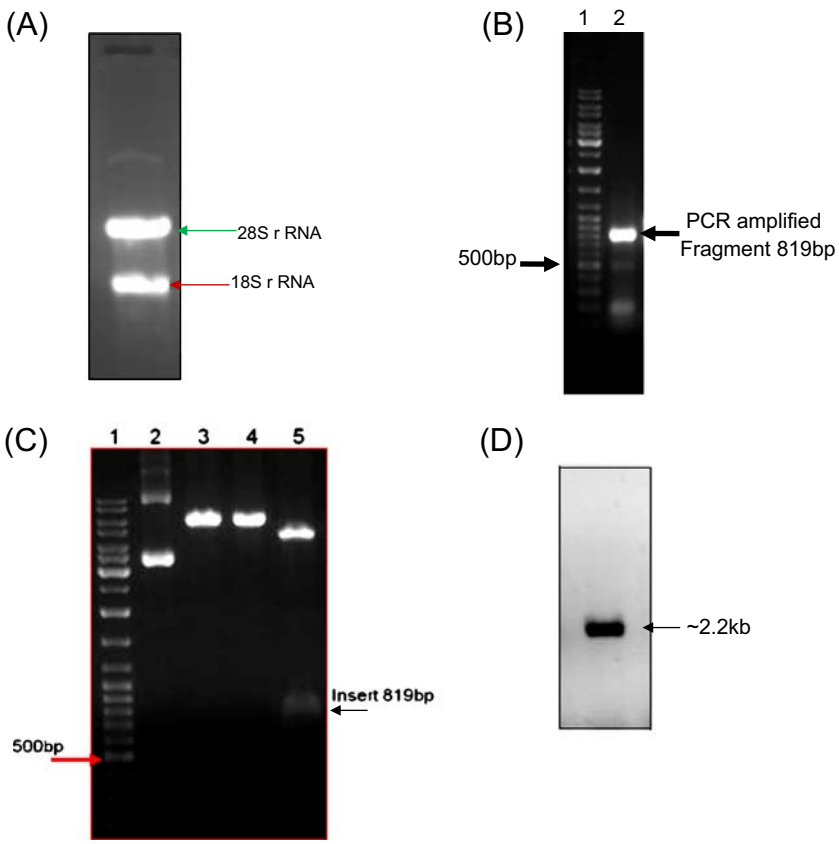


Fig. 3 Molecular cloning of starfish MPR 46. **a** Total RNA isolated from starfish gonad tissue. **b** PCR amplification of full length starfish MPR 46. Amplified product was subjected to 1% agarose gel electrophoresis. *Lane 1*, standard DNA ladder, *Lane 2*, amplified product (819 bp). **c** Restriction digestion analysis. *Lane 1* DNA ladder (100 bp-10 kb), *lane 2* Vector alone, *lane 3* and *4* single digestion with (EcoRI) and Hind III respectively, *Lane 5* Vector digested with EcoRI and Hind III [(←) fragment released from the vector after digestion with EcoRI and Hind III]. **d** Northern blot analysis. Fifteen microgram of total RNA isolated from starfish gonad tissue was subjected to denaturing 1% agarose gel electrophoresis, transferred to hybond-N-nylon membrane and hybridized with ^{32}P labeled starfish MPR 46 full length cDNA clone (819 bp)

the degenerative conserved sequence primers shown under methods. The sense and antisense, primers given in bold resulted in amplification of the fragment (Fig. 3b, lane1), the amplified fragment was cloned into TA vector (pTZ57R) and transformed into DH5α *Escherichia coli* cells. Cloned plasmid DNA was isolated, digested with EcoRI and Hind III, resulting in release of 819 bp insert (Fig. 3c lane 5). In northern blot analysis the transcript size of the starfish MPR 46 gene was found to be ~2.2 kb (Fig. 3d). The amino acid sequence deduced from the starfish MPR 46 cDNA clone was aligned with other known vertebrate sequences by multiple sequence alignment. In Fig. 4 the sequence of the starfish protein obtained has been numbered. The protein consists of a short signal peptide (1–20 amino acids), a N-terminal region (21–182 amino acids), a transmembrane domain (183–202 amino acids) and a cytoplasmic tail (203–273 amino acids). The extent of similarity of the starfish protein to other known vertebrates varied differently. It exhibited 46% similarity with human, 50% with the bovine, 54% with the mouse, 48% with the goat, 60% with the chicken, 54% with the *xenopus*, 25% with the killifish and 52% with the zebra fish sequences. The cysteine residues responsible for the proper folding of the protein are conserved from starfish to mammals. It is also known that amino acids such as the glutamine, histidine, arginine and tyrosine (indicated as $ in Fig. 4) in the extra cytoplasmic domain are important for the mannose 6-phosphate binding in the vertebrates. Interestingly these residues are also conserved in the starfish protein sequence (Gln 88, His 124, Arg 130, and Tyr 162). Additionally, an aspartic acid residue is also found in the starfish sequence at position 120 that may be involved in the metal binding. Furthermore, from our earlier studies it is clear that the chicken, fish and mollusc MPR 46 proteins bind phosphomannan gel in the absence of salt and in the presence of three divalent metal ions [5, 6] although it is still not clear why the MPR 46 protein in these species binds under these conditions, only. The starfish protein exhibits similar property as the chicken, fish and mollusc receptors. The transmembrane domain as well as the cytoplasmic tail of the starfish protein is also highly conserved. In particular the endocytosis signals (*YRGY* amino acids 251–254) the acidic cluster dileucine motif (DxxLL, 267–271) which are known to interact with the GGAs (Golgi associated gamma adaptin ear containing ARF binding proteins) for recycling are highly conserved in the starfish sequence also.

Expression and functional characterization of starfish MPR 46

The starfish MPR 46 cDNA was cloned into the pcDNA6V5/His expression vector for analysing the bio-

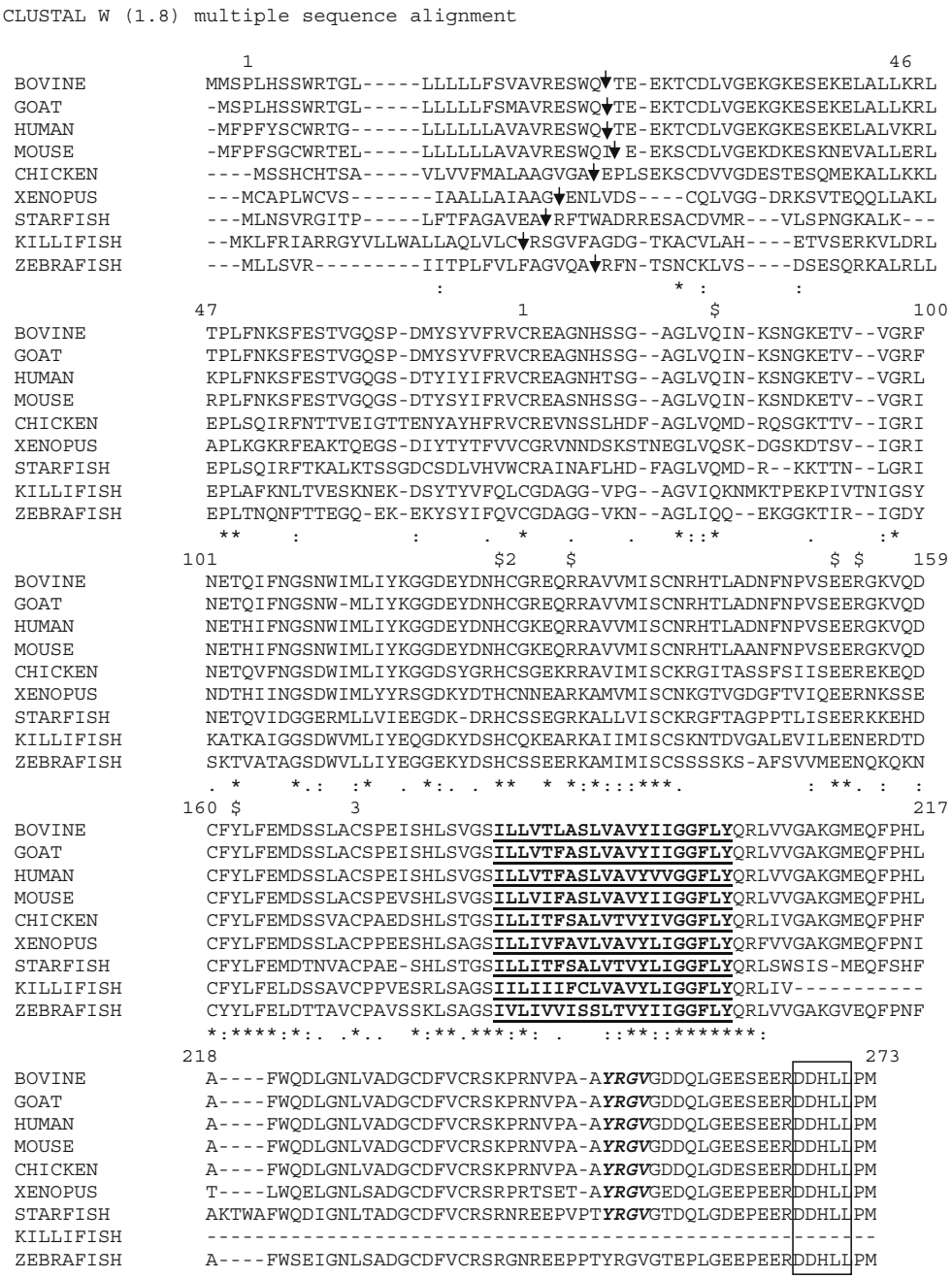


Fig. 4 Multiple sequence alignment of amino acids of MPR 46 protein from different species. The starfish sequence is numbered. *Arrow* indicates the signal peptide cleavage site predicted by the signal 3.0 server (http://www.cbs.dtu.dk/services/SignalP/); *Dollar sign* indicates the conserved M6P ligand binding residues; *Number sign* indicates predicted N-glycosylation sites predicted by the NetNGlyc 1.0 server; 1,2,3 indicate the conserved cysteine residues; the underlined sequence represents the transmembrane domain; *bold italicized* characters mark the tyrosine dependent endocytosis motif *YRGV*; *Boxed sequence* indicates the dileucine motif interacting with sorting GGAs; *Asterisk* marks identical amino acid residues; (*colon*) related amino acid residues; (*dot*) predominantly the same amino acid residue

chemical and functional properties of the receptor. Stable transfections were made in MPR-deficient mouse embryonic cells ($mpr^{(-/-)}$ MEF) that missort up to 98% of soluble lysosomal enzymes to the medium due to the lack of sorting MPRs. In these cells, the missorting of lysosomal enzymes, causing accumulation of non-degraded material (inclusions), can be rescued by the expression of a starfish receptor protein that re-establishes functional sorting. The subcellular distribution of starfish MPR 46 in the transfected cells was studied by immunocytochemical analysis. The results revealed an intracellular distribution of the starfish MPR 46 protein as detected by MSC1 antibody (Fig. 5f). The expressed starfish MPR 46 was localized in pre-lysosomal structures and colocalized with the lysosomal marker protein LAMP1 (Fig. 5g).

Biochemical properties of expressed starfish MPR 46

To analyze whether starfish MPR 46 binds mannose-6-phosphate residues, we prepared membrane fractions from starfish MPR 46-expressing $mpr^{(-/-)}$ MEF cells as described under materials and methods. The membrane extracts were passed over a multimeric mannose-6-phosphate phosphomannan ligand matrix. Unbound frac-

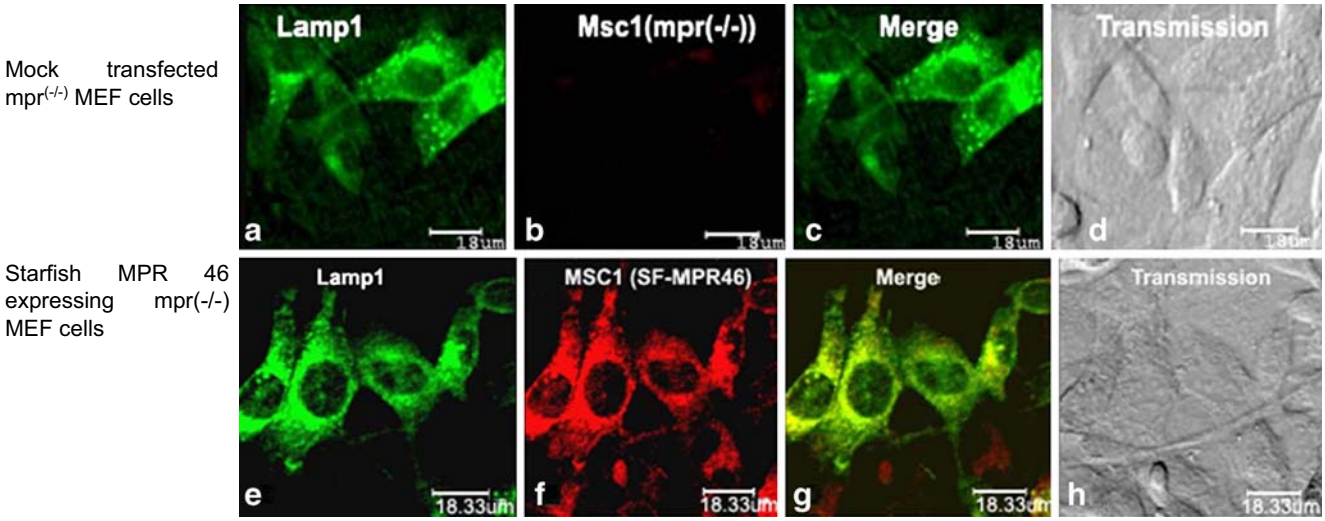


Fig. 5 Subcellular localization of MPR 46 protein. **a** and **e** incubation with LAMP-1 followed by FITC (*green*) conjugated secondary antibody, of mock transfected and mpr$^{(-/-)}$ MEF cells expressing starfish MPR 46 respectively. **b** and **f** incubation with MSC1 antibody followed by Cy3 (*red*) conjugated secondary antibody, of mock transfected mpr$^{(-/-)}$ MEF cells and starfish MPR 46 expressing cells respectively. **c** merged image of **a** and **b**; **g** merged image of **e** and **f**. **d** and **h** are the corresponding transmission images

tion, wash, glucose-6-phosphate and mannose-6-phosphate elutions were subjected to SDS-PAGE analysis and the proteins detected by silver staining. From (Fig. 6a), it is evident that the expressed protein could be specifically eluted from the PM gel using mannose 6-phosphate. In addition to the monomeric receptor band, the dimeric protein band was also detectable. The MPR 46 displays similar pattern in the zebra fish, goat, [9, 13]. Furthermore, by western blot analysis it has also been shown that the expressed starfish protein can be recognized by the goat MPR 46 antiserum, MSC1 antibody and 218 peptide antiserum [Fig. 6b–d]. With the 218 antiserum, both the monomeric and the dimeric bands could also be seen.

Sorting of phosphorylated ligands

The efficiency of sorting mannose-6-phosphorylated ligands by the expressed starfish protein was analysed, using the secretions obtained from the ^{35}S methionine labeled mock transfected and transfected cells. These were separately passed through receptor-affi-gel column as described under methods. After extensive washing, bound ligands were eluted with 5 mM mannose-6-phosphate (M6P), separated by SDS-PAGE and protein bands detected by fluorography. From Fig. 7a, it is evident that in the mock transfected cells there are a large number of bands visible in the secretions, while in the transfected cell secretions there

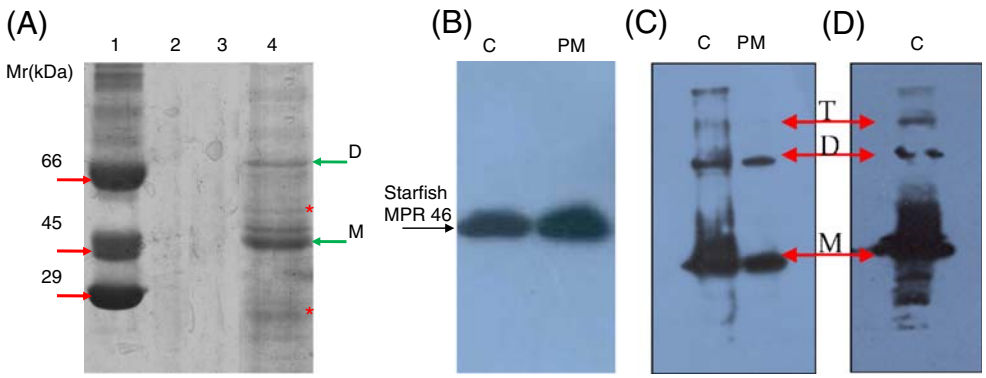


Fig. 6 Biochemical characterization of expressed starfish MPR 46. **a** Purification of expressed starfish protein on PM gel. Proteins were separated on 10% SDS-PAGE and silver stained; *Lane 1* Molecular weight markers, *lane 2* wash, *lane 3* glucose-6-phosphate eluate, *lane 4* mannose-6-phosphate eluate showing monomeric (*M*) and dimeric (*D*) forms. **b**–**d** Western blot analysis of the expressed protein. *C* and *PM* refer to the cell lysate and the affinity purified receptor from cells expressing the starfish protein separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with **b** goat MPR 46 antiserum, **c** MSC1 antibody and **d** 218 peptide antiserum. Blots were developed with ECL reagent; both the monomeric (*M*) and dimeric (*D*) forms were observed in cell lysate as well as PM elution (B&D). T (higher oligomer)

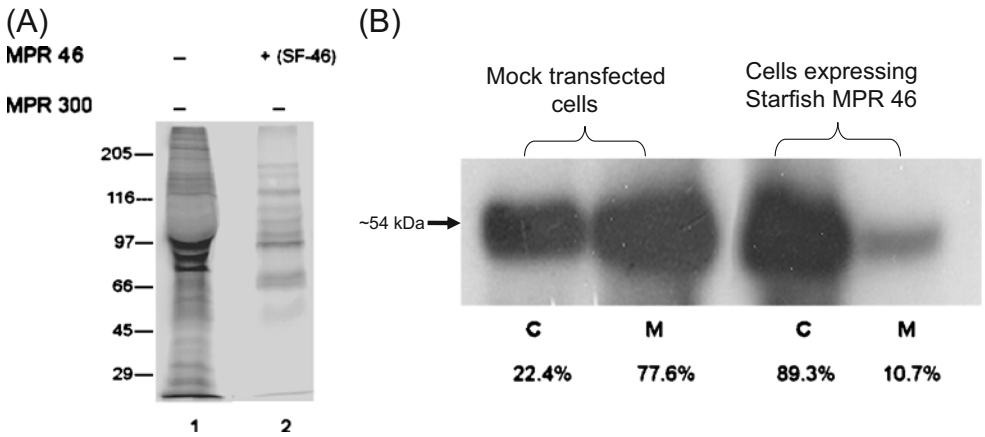


Fig. 7 **a** Secretion of M6P-containing polypeptides in mpr$^{(-/-)}$ MEF fibroblasts expressing starfish MPR 46. Cells expressing SFMPR46 were tested for their ability to retain the missorting of lysosomal enzymes; cells were labelled with [^{35}S] methionine. The labeled phosphorylated ligands secreted in the culture medium were purified on MPR affinity gel and characterized by SDS-PAGE and fluorography. *Lane 1* secretions from mock transfected cells, *lane 2* secretions from starfish MPR 46 transfected cells. **b** Biosynthesis of arylsulfatase A in mock transfected and mpr$^{(-/-)}$ MEF fibroblasts expressing starfish MPR 46. Cultured fibroblasts were metabolically labeled with [^{35}S] methionine. Arylsulfatase A was immunoprecipitated from equal amounts of trichloroacetic acid-insoluble radioactivity of cells and corresponding aliquots of the secreted culture media and subjected to SDS-PAGE and fluorography. The relative level of newly synthesized arylsulfatase A and the percentage of newly synthesized protein detectable in the cytosol (*C*) and culture medium (*M*) were calculated by densitometry analysis and are shown in the figure

are only a few bands, suggesting that the transfected protein is able to retain the phosphorylated ligands.

Sorting of newly synthesized arylsulfatase A and cathepsin D in MPR-deficient fibroblasts expressing starfish MPR 46

To determine the sorting of newly synthesized lysosomal enzymes, cells were metabolically labeled with ^{35}S methionine as described under methods. From the secretions and the soluble cell extracts of the mock transfected and transfected cells, arylsulfatase A was immunoprecipitated. In mock transfected cells, 77.6% of arylsulfatase A was secreted out while in transfected cells only 10.7% of the labeled enzyme was secreted in the culture medium and 89.3% was sorted to lysosomes efficiently (Fig. 7b). This clearly demonstrates that the bulk of newly synthesized

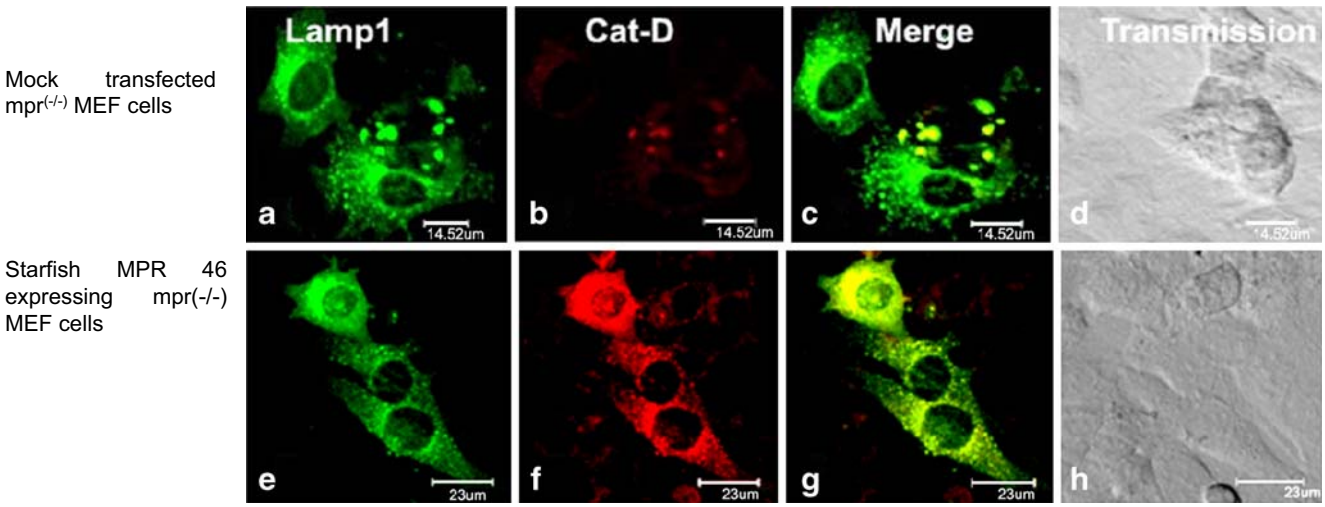


Fig. 8 Intracellular retention and sorting of cathepsin D. **a** and **e** incubation with LAMP-1 followed by FITC (*green*) conjugated secondary antibody, of mock transfected and mpr$^{(-/-)}$ MEF cells expressing starfish MPR 46 cells respectively. **b** and **f** incubation with cathepsin D antiserum followed by Cy3 (*red*) conjugated secondary antibody, of mock transfected mpr$^{(-/-)}$ MEF cells and starfish MPR 46 expressing cells respectively. **c** Merged image of **a** and **b**; **g** merged image of **e** and **f**, clearly shows the extensive co-localization of LAMP-1 and cathepsin D, thus supporting the function of starfish MPR 46 protein to bind and target cathepsin D to lysosomes. **d** and **h** are the corresponding transmission images

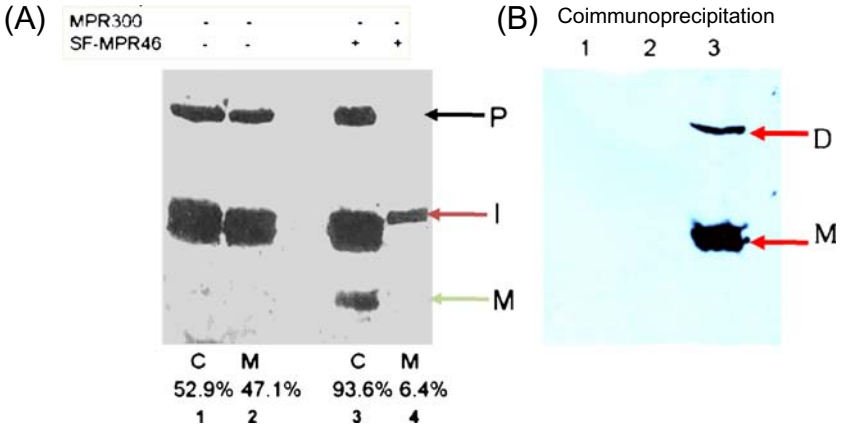


Fig. 9 Quantification of cathepsin D level. **a** Quantification of cathepsin D protein present in the cell lysate (C) and the 12 h culture medium (*M*) from the mock transfected mpr$^{(-/-)}$ MEF cells (*lanes 1* and *2*). Quantification of cathepsin D protein present in starfish MPR 46 protein expressing mpr$^{(-/-)}$ MEF cell lysate (C) and the 12 h culture medium (*M*; *lanes 3* and *4*). **b** Co-immunoprecipitation of the cell lysates prepared from mpr$^{(-/-)}$ MEF cells transfected with starfish MPR 46 using β-hexosaminidase antiserum (details given in text). *Lane 1*, cell lysate with preimmune serum, *lane 2*, cell lysate in presence of 5 mM M6P, *lane 3*, cell lysate in the absence of 5 mM M6P. The membrane was probed with MSC1 antibody. M and D represent the monomeric and the dimeric forms of the starfish MPR 46 receptor protein

arylsulfatase A is sorted to lysosomes in starfish MPR 46 expressing fibroblasts. Furthermore, in the transfected cells, in parallel to arylsulfatase A, sorting of cathepsin D and its colocalization with LAMP1 protein (a lysosomal membrane marker protein) was also demonstrated by immunofluorescence using cathepsin D antiserum. Compared to the mock transfected cells (Fig. 8, upper panel), in the transfected cells (Fig. 8, lower panel) the concentration of the cathepsin D is higher.

Cathepsin D in lysosomes of starfish MPR 46 expressing fibroblasts

The steady-state concentration of cathepsin D varied between 52.9% and 93.6% in mock transfected and transfected cells as analysed by western blotting (Fig. 9a). In starfish MPR 46 expressing fibroblasts, the precursor, intermediate forms and 30-kDa mature forms of cathepsin D are retained intracellularly (93.6%) and in secretions only the intermediate form appears with low concentration (6.4%). In the mock transfected cells the precursor form and intermediate forms are detectable intracellularly as well as in secretions almost with similar concentrations and the mature form is barely detectable. The specific interaction of the receptor with lysosomal enzyme was further confirmed by co-immunoprecipitation experiment with β-hexosaminidase antiserum, followed by blotting and detection using receptor antibody. Preimmune serum was used as a control (Fig. 9b lane 1). Mannose-6-phosphate dependent binding was also analysed in presence and absence of the sugar (Fig. 9b lane 2). The specific identification of the starfish MPR 46 monomeric and dimeric forms (identified with the MSC1 antibody) indicates the mannose-6-phosphate dependent binding of starfish MPR 46 protein with the β-hexosaminidase (Fig. 9b lane 3).

Discussion

The biogenesis of lysosomes requires the correct sorting of >50 acid hydrolases from their site of synthesis in the endoplasmic reticulum to their final destination in lysosomes. The 46 kDa cation-dependent mannose-6-phosphate receptor (CD-MPR) and the 300 kDa cation-independent MPR (CI-MPR) divert these soluble enzymes from the secretory pathway by delivering their cargo bearing mannose-6-phosphate on N-glycans from the *trans*-Golgi network (TGN) to endosomes. Mammalian mannose-6-phosphate receptors (MPR 46 and 300) are homologous proteins with distinct functions which have been well characterized. Only MPR 300 protein has been shown to be a multifunctional protein [20]. From the last few years we have been working on MPRs in non-mammalian vertebrates and invertebrate species to establish their phylogenetic origin, understand the evolutionary history and functional significance of these proteins in the animal kingdom. So far we have been able to characterize the goat, some non-mammalian vertebrate receptors, and the mollusc receptors. Extensive studies carried out recently established the evolutionary conservation of the vertebrate MPR 46 protein [9]. Among the non-mammalian vertebrates, it has been shown that the chicken MPR 300 is also a multifunctional protein, and the Zebra fish MPR 300 can bind mannose-6-phosphate containing ligands [21, 22]. There is a single report that shows the teleost fish can bind IGF-II [23]. We cloned the full length MPR 300 gene for

the fugu fish and found that the m6p binding sites are highly conserved throughout the vertebrates. It still remains to be established whether the fugu receptor binds IGF-II or not. Though we purified the mollusc receptors [6], in the *D. melanogaster*, only a lysosomal enzyme receptor protein (LERP) could be identified by us. This shows partial homology to the human MPR 300 protein, but fails to bind on the multivalent phosphomannan gel. Furthermore, unlike the mammalian and other known putative MPR 300 proteins that exhibit a molecular mass of 300 kDa, LERP shows a molecular mass of 150 kDa, and possibly represents a truncated form of the mammalian homologue. LERP contains five repeating units in its luminal domain that display a homology of 23–29.5% at the amino acid level to those of the human MPR 300. However, the residues known for mannose-6-phosphate binding are lacking in the LERP [11]. No MPR 46 homologue is known in the *Drosophila*. Additionally in other species, such as the prawn (Arthropoda) and earthworm (Annelidae) also, only MPR 300 like proteins could be detected and no MPR 46 protein could be detected [12].

Although the MPR clearly has a major role in lysosomal enzyme sorting in the vertebrate cells, its role in the invertebrates is not understood. In lower eukaryotes, such as *Saccharomyces*, *Trypanosoma*, and *Dictyostelium*, lysosomal enzymes are targeted without the aid of identifiable MPRs. The slime mold *Dictyostelium discoideum* produces a novel methyl-phosphomannose sequence on some of its lysosomal enzymes that can be recognized *in vitro* by the mammalian CI-MPR (not by the CD-MPR). However, despite the presence of a α-*N*-acetyl glucosamine phosphotransferase (GlcNAc-P-T) that recognizes α-1–2 linked mannose residues, no receptor for the phosphorylated mannose residues has been found in these organisms. In contrast to this situation, the protozoan *Acanthamoeba* produces a GlcNAc-P-T that does show specific recognition of lysosomal enzymes [24, 25]. Since we have already established that the vertebrate receptors are highly conserved proteins, and identified the putative receptors in the molluscs, it became necessary to extensively characterize the invertebrate receptors (echinodermates and molluscs). Sequence by cloning and functional characterization of the invertebrate receptors (echinodermates and molluscs) should conclusively establish the functional significance and phylogenetic origin of the two receptors, as well as their physiological roles under normal and pathological conditions. As a first contribution to gain new insights into the invertebrate receptors, in the present study, we set out to purify the receptors from the highly evolved invertebrates, echinodermates that fall above the molluscs in the evolutionary tree. We further cloned the gene for the starfish MPR 46 protein and studied its function by expressing it in MPR-deficient mouse embryonic cells ($mpr^{(-/-)}$ MEF). The phosphomannan binding ability of the expressed cDNA and the transcript size of ~2.2 kb obtained by northern analysis suggest that the expressed cDNA represents the MPR 46 protein. The amino acid residues critical for ligand binding by the MPR proteins have been identified [20]. Glutamine, histidine, arginine and tyrosine are the four important amino acids that have been identified and characterized to be important in ligand binding in mammalian MPR 46 proteins and these are also conserved in the starfish protein. Additionally from the crystal structure of bovine MPR 46 protein, it is also known that two cysteine residues, histidine, arginine, aspartic acid and glutamic acid are important for interaction with Mn^{+2} [1, 26]. We found that in starfish sequence, these residues are also highly conserved like in other vertebrate species and the aspartic acid at position 120 might play a crucial role in metal binding.

A key function of MPRs is to segregate newly synthesized acid hydrolases from secretory proteins at the TGN. In both MPRs this sorting signal is composed of a cluster of acidic residues followed by a dileucine motif at the carboxy terminus of the cytoplasmic domain [referred to as an acidic cluster-dileucine motif (DxxLL)] and also involves several binding sites for AP-1 (Adapter protein-1) [27]. Carboxy-terminal DxxLL motifs and conserved AP-1, GGAs binding sites are present in the cytoplasmic tails of starfish MPR 46 also. Following delivery of acid hydrolases, mammalian MPRs do not enter lysosomes, instead are recycled from late endosomes to the TGN a process mediated by the protein TIP47 (tail interacting protein) [27]. TIP47 has been shown to recognize a phenylalanine–tryptophan motif (223–224) in the cytoplasmic tail of the mammalian CD-MPR, and this motif is also present in the starfish MPR 46. Heterologous expression of the starfish MPR 46 in mammalian cells revealed an intracellular distribution (Fig. 6f) similar to the goat MPR 46 [13]. This distribution fits very well to the demonstrated lysosomal sorting function of the starfish receptor. The common distribution and the common sorting function both provide evidence that starfish MPR 46 protein functions like its mammalian counterpart, recognized by key organizers responsible for this specific vesicular sorting pathway. These data provide strong evidence to suggest that in the model organism, starfish (invertebrate) the putative lysosomal sorting receptors behave like the mammalian proteins, giving new insights towards the understanding of the importance of lysosomal enzyme sorting process in the invertebrates. This was further supported by additional experiments, where we found that an antiserum to the mollusc α-fucosidase enzyme showed immunological cross-reactivity with a protein from the soluble extracts of the starfish (both from the total animal extract as well as from the gonads) suggesting that the starfish may have

a related enzyme (data not shown). Additionally, β-hexosaminidase, arylsulfatase A, α-galactosidase, α-mannosidase and cathepsin D activities were also detectable in the soluble extracts (data not shown). Furthermore, it was found that in a far western analysis, the enzyme from whole animal tissue and gonads specifically interacts with the purified starfish MPR 300 protein (data not shown). We earlier found that the MPR 300 protein from molluscs interacts specifically with the mollusc α-fucosidase [10].

In summary, the results of our study clearly demonstrate that (1) the highly evolved invertebrate, starfish, contains the putative MPR proteins (MPR 46 and 300), that can be affinity purified on the multivalent phosphomannan gels, like the mammalian and non-mammalian vertebrate receptors, (2) the biochemical and immunological properties of the starfish receptors resemble the mollusc, mammalian and other receptors characterized, (3) the star fish MPR 46 exhibits high degree of structural homology to other vertebrate receptors, (4) the starfish also contains a glycosylated α-fucosidase enzyme that appears to be cross-reacting with the mollusc enzyme antiserum, (suggesting antigenic similarities among these invertebrate enzymes) and (5) starfish MPR 46 expressed in immortalized mouse embryonic fibroblasts cells ($mpr^{(-/-)}$ MEF), which are deficient in MPR 46 and MPR 300 show a clear evidence for the functional significance of starfish MPR 46 protein. This is the first report on the functional characterization of MPR 46 from the starfish. It is unknown whether the mannose-6-phosphate-dependent sorting system of lysosomal enzyme recognition is established identically in the invertebrates and mammals. This has to be addressed by an extensive characterization of the components of the lysosomal system in starfish, *i.e.* by cloning and characterization of lysosomal enzymes, the sorting GGAs and the second MPR, the MPR 300. The new data obtained on the starfish protein has now laid the foundation to clone the genes for the mollusc receptors and to establish the structural and functional significance of the receptors in the invertebrate species.

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