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Biochemical and functional characterization of cation dependent (Mr 46,000) goat mannose 6-phosphate receptor

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Abstract The Mannose 6-phosphate receptor (MPR's) proteins are important for transporting lysosomal enzymes from *trans*-golgi to the pre-lysosomal compartment. These are conserved in the vertebrates from fish to mammals. We have cloned the full length cDNA for the goat MPR 46 protein and compared its sequences to the other known vertebrate MPR 46 proteins. In the present study the full-length cDNA for the goat MPR 46 protein was expressed in MPR deficient cells. The expressed protein was purified on the multivalent phosphomannan gel in the presence of divalent metal ions. The apparent molecular mass of the expressed protein was found to be ~46 kDa and also exhibits oligomeric nature as observed in the other species, by using an MSC1 antibody (that recognizes the MPR 46 from molluscs to mammals) as well as with a peptide specific antibody corresponding to amino acid residues (218–237) of the cytoplasmic tail of human MPR 46 protein. Furthermore the distribution of the expressed protein was visualized by immunofluorescence using MSC1 and LAMP1 antibody. Additionally in the goat MPR 46 expressing cells, the sorting function of the expressed protein to sort cathepsin D to lysosomes was studied by confocal microscopy using cathepsin D antiserum and LAMP1 antibody. The binding of goat MPR 46 to cathepsin D was shown in far Western blotting and the mannose 6-phosphate dependent binding was shown by co-immunoprecipitation.

Keywords Mannose 6-phosphate receptor · MPR 46 · Goat · PCR · Vertebrate · Expression

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Introduction

In eukaryotic cells, newly synthesized lysosomal enzymes are transported to lysosomes by two homologous but distinct receptor proteins designated as Mannose 6-phosphate receptors (MPR 300 and 46, based on their apparent molecular mass). The receptor genes have been cloned from some mammalian species. The mammalian MPR 300 protein has 15 repetitive units in the extracytoplasmic domain, which share significant sequence similarity with each other, and also with the single unit that constitutes the extracytoplasmic domain of MPR 46. Furthermore, the repetitive domains of known MPR 300 proteins also exhibit 14–38% sequence homology [1, 2]. Other researchers cloned the full-length gene for the chicken MPR 300 and a partial clone for chicken MPR 46 [3, 4] and found that they are structurally related to the mammalian proteins. In view of this it is interesting to know and study the evolution of these receptors. In order to understand the evolution of these receptor proteins in the animal kingdom, we have previously identified the putative receptors from non-mammalian vertebrates such as the reptiles, amphibians, fish, [5, 6] and also cloned the receptor genes for the fugu pufferfish (*Takifugu rubripus*) [7] and the zebra fish [8]. Among the invertebrates the putative receptors were identified in the molluscs [9], echinodermates [unpublished data]. In *Drosophila melanogaster a* novel lysosomal enzyme recognition protein was discovered, which functions similar to mammalian MPR 300 [10]. In prawn and earthworm only MPR 300 like protein could be detected [unpublished data]. We have also characterized the goat receptors and cloned the genes for the same. The transmembrane domain and the cytoplasmic tail of the goat MPR 46 protein is highly conserved. The extracytoplasmic domain sequence also showed exten-

sive sequence homology with the other known species [11]. The potential residues essential for the M6P binding, cysteine residues that form the disulfide pairing in the amino terminal domain, dileucine motif DxxLL proposed to interact with sorting GGAs, endocytosis motif YRGV in the cytoplasmic tail are conserved.

To further understand the structure-function of the goat MPR 46 protein, the present study was carried out to (a) express the full length cDNA of the putative goat MPR 46 protein in MPR deficient cells, (b) analyze its expression, (c) determine the ability of the expressed protein to bind the multivalent phosphomannan gel, and also to the lysosomal enzyme cathepsin D, as the MPR 46 proteins are known to sort cathepsin D.

Materials and methods

Cells: MPR-deficient mouse embryonic fibroblast cells [mpr$^{(-/-)}$ MEF cells] used in the present study were kindly provided by Prof. Dr. Regina Pohlmann, University of Muenster, Germany. The following antibodies were available in the laboratory. MSC1 antibody (an affinity purified human MPR 46 cytoplasmic tail antibody). Two hundred eighteen antiserum (an antiserum raised against the synthetic peptide corresponding to residues 218–237 in the cytoplasmic tail of the human MPR 46). Mouse cathepsin D antiserum raised in a rabbit was a generous gift from Prof. Dr. Regina Pohlmann. LAMP1 monoclonal antibody was kindly provided by Prof. Dr. Stefan Hoening, University of Koeln, Germany.

Construction of expression vectors

The 840 bp full length goat MPR 46 cDNA clone that was originally cloned in pTZ57R vector [11] was taken out and cloned into mammalian expression vector pcDNA.6/V5-His A (Invitrogen). Polymerase chain reaction (PCR) was performed using sense (5′-GG*A ATT CCC ACC ATG ATG TCC CCC CTC CAC-3′) and anti-sense (5′-CCG C*TC GAG TCA CAT TGG TAA TAA GTG GTC-3′) primers flanking *EcoRI and XhoI restriction sites. The amplified fragment and pcDNA.6/V5-His A vector was digested with EcoRI and XhoI restriction enzymes (MBI Fermentas). The digested fragment and linearized vector was excised, extracted using QIAEX II gel extraction kit, and were taken in 1:3 molar ratio for ligation reaction using T4 DNA Ligase (MBI Fermentas) according to the manufacturers instructions. Ligated product was transformed in to DH5α. Colony PCR was carried out to identify the colony harboring the gene of interest. The plasmid DNA was isolated from one of the colony using Eppendorf fast plasmid mini kit and the presence of MPR 46 gene was confirmed by restriction digestion. The sequence and reading frame was verified using automated DNA sequencing (Bioserve, India).

Cell culture and transfection

Mouse embryonic fibroblasts (mpr$^{(-/-)}$ MEF) deficient in MPR 46 and MPR 300, were grown in Dulbecco's minimal essential medium supplemented with L-Glutamine 5 mM and 10% fetal calf serum (FCS). Transfection with pcDNA.6/ V5-His A vector construct in to these cells was done as described [12].

Preparation of cell lysate and extraction of membrane proteins from the transfected cells The goat MPR 46 and mock transfected (vector alone) cells were scraped with the help of a cell scraper from the petri plate, and the cell pellet was collected by centrifugation in a Biofuge stratos centrifuge, using a microliter rotor at 2,991×g for 10 min. The cells were then lysed in 20 mM Tris-HCl pH 7.4 buffer containing 600 mM KCl, 20% glycerol, 1% Triton X-100, 1 mM PMSF, 2 μg/ml leupeptin (Lysis buffer). The cell lysate was run on a 10% SDS-PAGE, proteins were transferred to nitrocellulose (NC) membrane and probed with MSC1 antibody to show the expressed goat MPR 46 protein. To obtain the membrane extract from the cells expressing goat MPR 46, the cell pellet was collected as described above and was suspended in 0.1 M sodium acetate buffer pH 6.0, containing 0.2 M NaCl, 1 mM PMSF, 5 mM iodoacetic acid, 1 mM EDTA, sonicated thrice for 35 s each time with an interval of 1 min, incubated for 20 min on ice, and centrifuged in a Beckman centrifuge, using a fixed angle 80Ti rotor at 161,280×g for 35 min. The pellet obtained at this step was dissolved in 50 mM imidazole-HCl buffer pH 7.0 containing 0.5% Triton X-100, sonicated as above, incubated for 20 min on ice, and recentrifuged as described above. The supernatant is designated as the total membrane extract and was used for the isolation of the expressed goat MPR 46 protein by Sepharose-divinlysulfone-phosphomannan gel (PM gel) chromatography.

Affinity chromatography

The fraction of membrane proteins obtained as mentioned above was applied on to PM gel at 4°C pre-equilibrated with buffer containing 50 mM imidazole-HCl pH 7.0, 5 mM sodium β-glycerophosphate, 150 mM NaCl, 0.05% Triton X-100, 10 mM $MnCl_2$ (column buffer) [5]. The flow rate of the column was maintained at 30 ml/h, and the gel was washed extensively with the same buffer. 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. The mannose 6-phosphate eluates were pooled and concentrated by acetone precipitation, aliquots analyzed by SDS-PAGE (under reducing and non-reducing conditions), Western blotting and far Western blotting.

Divalent cation dependence of the purified receptor obtained above to bind PM gel was analyzed by performing the affinity chromatography as mentioned above by excluding the divalent metal ions in the column buffer.

SDS-PAGE and Western blot analysis

Samples were analyzed by SDS-PAGE under reducing conditions as described [13]. Gels were stained using the silver staining method [14]. For immunological detection, aliquots of the purified proteins were electrophoresed, and the proteins were transferred to nitrocellulose membrane as described [15]. The membrane was placed in blocking solution containing 5% bovine serum albumin in phosphate buffer pH 7.4, containing 0.15 M NaCl and 0.2% tween-20 (PBST). The MPR 46 was detected using MSC1 antibody (2 μg/ml), and 218 antiserum (1:500 dilution). In case of 218 antiserum, alkaline phosphatase (ALP) conjugated goat anti-rabbit IgG (1:5,000 dilution in PBST) was used as the secondary antibody. The blot was then developed with BCIP-NBT substrate in ALP buffer and the bands were visualized and photographed. Horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:5,000 dilution) was used as a secondary antibody for detecting the blot probed with MSC1 antibody. The blot was developed with enhanced chemiluminescence (ECL) reagent.

Immunofluorescence using confocal microscopy

mpr$^{(-/-)}$ MEF cells expressing goat MPR 46 were analyzed for the MPR 46 protein by confocal microscopy using MSC1 antibody. After transfection, the cells were fixed in 4% formaldehyde in PBS buffer incubated at room temperature for 6 min, washed, and then permeabilized with 0.2% saponin in PBS for 6 min at room temperature. The cells were washed and blocked with 5% BSA in PBS for 30 min at room temperature. The cells were then incubated with LAMP1 antibody (1:100 dilution) followed by MSC1 antibody 2 μg/ml in 5% BSA in PBS for 1 h at room temperature. The cells were washed 5–6 times with PBS, blocked with BSA as above, incubated with fluorescent tagged anti-rabbit IgG-Alexa fluor 594 (molecular probes, Invitrogen) followed by anti-mouse IgG-FITC (Bangalore Genei) for 1 h at room temperature. Finally the cells were washed extensively and observed under confocal microscope. The mpr$^{(-/-)}$ MEF cells expressing goat MPR 46 were analysed for the cathepsin D retention using cathepsin D antiserum by immunofluorescence. Lysosomes were labelled by LAMP1 antibody. After fixation and permeabilization the cells were incubated with cathepsin D antiserum (1:250 dilution) and LAMP1 antibody (1:100 dilution) in 5% BSA, followed by incubation with anti-rabbit IgG-Alexa fluor 594 followed by anti-mouse IgG-FITC (Bangalore Genei).

Far Western blotting and co-immunoprecipitation

The medium collected from the cultured mpr$^{(-/-)}$ MEF cells was precipitated by addition of 50% ammonium sulfate and the protein pelleted by centrifugation at 17,226×g for 20 min. The pellet was suspended in 10 mM Tris-HCl pH 7.4, containing 150 mM NaCl (TBS) and dialyzed against TBS. Aliquot of the dialyzed sample was then separated on 10% SDS-PAGE and analyzed by Western blot [15] and far Western blot as described [16]. The Western blot and far Western blot analysis was carried out using the anti-cathepsin D antiserum and MSC1 antibody, respectively. In both cases, the detection was carried out by alkaline phosphatase conjugated secondary antibodies. For co-immunopreciptation of cathepsin D and goat MPR 46, 100 μl each of transfected cell lyaste was taken in to three different tubes. To each tube 900 μl of 20 mM Hepes pH 7.4 buffer containing 300 mM NaCl, 4 mM $MgCl_2$, 3 mM $CaCl_2$, 1 mM PMSF, 2 μg/ml leupeptin was added. Tube 1 received 1 μl of preimmune serum. Tube 2, was preincubated for 3 h with 5 mM M6P, while tube 3 did not receive M6P. 1 μl of cathepsin D antiserum was added to tube 2 and 3. The tubes were kept for rotation overnight at 4°C. To each tube, 20 μl of protein A-agarose (Bangalore Genei), was added and rotated for 4 h at 4°C. The reaction tubes were then briefly centrifuged in a table top centrifuge, washed with phosphate buffer pH 7.4, containing 0.15 M NaCl and 0.2% tween-20 (PBST) 6–7 times. The pellet was cooked in 2 X SDS-PAGE sample buffer and loaded on a 10% SDS-PAGE under reducing conditions. The proteins after separation were transferred to nitrocellulose membrane. The membrane was probed with MSC1 antibody, processed as described above and the protein bands were detected using ECL reagent.

Results

Vector construction, transfection and analysis of expressed MPR 46 protein Amplification of the goat MPR 46 cDNA was carried out using the primers as described in materials and methods. The amplified product corresponding to 840 bp in length (Fig. 1a lane 2) was cloned into the mammalian expression vector. The presence of insert in the expression vector was confirmed by restriction digestion

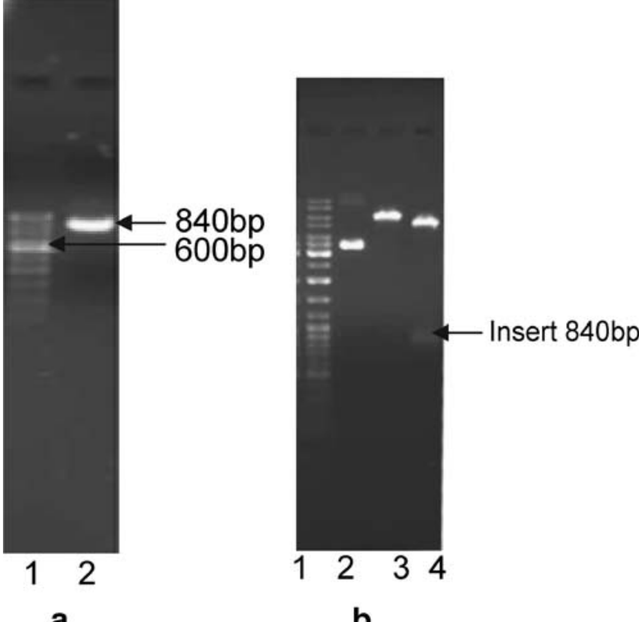

Fig. 1 0.8% agarose gel electrophoresis **a** Lane 1, 100 bp ladder, lane 2, specific amplification of 840 bp. **b** lane 1, mixed DNA ladder, (10,000–100 bp) lane 2, undigested goat 46 in pcDNA.6/V5-His A, lane 3, single digested goat 46 in pcDNA.6/V5-His A (with EcoRI), and lane 4, double digested goat 46 in pcDNA.6/V5-His A (with EcoRI and XhoI)

with EcoRI and XhoI. (Fig. 1b lane 4), sequence and reading frame was verified by automated DNA sequencing (data not shown). Transfections were made in mpr$^{(-/-)}$ MEF cells that missort up to 80% of soluble lysosomal enzymes to the medium due to the lack of MPR 46 and MPR 300 proteins. The deficiency results in abnormal size and number of intracellular storage lysosomes that cannot degrade ingested materials. In these cells, the missorting of lysosomal enzymes can be rescued by the expression of a receptor protein that re-establishes functional sorting [12].

The cell lysate from the mpr$^{(-/-)}$ MEF cells that were transfected with the expression vector containing goat MPR 46, and mock transfected cells were subjected to Western blot analysis using MSC1 antibody, and the blot was developed with BCIP/NBT substrate. The mock transfected cell lysate did not show any bands (Fig. 2 lane 1), but the lysate from goat MPR 46 transfected cells showed monomeric and dimeric bands of MPR 46 (Fig. 2 lane 2). Membrane fractions from the transfected cells were prepared and subjected to affinity chromatography on PM gel. Bound protein was specifically eluted with 5 mM M6P, and analyzed on SDS-PAGE (Fig. 3a). From the results it is evident that the putative receptor was found in the mannose 6-phosphate eluate and exhibited a molecular mass similar to the purified MPR 46 receptor from goat liver tissue, in addition to the monomeric form, a dimeric form of MPR 46 has been observed (Fig. 3a, lane 4). The oligomeric nature was observed both under non-reducing (Fig. 3b lane 2) and reducing conditions (Fig. 3b lane 3). It is known from our earlier studies that the chicken and human cell MPR 46 protein exhibit oligomeric nature. The authenticity of the receptor was further confirmed by immunoblotting using an MSC1 antibody wherein the purified goat liver MPR 46 protein served as a positive control (Fig. 3c lane 1 and 2) and 218 antiserum, respectively, (Fig. 3d). In the absence of divalent metal ions the receptor was completely recovered in the unbound fraction suggesting that PM gel binding ability depends on divalent metal ions (data not shown).

Localization, interaction of the lysosomal protease cathepsin D with expressed Goat MPR 46 protein The distribution of the expressed goat MPR 46 protein was detected by immunofluorescence using MSC1 and LAMP1 antibody (Fig. 4). The sorting function of the expressed protein to sort lysosomal protease cathepsin D to lysosomes in the goat MPR 46 transfected mpr$^{(-/-)}$ MEF cells was demonstrated by confocal microscopy using the cathepsin D antiserum (Fig. 5f). The lysosomes in the same cells were visualized using LAMP1 antibody (Fig. 5e). Merged image (Fig. 5g) of both the images (Figs. 5e and f) showed extensive co-localization of cathepsin D and LAMP1. The goat MPR 46 expressing MEF cells clearly improved intracellular steady state concentrations of cathepsin D as shown in Fig. 5f, which was missorted to the medium by the MEF cells that were deficient for both the MPRs as shown in Fig. 5b. The presence of cathepsin D in mpr$^{(-/-)}$ MEF secreted medium was detected by immunoblotting as shown in Fig. 6a. The interaction of expressed goat MPR 46 protein with cathepsin D was confirmed by far Western blotting as shown in Fig. 6b. The specific interaction was further confirmed by co-immunoprecipitation experiment with cathepsin D antiserum. Preimmune serum was used as a control (Fig. 6c lane 1). Mannose 6-phosphate dependent

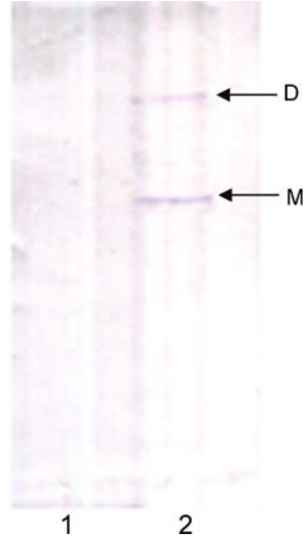

Fig. 2 Western blot analysis of the mock transfected and transfected cell lysates. Lane 1, mock transfected cell lysate, lane 2, transfected cell lysate, separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with MSC1 antibody (2 μg/ml). Both the monomeric (M) and dimeric (D) forms were observed

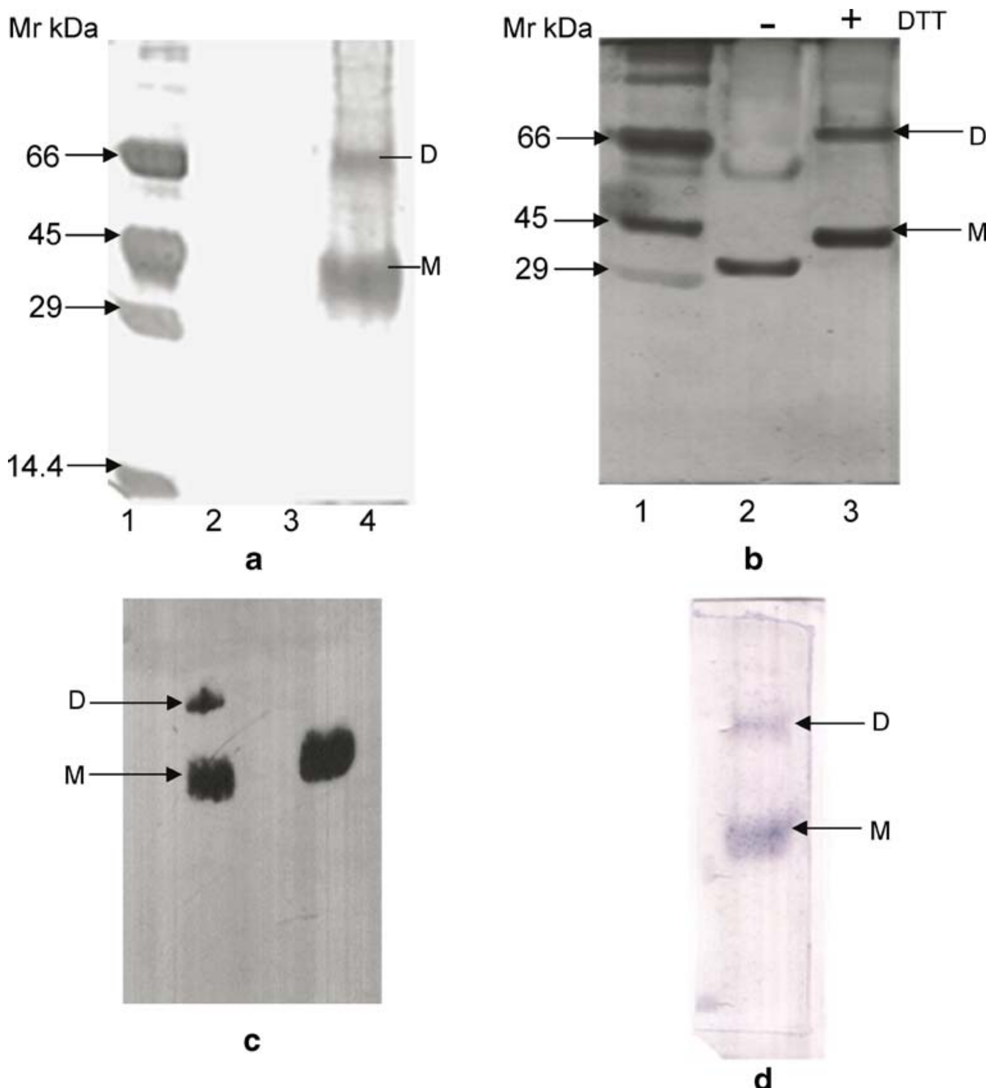

Fig. 3 Purification of expressed goat MPR 46 protein on PM gel. **a** Proteins were separated on 10% SDS-PAGE and silver stained. Lane 1, Molecular weight markers, lane 2, wash, lane 3, Glucose 6-phosphate eluate, lane 4, mannose 6-phosphate eluate showing monomeric (M) and dimeric (D) forms. **b** Lane 1, Molecular weight markers, lane 2, mannose 6-phosphate eluate of goat 46 from the PM Gel under non-reducing conditions, lane 3, mannose-6-phosphate eluate of goat 46 from the PM Gel under reducing conditions. **c** Western blot analysis of the mannose 6-phosphate eluate of goat 46 from the PM Gel, probed with MSC1 antibody. Lane 1, Mannose 6-phosphate eluate showing monomeric (M) and dimeric (D) forms. Lane 2, goat MPR 46 purified from liver tissue serving as a positive control. **d** Western blot analysis of the mannose-6-phosphate eluate of goat 46 from the PM Gel, Probed with 218 peptide specific antiserum

binding was also analysed (Fig. 6c lane 2). The specific identification of the goat MPR 46 monomeric and dimeric forms indicates the interaction of goat MPR 46 protein with the cathepsin D (Fig. 6c lane 3)

Discussion

Lysosomal biogenesis is important in many physiological and pathological processes. In mammals it has been shown that two distinct but homologous receptors designated as the MPR 300 and 46, mediate the transport of the lysosomal enzymes to lysosomes. Overexpressions of the CD-MPR in various cell systems have indicated that the CD-MPR could function in intracellular transport of lysosomal enzymes [17, 18]. Our laboratory first purified and characterized the goat MPR proteins and has also developed an ELISA method to quantify the receptors [19]. Furthermore to understand the structural similarities of the goat receptors to that of other known mammalian and non-mammalian receptors, we have also obtained a full-length clone for the goat MPR 46 and a partial cDNA clone for the goat MPR 300 protein [11]. Results of these experiments revealed that the goat receptor is homologous to the other known vertebrate receptors particularly the bovine receptor. Table 1 summarizes the homology of the goat receptor to the other known vertebrate receptors. The homology of the goat receptor is significant particularly with respect to the location of the arginine residue that is critical to the lysosomal enzyme binding, the sugar binding pocket as well as the metal binding regions. It is therefore of interest to express the goat MPR 46 in mpr$^{(-/-)}$ MEF cells to compare its properties to those reported previously for the bovine and human receptors and study its intracellular sorting of lysosomal enzymes and hence the present study was carried out to gain further insight into the biological function of the goat MPR 46 receptor. It is well established that the mammalian MPR 46 protein shows an absolute requirement

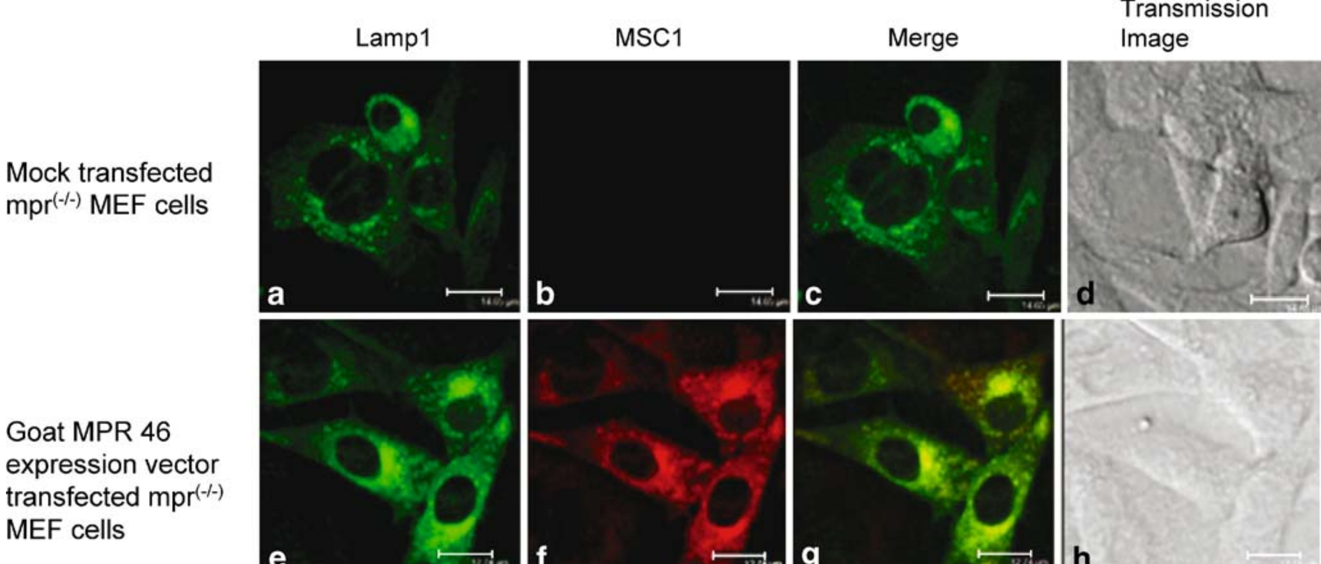

Fig. 4 Immunofluorescence with LAMP1 antibody in mpr$^{(-/-)}$ MEF cells (**a**) and in goat MPR 46 expressing cells (**e**). Immunofluorescence using MSC1 antibody in mpr$^{(-/-)}$ MEF cells (**b**) and in goat MPR 46 expressing cells (**f**). **c** merged image of (**a**) and (**b**), **g** merged image of (**e**) and (**f**). **d** and **h** are the corresponding transmission images

for divalent metal ions to bind on PM gel. Consistent with the earlier reports [20, 21] we have also found that the expressed goat MPR 46 protein binds to the PM gel only in the presence of divalent metal ions. The expressed protein in SDS-PAGE analysis exhibited a molecular mass of ~46 kDa, in addition to this monomeric form, the dimeric form of the receptor could also be detected as has been reported earlier for chicken and human MPR 46 protein [4, 22]. Both these forms were also recognized by the MSC1 antibody as well as the 218 antiserum used, which are known to recognize the oligomeric forms of the receptor [22]. The oligomeric nature was also supported by the results obtained in the SDS-PAGE analysis of the receptor in the presence and absence of reducing agent. From our result it is apparent that there is a distinct shift in the mobility of the receptor under reducing conditions compared to non-reducing

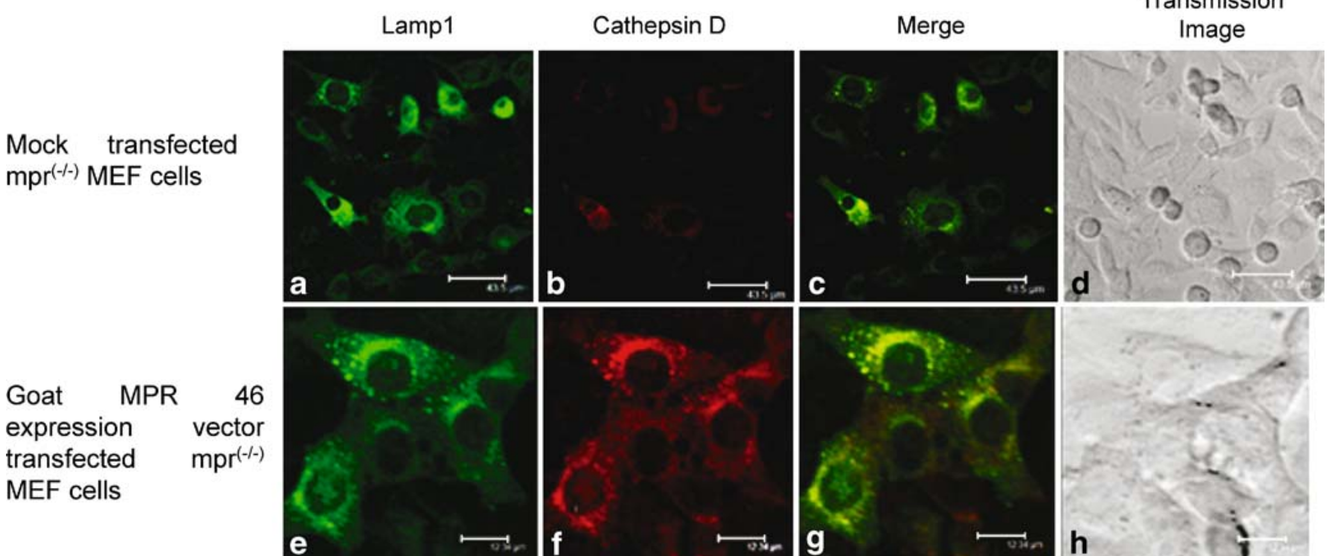

Fig. 5 Sub cellular distribution and localization of cathepsin D in goat MPR 46 expressing cells visualized using confocal microscopy. Immunofluorescence using LAMP1 antibody in mpr$^{(-/-)}$ MEF cells (**a**) and in goat MPR 46 expressing cells (**e**). Immunofluorescence using cathepsin D antiserum in mpr$^{(-/-)}$ MEF cells (**b**) and in goat MPR 46 expressing cells (**f**). **c** merged image of (**a**) and (**b**), **g** merged image of (**e**) and (**f**). **d** and **h** are the corresponding transmission images. Extensive co-localization of cathepsin D and LAMP1 is observed in cells expressing goat MPR 46

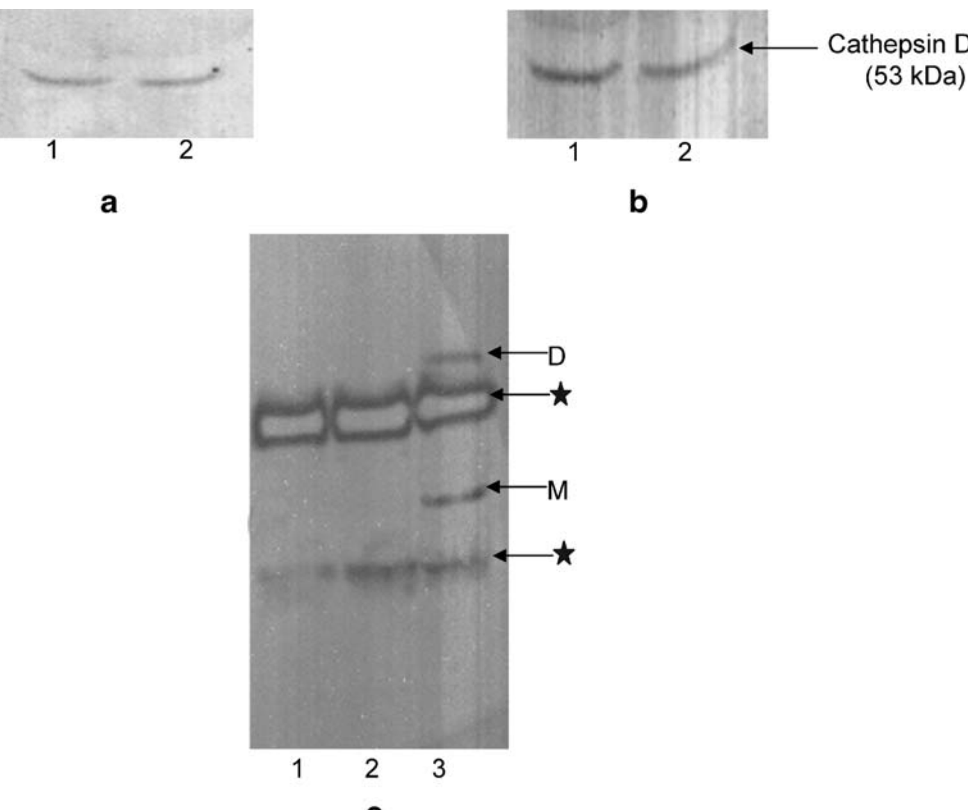

Fig. 6 **a** Western blot of mpr$^{(-/-)}$ MEF cells secreted medium probed with cathepsin D antiserum. **b** Far Western blot of mpr$^{(-/-)}$ MEF cells secreted medium was separated on 10% SDS-PAGE, transferred onto NC membrane and incubated with purified expressed goat MPR 46. Bound MPR 46 to cathepsin D was detected by using MSC1 Ab. Lanes 1 and 2 are duplicates of the proteins obtained from the mpr$^{(-/-)}$ MEF cells secreted medium. **c** Co-immunoprecipitation of the cell lysates prepared from mpr$^{(-/-)}$ MEF cells transfected with goat MPR 46 using cathepsin D antiserum (details given under methods). 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.★ Indicated in the figure shows the non-specific signal due to the IgG heavy chain and light chain, M and D represent the dimeric and monomeric forms of the receptor protein

conditions, apparently due to the opening up of the disulfide bridges. The expressed protein in the cells was observed by immunofluorescence using confocal microscopy. In a recent study we have demonstrated the expression of the Zebra fish MPR 46 protein in mpr$^{(-/-)}$ MEF cells [8]. With these studies it has been shown that the expressed Zebra fish MPR 46 sorts the cathepsin D. In order to verify that the expressed goat receptor protein is capable of

Table 1 Similarity of the various regions of the goat MPR 46 protein with other known vertebrate MPR proteins

Species	Size of mature protein (kDa)	CDNA clone size	Transmembrane domain	Ligand binding region sequences and / conserved residues in cytoplasmic tail (italics)	Potential glycolsylation sites / conservation of cysteins (italics)	Size of RNA transcript (kb)
HUM	46	0.837	ILLVTFASLVAVYVVGGFLY	DNHCGKEQ**RR**AV ***YRGV*** / *DXXLL*	conserved/yes	2.3
BOV	46	0.840	ILLVTFASLVAVYVVGGFLY	DNHCGREQ**RR**AV ***YRGV*** / *DXXLL*	conserved/yes	2.3
MOU	46	0.837	ILLVTFASLVAVYVVGGFLY	DNHCGKEQ**RR**AV ***YRGV*** / *DXXLL*	conserved/yes	2.3
GOAT	46	0.840	ILLVTFASLVAVYVVGGFLY	DNHCGREQ**RR**AV ***YRGV*** / *DXXLL*	conserved/yes	2.3
CHI	46	Partial	ILLVTFASLVAVYVVGGFLY	– ***YRGV*** / *DXXLL*	conserved/yes	3.3
XEN	46	Partial	ILLVTFASLVAVYVVGGFLY	DTHCNNEA**R**KAM ***YRGV*** / *DXXLL*	conserved/yes	not known
KIL	46	Partial	ILLVTFASLVAVYVVGGFLY	DSHCQKEA**R**KAll ***YRGV*** / *DXXLL*	conserved/yes	not known
ZEB	38	0.84	ILLVTFASLVAVYVVGGFLY	DSHCSSEE**R**KAM ***YRGV*** / *DXXLL*	conserved/yes	not known

HUM human, *BOV* bovine, *MOU* mouse, *CHI* chicken, *XEN* xenopus, *KIL* killi fish, *ZEB* zebrafish, *R* : Conserved arginine residue in MPR 46 responsible for ligand binding. Conserved sequences in the cytoplasmic tail : *bold italics*: tyrosine dependent endocytosis motif : ***YRGV***., Conserved acidic diluecine motif in italics.

binding to the cathepsin D, an antiserum to cathepsin D was used in immunofluorescence studies. From our results it is clear that the expressed goat MPR 46 protein is capable of binding and sorting newly synthesized lysosomal enzymes was suggestive by the increase in the localization of the cathepsin D in the expressing cells compared to the mpr$^{(-/-)}$ MEF cells (compare panel 5b and 5f). This was also supported by the far Western blot analysis and co-immunoprecipitation, which confirmed the specific interaction of goat MPR 46 to the cathepsin D which is dependent upon M6P.

In summary our results clearly demonstrate the following (1) the full length goat MPR 46 cDNA can be expressed in mpr$^{(-/-)}$ MEF cells (2) the expressed protein can be affinity purified on a PM gel in the presence of divalent metal ions and exhibits typical biochemical and immunological characteristics similar to the well characterized mammalian receptors as evidenced by our results (3) the distribution of the expressed protein was observed by immunofluorescence (4) further evidence that the expressed protein is sorting lysosomal enzymes came from immunofluorescence studies using the cathepsin D (a known lysosomal enzyme) antiserum.

Our recent studies have clearly established that the putative MPR 46 proteins are highly conserved throughout vertebrates with similar ligand binding properties [8]. In view of the structural similarities the goat receptor exhibits with other well characterized MPR 46 proteins, and the results of the present study establishing the functional property of the goat receptor viz; PM gel binding, cation dependence to bind on PM gel, as well as specific sorting of cathepsin D provide evidence that the goat protein studied here is the putative lysosomal enzyme targeting receptor. In addition to these, it would be interesting to analyze what other lysosomal enzymes can the goat MPR 46 receptor bind and sort.

Our future studies focus on large scale purification of the goat MPR 46 protein and attempt crystallization of the protein. Furthermore, prepare a goat MPR 46 receptor affigel and use the same to isolate, purify and characterize lysosomal enzymes that can bind the MPR 46 receptor from mammals, birds and fish. These would pave way to understand the biogenesis of lysosomes in the non-mammalian vertebrates such as the birds and fish, which is poorly understood.

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