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Role of cation independent mannose 6-phosphate receptor protein in sorting and intracellular trafficking of lysosomal enzymes in chicken embryonic fibroblast (CEF) cells

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Abstract Delivery of soluble lysosomal proteins to the lysosomes is dependent primarily on the mannose 6-phosphate receptors (MPRs) in mammals. However, in non-mammalian cells the role of MPR300 in sorting and trafficking of acid hydrolases to lysosomes is not fully understood till now. In this paper, we tested the role of MPR300 in sorting and trafficking of lysosomal enzymes in CEF cells using a small interfering RNA (siRNA) technology. Inactivation of MPR300 resulted in the secretion of large amounts of newly synthesized hydrolases into the medium and also inhibited the endocytosis of mannose 6-phospharylated ligands. Knockdown of MPR300 in CEF cells results in missorting of fucosidase and arylsulfatse A enzymes into the medium. The results indicated that the MPR300 in CEF cells plays a key role in sorting and trafficking of these soluble hydrolases.

Keywords Chicken embryonic fibroblasts (CEF) · MPR300 · Silencing · siRNA · Mannose 6-phosphate · Lysosomal enzymes sorting · Endocytosis · LAMP1 · RNAi

Introduction

Mammalian cells contain two distinct mannose 6-phosphate (M6P) binding proteins which are transmembrane glycoproteins: the 300-kDa cation-independent M6P/IGF-II receptor (M6P/IGF-IIR or MPR300) and the 46-kDa cation-dependent M6P receptor (MPR46). While the former appears to be implicated both in targeting of newly synthesized lysosomal enzymes and endocytosis of extra cellular lysosomal proteins, the latter has been shown so far only to participate in intracellular trafficking of lysosomal enzymes to lysosomes [1]. Lysosomal enzymes are synthesized as latent proenzymes in the rough endoplasmic reticulum (ER) [2]. They are co-translationally glycosylated in the rough ER on some asparagine residues by addition of N-linked oligosaccharides from a lipid dolichol intermediate [3]. They are then transferred to the *cis*-Golgi. In this compartment, owing to the sequential action of two enzymes phosphomannosyl residues are exposed on the oligosaccharide moiety, resulting in the formation of the mannose 6-phosphate (M6P) recognition marker. The so formed phosphomonoester residues are specific to lysosomal hydrolases and allow these proteins to be sorted from other proteins, through their strong interaction with M6P receptors (MPRs) in the *trans*-Golgi network [4]. These receptor–ligand complexes are transported to the *endo* lysosomal compartment where the acidic pH leads to the dissociation of lysosomal enzymes from the MPRs. The receptors then recycle back to the Golgi apparatus; the major part of enzymes that follows this targeting pathway reaches the lysosomes. However, a small proportion of phosphorylated lysosomal polypeptides are escaped from this pathway and released from cells. These secreted enzymes can interact with MPR300 present on the plasma membrane and can therefore be internalized and targeted to lysosomes. Besides acid hydrolases, the MPR300 can serve as a receptor for other mannose 6-phosphorylated ligands such as the precursor form of transforming growth factor

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β1 (TGF-β1), proliferin, thyroglobulin and granzyme B. This receptor has been shown to interact with non-mannose 6-phosphorylated ligands such as the, plasminogen and urokinase receptor, IGF-II, retinoic acid, and Heparanase [5, 6].

In the last few years the major thrust of research in our laboratory has been to first identify the mammalian homologues of the MPR proteins, and sequence these proteins to understand their structure, function and physiological significance, to eventually understand and establish their phylogenetic origin. Our studies extended from mammals to invertebrates [7–14]. The chicken MPR300 was sequenced and its function determined in COS 7 cells its exact physiological role in sorting of lysosomal enzymes to lysosomes have not yet been defined. Sequencing study revealed that a 8767-bp full-length chicken cDNA encodes a protein of 2470 amino acids that includes a putative signal sequence, an extracytoplasmic domain consisting of 15 homologous repeat sequences, a 23-residue transmembrane sequence, and a 161-residue cytoplasmic sequence. Overall, it shows 60% sequence identity with human and bovine MPR300 proteins and all, but two of 122 cysteine residues are conserved [15]. Matzner *et al.*, also provided evidence for the two MPR proteins in CEF cells as well as in different tissues in chicken. In a recent study we have cloned the full length gene for the chicken MPR46 protein and showed it to be structurally related to other known vertebrate MPR46 proteins [16]. In mammals analysis of primary fibroblasts lacking either the MPR46 or the MPR300 or both MPRs has indicated that one MPR is not sufficient to carry out transport of the bulk of the newly synthesized lysosomal enzymes [17]. In order to precisely understand the role of chicken MPR300 in intracellular sorting and trafficking of lysosomal enzymes *in vivo,* we used chicken embryonic fibroblasts cells and carried out a detailed analysis of the sorting capacity of MPR300. We have employed the recently developed siRNA (small interfering RNA)-dependent gene-specific silencing technology, to which we have introduced several modifications. RNA interference (RNAi) is a phenomenon describing double-stranded (ds) RNA-dependent gene-specific post-transcriptional silencing, which can potentially result in the inhibition of specific protein synthesis. Very recently, it has been reported that in mammalian cells, 21- or 22-nucleotide (nt) RNA sequences with 2-nt 3′ overhangs (siRNAs), exhibit an RNAi effect [18, 19]. As a result, siRNAs have become powerful tools for studying function of essentially any gene whose sequence is known. The present study provides new insights about the functional significance and physiological role of MPR300 protein in intracellular sorting of lysosomal enzymes in CEF cells.

Materials and methods

Antibodies and reagents

Affinity purified antibodies to the goat MPR300 protein was available in the laboratory. DMEM, trypsin-EDTA, penicillin-streptomycin, Blasticidin S, were purchased from Sigma. Cy3 coupled anti-mouse and anti-goat IgG were purchased from Chemcon, India. FBS was purchased from JRH Bioscience and psilencer 1.0U6 was purchased from Ambion. ^{32}P dCTP was from BRIT, India, EcoRI and ApaI were purchased from MBI Fermentas, India. Chemiluminescence reagent was from Pierce chemical company. MPR300 localization in CEF cells was detected by affinity purified antibodies to the goat MPR300 protein obtained as described earlier [20]. Mouse lysosome-associated membrane protein (LAMP) 1 was detected using monoclonal anti-mouse LAMP1 antibodies. Immunoprecipitation of α- fucosidase was done with antibodies against *unio* α- fucosidase enzyme that were raised in our laboratory as described [21]. Arylsulfatse A was detected using a rabbit antiserum [22].

Designing of MPR300 siRNA

Construction of plasmids that contain DNA templates for the synthesis of siRNAs was done as described in [23]. The theoretical RNAi target sites of chicken MPR300 were searched via an online software (http://www.ambion.com/techlib/misc/siRNA_finder.html) based on its cDNA sequence (GenBank accession No. NM_204970.1). Briefly, siRNA target sites were typically chosen by scanning an mRNA (National Center for Biotechnology Information access number: NM_204970.1). Sequence for AA dinculeotides, recording the 19 nucleotides immediately downstream of the AA, and then comparing the potential siRNA target sequences were analyzed by BLAST research to ensure that they did not have significant sequence homology with other genes. Then the chicken MPR300 sequence was inserted into double-strand hairpin siRNA insert frame (designed by Ambion): strand 1, 5′-N (19) (sense) TTCAAGAGA N (19) (antisense) TTTTTT-3′ (53 bp); strand 2, 3′-CCGG N (19) (antisense) AAGTTCTCT N (19) (sense) AAAAAATTAA-5′ (61 bp); two strands of oligonucleotides were separately synthesized (Sigma-Aldrich). The MPR300 hairpin siRNA insert was made by annealing the two oligonucleotide strands together. The annealing reaction was performed by mixing 2μl of each oligonucleotide with 46μl of annealing buffer (100 mM K-acetate, 30 mM HEPES-KOH pH 7.4, and 2 mM Mg-acetate) and incubating the mixture at 90°C for 3 min, followed by 37°C for 1 h. The vector pSilencer 1.0-U6 (Ambion) was digested with EcoRI and ApaI (MBI, Fermentas), and the hairpin siRNA was ligated into the

vector following the manufacturer's instructions. The sequence of the insert was confirmed by automated sequencing and by analyzing the fragments generated from digestion with HindIII. These vectors produce a short hairpin RNA with the linker sequence (TTCAAGAGA) that forms a looped structure, the linker being processed by Dicer, to generate a MPR300 specific siRNA. We designed four different types of DNA templates for synthesizing MPR300 specific siRNA, the following templates were used for the silencing of MPR300 in CEF cells.

CHK-NSK-MPR300 FOR pSilencer-1.0-U6—Ampicillin

Sense strand spacer anti-sense strand Terminal

5'-CAATATTGCGCCTTAAGTG**TTCAAGAGA**CACTTAAGGCGCAATATTGTTTTTT-3',

5'-AATTAAAAAACAATATTGCGCCTTAAGTG**TCTCTTGAA**CACTTAAGGCGCAATATTGGGCC-3'

Cell culture, transfection and selection

Chicken embryonic fibroblast cells (CEF cells) were maintained at 37°C (5% CO_2) in DMEM medium as described in [24]. CEF cells were grown to about 60 to 80% confluency (in 6-cm—diameter Petri dishes) and then cotransfected with 10 μg of pSilencer 1.0-U6 and 1 μg of pEF6/V5-His TOPO vector (Invitrogen; the plasmid containing the blasticidin-S-selectable marker gene) by calcium precipitation method [25]. Transfected cells were selected for blasticidin resistance (10 μg /mL). The effect of siRNA was assessed at both RNA and protein levels by RT-PCR, immunoprecipitation, immunofluorescence and western bot. Analysis in all experiments was done after 48 h of post-transfection.

RT-PCR

Total RNA was extracted from CEF cells transfected with MPR300 siRNA using Qiagen RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Contaminating genomic DNA was removed with RNase-free DNase I (Qiagen). RT-PCR was performed with MBI-Fermentas RT-PCR kit according to the manufacturer's instructions. The primers used in the PCR are as follows: (chicken MPR300, forward) 5′-GGCTGCTCTGTGACTGATGA-3′ (chicken MPR300, reverse) 5′- TTCCAAAGGATGACACACCA-3′ (β-actin, forward) 5′-TAAAACGCAGCTCAGTAACAGT CGG-3′; (β-actin, reverse) 5′-TGCAATCCTGTGGCATC CATGAAAC-3′.

Metabolic labeling of cells and immunoprecipitation

Metabolic labeling with [^{35}S] methionine and immunoprecipitation was done as described [10].

Immunofluorescence using confocal microscopy

Knockdown of MPR300 gene expression in CEF cells by specific siRNAs was observed by confocal microscopy using goat MPR300 antibody. After transfection with pSilencer 1.0-U6 [with DNA templates which can synthesize specific siRNA], 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 with PBS and blocked with 5% BSA in PBS for 30 min at room temperature. The cells were then incubated with LAMP1 antibody (1:100 dilutions) and with MPR300 antibody 2 μg/ml in 0.1% BSA in PBS for 1 h at room temperature. The cells were washed 5–6 times with PBS, blocked with BSA as above, and incubated with fluorescent tagged anti-rabbit IgG-Cy3 and anti-mouse IgG-FITC (Chemicon, India) for 1 h at room temperature. Finally the cells were washed extensively 5–6 times with PBS and observed under confocal microscope for localization of MPR300.

SDS-PAGE and Western blotting

Lysates from siRNA transfected cells were prepared and analysed by SDS-PAGE and Western blotting as described in [26].

Lysosomal enzyme assays

Lysosomal enzyme activities in the secreted medium as well as in the soluble fraction were determined as described previously using the corresponding p-nitro-phenyl derivatised chromogenic substrates [17, 27].

Chromatography on MPR46 and MPR300 affinity gel

MPR46 and MPR300 proteins were purified from goat liver as described [20] and coupled to Affi-gel 10 (Bio-Rad) according to the manufacturer's instructions. To characterize the level of M6P containing polypeptides intracellular and those secreted out into the medium, the control cells and MPR300 silenced cells, were metabolically labeled with [^{35}S] methionine, after labeling the secreted media and soluble extract were subjected to MPR affinity chromatography as described [28]. 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; further characterized by SDS-PAGE (10%) and fluorography.

Endocytosis of M6P-containing [^{35}S] polypeptides

The labeled [^{35}S-methionine)] M6P-containing polypeptides were purified on receptor-affinity column containing a mixture of both MPRs. The M6P eluates from this column were concentrated by acetone precipitation at −80°C and dialyzed against DMEM. siRNA treated and control cells were incubated for up to 30 min with 0.5 ml DMEM containing 50,000 cpm of purified M6P-containing [^{35}S] polypeptides. After the incubation, cells were washed and lysed as described above and after centrifugation for 20 min at 100×g, the supernatant and the pellet were counted in a scintillation counter.

Results

Effect of MPR300 inactivation on lysosomal trafficking

Selection of siRNA transfectants CEF cells were transfected with pSilencer 1.0-U6 generating short hairpin siRNA and co-transfected with pEF6/V5-His TOPO expression vector carrying selectable blasticidin S marker as described in the materials and methods section. Transfected cells were selected for blasticidin S resistance and characterized by the presence of the insert in the genomic DNA (data not shown). Using this approach we have created stable CEF cells transfected with the pSilencer 1.0 U6 vector that contained a DNA template oligonucleotide (CHK-NSK-MPR300), which can synthesize MPR300 gene specific siRNA. The sequence included two complementary regions that permit the refolding of the transcribed RNA into a double-stranded region and a connecting stem (shRNA). The production of shRNA triggers dicer cleavage and leads to the silencing of MPR300 mRNA. To verify the expression of the shRNA and the knockdown of MPR300, RT-PCR, and immunoprecipitation were conducted as for the *in vitro* siRNA experiments. Both analyses showed that the levels MPR300 expression were decreased. These results were further confirmed by immunofluorescence and confocal microscopy. First, we isolated total RNA from transfected and mock transfected (control) cells using a Qiagen's RNeasy kit (Qiagen), and performed semi quantitative RT-PCR. Our results showed that the designed siRNA successfully inhibited the expression of MPR300 mRNA in CEF cells. To verify that transfection did not inhibit the expression of unrelated proteins we examined the level of β-actin mRNA. β-Actin is expressed constitutively in most cells. The levels of β-actin transcripts were measured by RT-PCR in normalized samples of transfected or mock transfected cells. Our results showed that β-actin transcripts were not affected by the siRNA treatment (Fig. 1A). Cells transfected with silencing vector were analyzed by immunoprecipitation to observe the expression level of MPR300 protein. The results showed an efficient down-regulation of the MPR300 in chicken embryonic fibroblast cell lines (Fig. 1B). To verify the RT-PCR and immunoprecipitation results, further immunofluorescence or confocal microscopy was done for confirmation of knock-down of MPR300. Confocal microscopy demonstrated that MPR300 siRNA abolished the immunostaining of the anti-MPR300 antibodies (Fig. 1C, lower panel).

Western blot and quantitative analysis of MPR300 silencing

The efficiency of silencing was quantitatively determined by western blot analysis was used to compare the knockdown of MPR300 in silencing cells at different times (0, 2, 4, 6,8,10 and 12 h) after 48 h of post-transfection with pSilencer 1.0U6-CHK-NSK-MPR300. Figure 2 shows that silencing of MPR300 was observed at 60 h of post transfection. These results confirm that the siRNA has efficiently induced MPR300 silencing at the post-transcriptional level and at a time course.

Sorting of phosphorylated ligands

In order to investigate the sorting of the phosphatylated ligands as a function of MPR300.The fibroblasts knockdown for MPR300 were first tested for their ability to transport lysosomal enzymes to lysosomes. The different cells were pulsed with [^{35}S] methionine, chased and the labelled phosphorylated ligands secreted in the culture medium were purified on MPR affinity columns and quantitated. On average, MPR300 knockdown fibroblasts secreted about ten times more phosphorylated ligands when compared with immortalized fibroblasts expressing physiological levels of the two MPRs (5%) taken as controls.

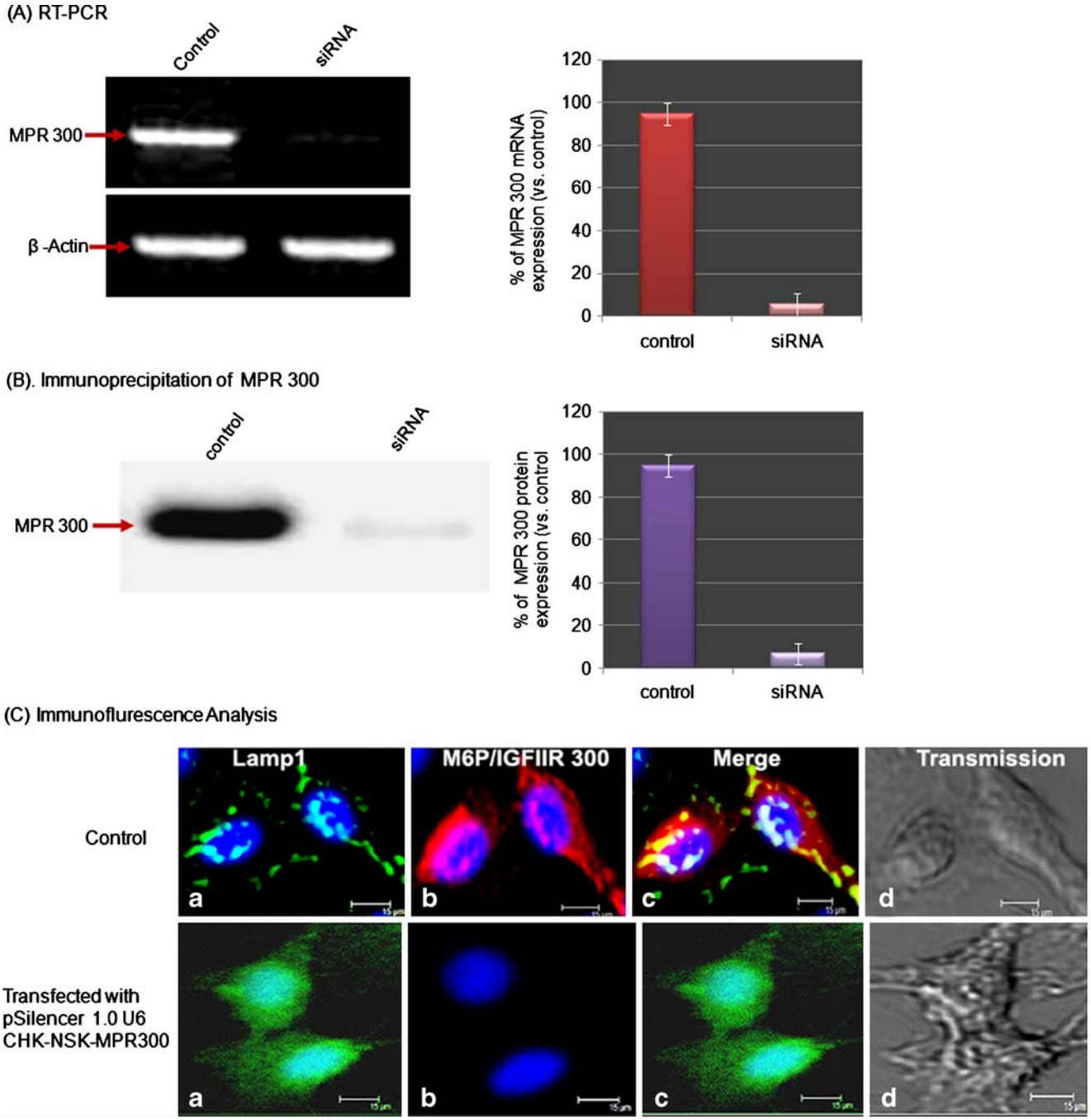

Fig. 1 **A** RT-PCR. To confirm the effect of MPR300 siRNA, total RNA extracted from CEF cells transfected with silencing vector was analyzed by semi-quantitative RT-PCR. β-Actin was used as internal loading control. **B** siRNA mediated down-regulation of MPR300 in CEF cells. MPR300 specific siRNA was synthesized by transfecting the psilencer-CHK-MPR300-siRNA. The cells were incubated for 56 h. The down regulation of MPR300 was analyzed by Immunoprecipitation analysis. Lane 1 control, lane 2 is siRNA treated cells. **C** RNAi mediated MPR300 silencing in CEF cells. To selectively silence MPR300, CEF cells were stably transfected with either psilencer 1.0 U6 CHK-MPR300 siRNA and selected with blasticidin S. Cells were grown on cover slips and the immunofluorescence staining of MPR300 in the clones expressing MPR300 siRNA was done as described in materials and methods. Nuclei were visualized using DAPI. CEF cell transfected with MPR300 small interference RNA (siRNA) vector abolished the perinuclear staining of MPR300 antibody (Cy3, red). The overlay shows green fluorescence only, produced by the anti-LAMP1 antibody (a lysosomal membrane marker protein). Cell transfected with vector alone (control) immunostained with anti-LAMP1 (green) and anti-MPR300 (red) antibodies. (*Bars.15 μm*)

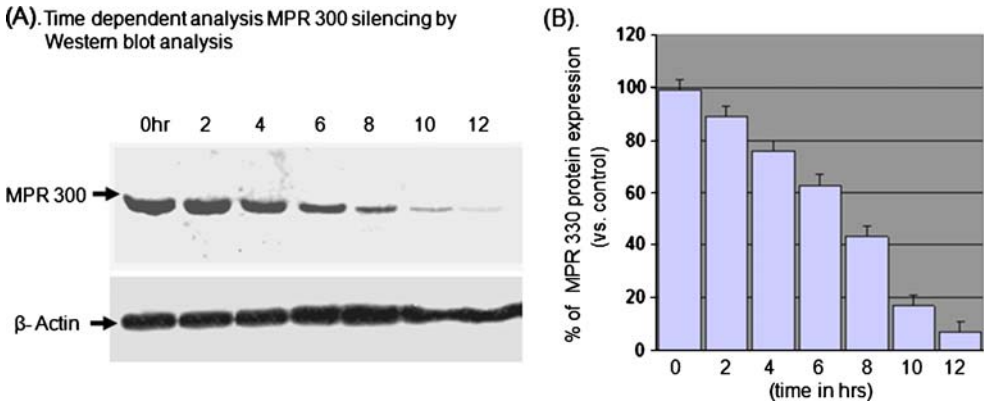

Fig. 2 Western blot analysis of MPR300 protein. CEF cells were transfected with MPR300 siRNA. Forty-eight hours post-transfection, the cell lysates were analyzed by Western blotting with anti-MPR300, and β-actin (loading control) antibodies. Right panel shows the percent of expression level of MPR300 as quantified by Image J software. Mean value±S.E.M. (n=3)

(Fig. 3A). Figure 3B shows that upon decreasing in expression of the MPR300 in siRNA treated CEF cells increase the secretion of phospharylated ligands into the culture medium.

Effect of down-regulation of MPR300 on lysosomal hydrolases sorting to lysosomes

The consequence of silencing the MPR300 in CEF cells was observed by analyzing the intracellular and extracellular activities of six soluble lysosomal enzymes known to be transported to lysosomes via MPRs in mammals (β-hexosaminidase, arysulfatse A, α-fucosidase, α-mannosidase, α-galactosidase and cathepsin D whose concentrations were measured after 60 h of post-transfection. Analysis was performed in triplicate average mean values are presented here. Silencing resulted in a reduction of six soluble lysosomal enzymes activities in soluble fraction in between 7–10% when compared to the control (Fig. 3C). Under the same conditions the fraction of activities of these enzymes that accumulated by secretions into the medium increased upto 80–93% in MPR300 silenced fibroblasts when compared with control (Fig. 3D). These results suggest that the loss of MPR300 by siRNA results in missorting of lysosomal enzymes into the culture medium. CEF cells silenced for MPR300 was tested for their ability to retain M6P containing ligands intracellularly. In this experiment we examined the levels of the M6P containing polypeptides intracellular as well as those secreted out into the culture medium in control and silenced cells. After metabolic labeling of the cells with [^{35}S] methionine, the secretions and soluble extracts were collected and passed over MPR300/46 receptor-affinity column. After extensive washing, bound ligands were eluted with 5 mM Man-6-P; fixed concentrations of eluted fractions were characterized by SDS-PAGE and fluorography. The results shown in Fig. 3E and F demonstrates that the number of M6P containing polypeptides eluted from the receptor gel was very high in soluble fractions of control cells (Fig. 3E, lanes 1) and in similar conditions the number of M6P containing polypeptides were low in the soluble fraction of the MPR300 silenced cells (Fig. 3E, lane 2) as these cells secrete a large amount of Man 6-phosphorylated ligands into the culture medium, when compared to control (Fig. 3F, lanes 1 and 2). From the previous studies, it is known that MPR300 expressed at cell membrane mediates the endocytosis of M6P containing ligands; here we tested the effect of siRNA on the endocytosis functions of MPR300. From the graph shown in Fig. 3G reveals that internalization of purified Man 6-phospharylated ligands was inhibited in siRNA treated cells silenced for MPR300 when compared with controls. From these results, we conclude that fibroblasts lacking MPR300 expression missort a significant fraction of their newly synthesized lysosomal enzymes and inhibit the endocytosis of Man6-phospharylated ligands, indicating that in embryonic fibroblasts the MPR300 is responsible for most of the intracellular transport and endocytosis of soluble lysosomal enzymes.

Determination of missorting of newly synthesized α-fucosidase in MPR300 silenced fibroblasts

To further study the effect of silencing MPR300 in CEF cells, we also monitored the sorting of the specific enzyme α-fucosidase by immunoprecipitation experiments. Fibroblasts after 60 h of post transfection were metabolically labeled with [^{35}S] methionine for 8 h. After labeling, α-fucosidase was immunoprecipitated by taking fixed concentration of the soluble fraction and secretions from control and silenced cells. From Fig. 4A in the control cells 98.6% of the newly synthesized enzyme retained in soluble fraction and only 0.4% of the labeled fucosidase was recovered in the secretions, where as in MPR300 silenced fibroblasts (siRNA treated), the percentages were 20.8% and 79.2% respectively. This clearly demonstrates that the bulk of newly synthesized enzyme is secreted into

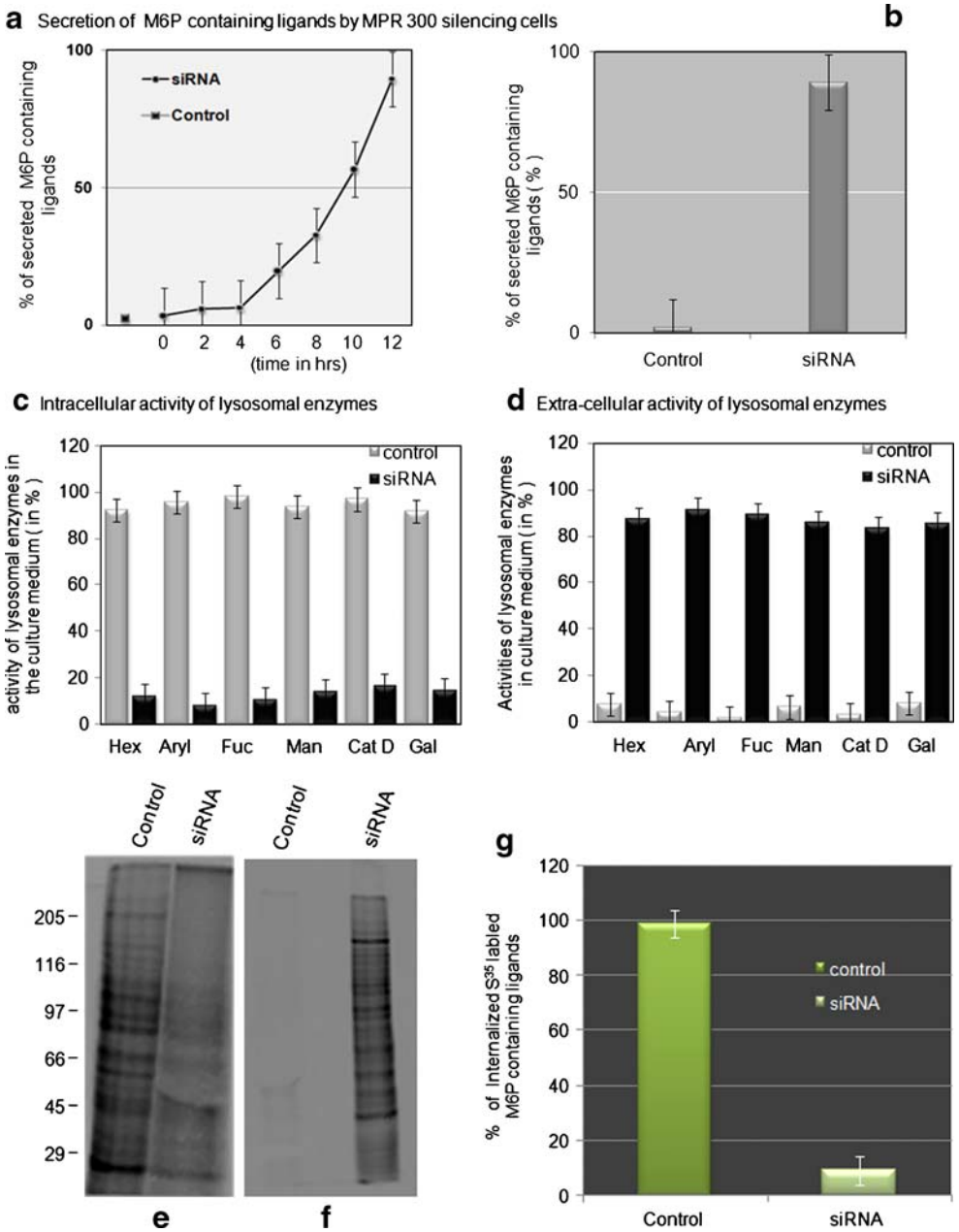

Fig. 3 Secretion of phosphorylated ligands by MPR300 silencing cells. **A** immortalized wild type fibroblasts taken as positive control. Cells were labeled with [^{35}S] methionine and chased. The secreted lysosomal enzymes were purified on MPR affinity columns and quantified. The values are expressed as means of two independent experiments. **B** Control and MPR300 silenced cells were labeled with [^{35}S] methionine and chased as described in methods. The secreted lysosomal enzymes were purified on MPR affinity columns and quantified. **C** Intracellular activity of lysosomal hydrolases in control and MPR-silenced chicken embryonic fibroblasts cells. **D** Activity of lysosomal hydrolases in secretions of control and MPR300 silenced CEF cells. The activity of lysosomal enzymes was determined in the secreted culture media and the soluble fraction of CEF cells. Fractions were collected after a 60 h culturing period as described under "materials and methods." The assays were done in duplicate; mean values are given. **E** Binding of Man-6-P-containing polypeptides in the soluble fraction as well as secretions of control and MPR-deficient CEF cells to immobilized MPR46 or MPR300 receptor column. Soluble fraction and secretions of metabolically labeled fibroblasts were passed over a MPR46/300-Affi-Gel column, at pH 6.5. The material bound to the column eluted with 5 mM M6P. Aliquotes of eluted sample was separated and analyzed by SDS-PAGE. Lanes 1 and 2 in figure **E** and **F** are samples from control and MPR300 silenced cells respectively. **G** Effect of MPR300 gene kockdown on endocytosis of M6P-containing polypeptides. The [^{35}S] M6P-containing polypeptides were isolated from secretions of siRNA treated CEF cells by affinity chromatography on MPR 46/300 Affigel 10 and incubated for up to 30 min with control and MPR300 silenced cells. Endocytosis function of MPR300 is indicated by the percentage of internalized man-phosphorylated ligands

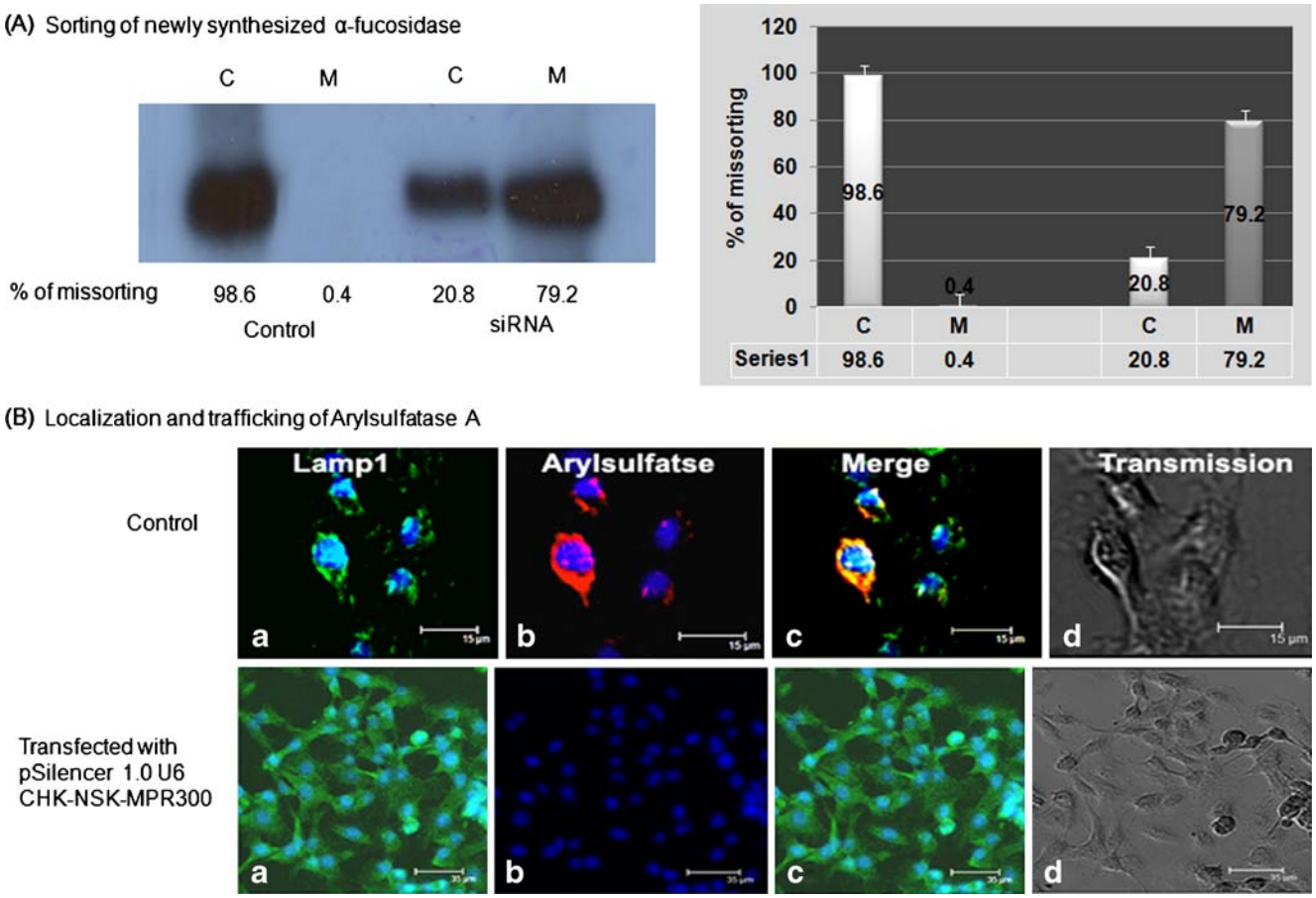

Fig. 4 **A** Effect of gene silencing of MPR300 on intracellular sorting of α-fucosidase in CEF cells. Immunoprecipitation of α-fucosidase. Equal amounts of protein were taken in each lane. The cells were labeled with [^{35}S] methionine for 1 h and then chased as indicated. α-fucosidase was immunoprecipitated from the cells (**C**) and culture medium (**M**). The bands were quantitated by densitometry. **B** Intracellular localization of ASA in control fibroblast cells and siRNA treated cells. Control fibroblast cells (upper panel) and siRNA treated cells (lower panel) were transfected with psilencer-CHK-MPR300-siRNA and stained with anti-ASA antibodies followed by Cy3 conjugated goat anti-rabbit IgG. The lysosomes of control fibroblast cells (A, upper panel) and siRNA treated cells (E, lower panel) were stained with anti- LAMP I antibody followed by FITC conjugated goat anti-mouse IgG. (C) is the merged image of (A) and (B). (G) Is the merged image of (E) and (F). Note that the intensity of the granular immunostaining for ASA in siRNA treated cells is decreased (red). (Scale Bar is *15- μm*)

the culture medium due to the silencing of the sorting receptor MPR300.

MPR300 regulates endogenous arylsulfatse A trafficking

After confirming the inhibitory effect of the MPR300 siRNA we tested the hypothesis that MPR300 is the sorting and trafficking receptor for arylsulfatase A. We predicted that knocking down MPR300 would affect the localization of Arylsulfatse A. To assess this prediction we immunostained CEF cells treated with MPR300 siRNA with anti-arylsulfatase A and anti-LAMP1. Therefore, cargo that requires MPR300 to be sorted to the lysosomes should be absent from or exhibit reduced immunostaining in the punctate structures for arylsulfatse A. Conversely, cargo that did not depend on MPR300 should continue to be visible arylsulfatse A in punctate structures. As predicted, arylsulfatse A was absent from punctate structures of siRNA treated cells (Fig. 4B, lower panel), these confirming a role of MPR300 in the trafficking of lysosomal enzymes to the lysosomal compartment.

Discussion

M6P-dependent transport of soluble lysosomal enzymes is a crucial step in the biogenesis of lysosomes. Newly synthesized lysosomal enzymes are carried to the lysosomes by vesicular transport from the endoplasmic reticulum, through the Golgi complex and endosomes. Initial transport steps are shared with proteins of the secretory pathway and apparently do not require specific signals. At the *trans* face of the Golgi complex, soluble lysosomal

enzymes bind M6P receptors by their M6P-recognition signal and are subsequently transported via clathrin-coated vesicles to late endosomes (also termed prelysosomes) where the enzyme release is triggered by the acidic interior [2]. The enzymes are then transported to the lysosomes by capillary movement and M6P receptors are either targeted to the cell surface or carried back to the Golgi complex [1]. In higher eukaryotes, the transport of soluble lysosomal enzymes involves the recognition of their mannose 6-phosphate signal by two receptors: the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (CI-MPR) and the cation-dependent mannose 6-phosphate receptor (CD-MPR). However, several lines of evidence suggest that the M6P/IGFII receptor is more efficient than the CD-M6P receptor in determining the intracellular sorting of newly synthesized lysosomal enzymes (Hille-Rehfeld, 1995). Other studies using gene-targeting technology defined the *in vivo* function of the MPRs. Mutant mice lacking the CD-MPR are viable and apparently have normal steady state levels of lysosomal enzymes [27, 29]. Mutant mice lacking the CI-MPR accumulate high levels of IGF-II and usually die prenatally [30–32]. This is directly attributed to over-stimulation by IGF-II, as CI-MPR-deficient mutants are completely viable in an IGF-II-deficient background, with the double mutants having a dwarf phenotype similar to that of the single IGF-II-deficient mutants. Despite the viability of the single MPR-deficient mice, the importance of the MPRs in lysosomal enzyme targeting *in vivo* was indicated by the finding that triple mutants lacking the CD-MPR, CI-MPR and IGF-II are not viable [31]. However, primary fibroblasts from embryos lacking both MPRs are almost totally impaired in intracellular lysosomal enzyme sorting and, as a consequence, accumulate undigested material in their endosomes/lysosomes [17]. This phenotype, similar to that of I-cell fibroblasts characterized by a lack of synthesis of the Man-6-P signal on lysosomal enzymes, clearly illustrates the importance of the two MPRs as major components of the Man-6-P-dependent targeting system in fibroblasts. Our laboratory has isolated and purified the MPR proteins from mammals, different non-mammalian vertebrates and invertebrates, and showed that they exhibit similar biochemical and immunological properties compared to mammals [7–14]. So far, it has remained unclear why higher eukaryotic cells express two different mannose 6-phosphate binding proteins. This can be answered only by comparative biochemical studies among the non-mammalian vertebrates, invertebrate species and related specific cell lines. The comparative approach also helps to uncover the individual contributions of the two receptors to normal physiology and to pathological processes. We recently found that the CEF cell MPR300 can also bind human IGF-II [24] and the chicken MPR46 protein is one of the lysosomal enzymes targeting receptors [16]. The precise cellular localization and functions of the CEF MPR300 have not been extensively characterized like the mammalian proteins. To assess the role of MPR300 in transport of lysosomal enzymes in CEF cells, the present study was undertaken. In this study, we applied a DNA vector based on a specific RNA interference approach to silence the MPR300 in CEF cells and to evaluate the biological consequences of this on lysosomal sorting and trafficking. Earlier, none other study has used this technology to study the MPRs function *in vivo*. We designed DNA templates for synthesizing specific siRNAs based on chicken MPR300 cDNA sequence to silence the MPR300 in CEF cells.

In our system, the amount of MPR300 protein, detected by immunoprecipitation and immunofluorescence analysis, was shown to be lower in psilencer 1.0 U6 siRNA CHK-MPR300 transfected cells. Our results are in line with what is known about the MPR300 in mammals, which has been discussed above. In our study, we observed that psilencer 1.0 U6 siRNA CHK-MPR300 transfected cells were characterized by silencing of MPR300 and subsequent missorting of lysosomal enzymes.

From the present study we can conclude the following. The silencing studies have proved the role of MPR300 in intracellular sorting of lysosomal enzymes to lysosomes and subsequently proves that the primary function of MPR300 is conserved in non-mammalians as in higher mammals. It was found that specific silencing of MPR300, but not of MPR46, with siRNA significantly showed missorting of lysosomal enzymes in CEF cells. Since we have silenced only the MPR300 gene in these cells, the missorting of lysosomal enzymes was only to the extent of about 60–85% of the control cells, as the MPR46 might have compensated for the transport of the remaining percentage of enzymes. Since MPR300 is also expressed at the plasma membrane of CEF cells, it is proposed that MPR300 is the major receptor protein for the internalization of lysosomal enzymes from the extracellular regions, through a M6P dependent manner. However, this effect cannot be seen from our experiments in the MPR300 silenced cells, emphasising the role of the cell surface MPR300 in internalization of the M6P containing ligands. To our knowledge this is the first report on the use of this advanced siRNA technology focusing to study of the lysosomal enzyme sorting function of the MPR300 in CEF cells. This study would eventually pave the way in understanding the functional significance and the multifunctional nature of this protein in non-mammalian species with respect to the mammalian protein as the mammalian protein is known to be a multifunctional protein capable of binding a number of ligands in a mannose 6-phosphate dependent manner and also ligands that do not contain mannose 6-phosphate [6]. The interesting questions to be addressed in the field of MPRs in particular among the non-mammalian vertebrate species, are

(a) what is the reason for a protein to attain so many ligand binding abilities, (b) what is the biological consequence of these binding abilities and (c) where has this function originated in the evolution? Further studies would eventually explore new insights about the role of MPR300 in its multifunctional nature *in vivo* using this advanced RNAi technology.

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