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Mannose 6-phosphate receptors in an ancient vertebrate, zebrafish

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Abstract The endosome/lysosome system plays key roles in embryonic development, but difficulties posed by inaccessible mammalian embryos have hampered detailed studies. The accessible, transparent embryos of *Danio rerio*, together with the genetic and experimental approaches possible with this organism, provide many advantages over rodents. In mammals, mannose 6-phosphate receptors (MPRs) target acid hydrolases to endosomes and lysosomes, but nothing is known of acid hydrolase targeting in zebrafish. Here, we describe the sequence of the zebrafish cation-dependent MPR (CD-MPR) and cation-independent MPR (CI-MPR), and compare them with their mammalian orthologs. We show that all residues critical for mannose 6-phosphate (M6P) recognition are present in the extracellular domains of the zebrafish receptors, and that trafficking signals in the cytoplasmic tails are also conserved. This suggests that the teleost receptors possess M6P binding sites with properties similar to those of mammalian MPRs, and that targeting of lysosomal enzymes by MPRs represents an ancient pathway in vertebrate cell biology. We also determined the expression patterns of the CD-MPR and CI-MPR during embryonic development in zebrafish. Both genes are expressed from the one-cell stage through to the hatching period. In early embryos, expression is ubiquitous, but in

later stages, expression of both receptors is restricted to the anterior region of the embryo, covering the forebrain, midbrain and hindbrain. The expression patterns suggest time- and tissue-specific functions for the receptors, with particular evidence for roles in neural development. Our study establishes zebrafish as a novel, genetically tractable model for in vivo studies of MPR function and lysosome biogenesis.

Abbreviations M6P: Mannose 6-phosphate · MPR: Mannose 6-phosphate receptor · CD-MPR: Cation-dependent MPR · CI-MPR: Cation-independent MPR · HPF: Hours post-fertilization · IGF: Insulin-like growth factor · TGN: *trans*-Golgi network

Introduction

In mammals, mannose 6-phosphate receptors (MPRs) play an important role in lysosome biogenesis by sorting newly synthesised acid hydrolases at the *trans*-Golgi network (TGN) (reviewed in Dahms and Hancock 2002; Ghosh et al. 2003). Two structurally related MPRs have been described [referred to as the cation-dependent MPR (CD-MPR) and cation-independent MPR (CI-MPR)], and it has been suggested that they arose from a common ancestor, with the CI-MPR resulting from multiple duplications of a single ancestral gene (Kornfeld 1992). Both types of MPR are present in chicken, reptiles, amphibians and fish, and putative MPRs have been reported in invertebrates (Nadimpalli and Von Figura 2002). The invertebrate M6P-specific proteins are not well characterized, and thus, we do not yet know how the MPR genes evolved.

In addition to their involvement in lysosome formation, the MPRs have other biological activities. In particular, the CI-MPR is an endocytic receptor and interacts with M6P-containing ligands that do not have acid hydrolase activity (Dahms and Hancock 2002). These include many peptides that are important in embryonic development such as TGF- β , proliferin and leukaemia inhibitory factor (LIF). The CI-MPR interacts with several ligands through M6P-

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independent means. These include the foetal mitogen, insulin-like growth factor 2 (IGF2), which the receptor targets for degradation, and this activity is essential for normal mammalian development. The acquisition of an IGF2-binding site by the CI-MPR appears to have occurred after the divergence of marsupial and placental mammals from their common ancestor with egg-laying mammals, and it has been suggested that this acquisition was a major factor in driving the evolution of an imprinted CI-MPR in some mammals (Killian et al. 2000). However, a high-affinity IGF2 binding site has been described in the CI-MPR of a teleost fish (trout) (Mendez et al. 2001), raising the possibility that IGF2 binding was an ancestral property of the CI-MPR.

Recently, a *Drosophila* protein (lysosomal enzyme receptor protein, LERP) that is structurally and functionally related to the mammalian CI-MPR was identified (Dennes et al. 2005). LERP mediates lysosomal enzyme targeting and rescues the missorting of lysosomal enzymes that occurs in MPR-deficient mammalian cells. Interestingly, however, the residues that are involved in M6P recognition in mammalian MPRs are not conserved in the *Drosophila* protein, and LERP does not bind to the multimeric M6P ligand phosphomannan. The nature of the interaction between *Drosophila* LERP and mammalian lysosomal enzymes has not yet been elucidated. However, it may reflect an evolutionarily ancient aspect of lysosome biogenesis that predates the M6P-dependent trafficking of lysosomal enzymes seen in present-day mammals. It also raises the question of how, and when, the involvement of the M6P-specific system in lysosome biogenesis evolved.

Clearly, the structures and ligand-binding activities of the MPRs raise questions about their phylogenetic origin, evolutionary history and functional significance in vivo. These can only be answered by comparative studies that include non-mammalian vertebrates and invertebrates. We predict that the zebrafish, a genetically tractable non-mammalian vertebrate, will be pivotal in these comparisons. In this report, we compare the zebrafish CD-MPR and CI-MPR sequences with those of their mammalian orthologs. We also describe the expression patterns of the zebrafish MPRs during embryonic development.

Materials and methods

Genomic DNA was prepared from adult zebrafish by conventional protease K digestion and alcohol precipitation procedures. Total RNA was extracted from adult zebrafish by homogenization of approximately 100 mg of tissue in RNA-Stat 60 (Tel-Test, Friendswood, TX). cDNA was prepared by reverse transcription of 1–5 µg of DNase-treated total RNA (Superscript II, Life Technologies, Grand Island, NY). Polymerase chain reaction (PCR) amplification was performed with Platinum Taq DNA polymerase (Life Technologies) or with the Expand Long Template PCR system using buffer 3 (Roche, Alameda, CA) (primer sequences available upon request from authors). Amplified

fragments were sequenced with an automated ABI 377 sequencer (PE Biosystems, Foster City, CA) using the manufacturer's BigDye terminator cycle sequencing kit (Duke University's DNA sequencing facility).

Identification of zebrafish MPR cDNA sequences

Zebrafish *CD-MPR* cDNA (NM_213205) was identified by searching the National Center for Biotechnology Information (NCBI) database with the mouse CD-MPR sequence (NM_010749). For zebrafish *CI-MPR* sequence, we initially accessed the publicly available results of a Sanger Center 'Human BLAST against Zebrafish' search to identify zebrafish genomic trace files that showed similarity to the human *CI-MPR/IGF2R* (located on human chromosome 6; accession no. NM_000876). These data are available at <http://134.174.23.160/HumanblastZebrafish/>. Sequences that produced significant alignments were used to design oligonucleotide primers for PCR assays with zebrafish cDNA as template, and cDNA fragments corresponding to the majority of the zebrafish *CI-MPR* were amplified. During this process, the sequence of a genomic contig in linkage group 20 that contained all the zebrafish *CI-MPR* cDNA sequence became available (accession no. AL929119), and using this, we completed the experimental determination of the *CI-MPR* coding sequence. The zebrafish *CI-MPR* cDNA sequence has been deposited in GenBank (accession no. AY570286). We found no evidence for duplicate copies of either zebrafish MPR.

Sequence analysis

Signal peptide sequences, transmembrane domains, cytoplasmic tails and predicted sites of N-linked glycosylation were identified using the predictor programmes available at <http://www.cbs.dtu.dk>. Multiple sequences were aligned using the ClustalW programme at <http://www.ebi.ac.uk>. Accession numbers of compared CD-MPR sequences are as follows: bovine, P11456; human, NP_002346; and mouse, NM_010749. Accession numbers of compared CI-MPR sequences are chicken, AAC59718; echidna, AAL23910; bovine, NP_776777; human, NP_000867; kangaroo, AAF19160; mouse, NP_034645; opossum, AAL23909; platypus, AAF68173; and *Xiphophorus*, CAB94817.

Whole-mount in situ hybridization

cDNA fragments of the zebrafish *CI-MPR* transcript (772 bp, spanning exons 43–47) and of the *CD-MPR* transcript (596 bp, spanning the last two exons) were amplified by reverse transcriptase (RT)-PCR and subcloned into a pGEM-T easy vector (Promega). Digoxigenin-labelled sense and antisense riboprobes were generated, and in situ hybridization was performed on AB wild-type zebrafish embryos as described (Eivers et al. 2004). At later devel-

opmental stages, 18, 24, 36, 48 and 72 h post-fertilization (HPF), fixed embryos were treated with proteinase K (10 µg/ml) for 4, 8, 15, 45 and 60 min, respectively.

Results and discussion

The zebrafish MPRs

The open reading frame of the zebrafish *CI-MPR* (AY570286) encodes a protein of 2,459 amino acids: a signal peptide of 29 amino acids, a 2,258-amino-acid extracytoplasmic domain, a 23-amino-acid transmembrane region and a 149-amino-acid cytoplasmic tail. The extracytoplasmic domain resembles those of mammalian and chicken *CI-MPRs* in consisting of 15 repeating units (average length 147 amino acids), and containing an insert in repeat 13, similar to

the type II repeats found in the collagen-binding site of fibronectin (Dahms and Hancock 2002). The amino acid sequence of the mature zebrafish receptor is 51 and 53% identical to those of the human and chicken receptors, respectively, and the amino terminal portion is 61% identical to the partial *CI-MPR* sequence of the teleost *Xiphophorus* (Yerramalla et al. 2000). The zebrafish *CI-MPR* contains 118 cysteine residues in the extracytoplasmic domain, all of which are conserved in the human, bovine and chicken receptors (not shown). This region also contains 18 predicted sites for N-linked glycosylation, indicating that the molecular weight of the expressed protein is likely to be larger than predicted from the coding sequence (271 kDa). A search of the NCBI database revealed a zebrafish mRNA (accession no. NM_213205) encoding an open reading frame with striking similarity to mammalian *CD-MPRs* (overall amino acid identity 44–47%). The predicted zebrafish *CD-MPR*

Fig. 1 Conservation of carbohydrate recognition domains of zebrafish MPRs. **a** The extracytoplasmic domain of the zebrafish *CD-MPR* (NM_213205) was aligned with the indicated mammalian *CD-MPRs* using ClustalW analysis. The amino acid residues involved in recognition of M6P are indicated by the arrowheads above the alignment, and the Asp residue involved in coordination of Mn²⁺ is indicated by a filled circle.

b, c Repeats 3 (**b**) and 9 (**c**) of the bovine and zebrafish (AY570286) *CI-MPRs* were aligned using Clustal W [the position of the repeats was established using the signal sequence cleavage site as the amino terminus of repeat 1 and the conserved XLXXL motif as the carboxy terminus (Yerramalla et al. 2000)]. The five amino acids that are critical for ligand binding in each repeat of the bovine *CI-MPR* (Dahms and Hancock 2002) are indicated by arrowheads. Three small residues in repeat 3 and three bulky residues in repeat 9, associated with M6P phosphomonoester and M6P-OCH₃ phosphodiester binding, respectively (Dahms and Hancock 2002), are indicated in *bold*. (consensus line below the alignments: * indicates identical residues in all sequences; : indicates highly conserved residues; . indicates weakly conserved residues)

A

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Bovine      TEEKTCDLVGEKKGKESKELALLKRLTPLFNKSFESTVQGSPDMYSYVFRVCREAGNH 58
Human      TEEKTCDLVGEKKGKESKELALVKRLKPLFNKSFESTVQGSDTYIYIFRVCREAGNH 58
Mouse      TEEKSCDLVGEKDKESKNEVALLERLRPLFNKSFESTVQGSDTYSYIFRVCREASNH 58
Zebrafish   RFNTSNCKLVSD---SESQRKALRLLLEPLTNQNFT-TEGQEKEKYSYIFQVCGDAGGV 54
          : . . * . * . * . : * . : . : * * * * . : * * * * : * * . * . * . * .

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          ▼                               ●                               ▼
Bovine      SSGAGLVQINKSNGKETVVGRFNETQIFNGSNWIMLIYKGGDEYDNHCGREQRRVVMIS 118
Human      TSGAGLVQINKSNGKETVVGRNLNETHIFNGSNWIMLIYKGGDEYDNHCGREQRRVVMIS 118
Mouse      SSGAGLVQINKSNDKETVVGRINETHIFNGSNWIMLIYKGGDEYDNHCGREQRRVVMIS 118
Zebrafish   KN-AGLIQEQEG-KGTIRIGDYSKTVATAGSDWVLLIYEGGEKYDSHCSSEERKAMIMIS 112
          .. * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * .

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          ▼                               ▼
Bovine      CNRHTLADNFPVSEERGVQDCFYLFEMDSSLACSPESHLS 161
Human      CNRHTLADNFPVSEERGVQDCFYLFEMDSSLACSPESHLS 161
Mouse      CNRHTLAANFPVSEERGVQDCFYLFEMDSSLACSPESHLS 161
Zebrafish   C S S S K S A - F S V V M E E N Q K Q K N C Y Y L F E L D T T A V C P A V S S K L S 154
          * . : : * . * * * . * : * : * * * * : : . * . * : * *

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B

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          ▼
Bov3       SRVEASDSLFTYSEA---DEYTYLISICGGSQAPICN--KKDAAVCVQVKADSTQVKVA 404
Zeb3       --THGSTDTPYMAEAKNGKDYFFYLNVCGGTNAGQCFDDKGYISACQFKENGDKV-KIA 385
          : . . * . * . * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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          ▼                               ▼                               ▼
Bov3       GRPQNLTLYRSDGDLTLIYFGGEECSGGFQRMVSVINFECNQTAGNNGRGAPVFTGEVDCT 464
Zeb3       GRYQNQLTRYSDGDLTLIYPDGSRCSSTGFQRMIIINFECNATAGN--GQPVFTGESDCT 442
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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          ▼
Bov3       YFFTWDTKYACVHEKEALLCGVSDGKQRFDSLAL 498
Zeb3       YFDFWQTSYACV-EKEDLLCRVTDHKKRYDLSPL 475
          * . * * . * * * * * * * * * * * * * * * * * * * * * * *

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C

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          ▼
Bov9       IPLGLNDTVVRAGEYTYTYFRVCGELTSGVCPTSDKSKVISSCQEKRGPGQFGQKVAAGLFNQ 1309
Zeb9       ---AGQDYEVKSSSTYEHFAVCGPITTSVCLH-DASQSVSSCQVENQK--HRIAGIANQ 1279
          . . . * * : : . * * * * * * * * * * * * * * * * * * * * * * * *

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          ▼                               ▼                               ▼
Bov9       KLTYENGLVKMNYTGGDTCHKVYQRSTTIFFYCDRS--TQAPVFLQETSDCSYLFEWRTQ 1367
Zeb9       NLTFFDDGIIMINYTNGETCHKIYERSTAILFSCDHSRNPQKPDFIKETADCTYLFWHHTA 1339
          : * * : : * : * * * * * * * * * * * * * * * * * * * * * * * *

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Bov9       YACPPYDLTECSFKNEAGETYDLSSL 1393
Zeb9       LACPSFKTTTCSYNDGSGHSYDLSSL 1365
          * * * . . * * * : : . * . * * * * *

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et al. (2005) suggest that many of the residues that contribute to the IGF2 binding pocket of the mammalian CI-MPR, including the equivalent of Ile¹⁵⁷², are present in the *Drosophila* LERP. In view of these observations, experiments that address the ability of zebrafish CI-MPR to bind IGF2 are currently underway.

Expression of zebrafish MPRs during embryonic development

Zebrafish should be an excellent model organism for in vivo experiments designed to understand the reason(s) why animals have two types of MPR (and perhaps for examining IGF2-independent functions of the CI-MPR). As the first step in this approach, we used in situ hybridization to determine the expression patterns of the zebrafish receptors during embryonic development. Although there were individual differences in staining intensities, expression patterns of both receptors were generally similar. Thus, mRNAs for *CD-MPR* and *CI-MPR* were detected in the one-cell embryo and in all blastomeres prior to the midblastula transition (Fig. 4a–d), indicating that maternal mRNAs for both receptors are deposited in the developing oocyte. Expression of both receptors was detected in all cells of blastula-stage embryos (Fig. 4e–f), throughout gastrulation (50% epiboly and tail-bud stages) (Fig. 4g–j) and in the midsegmentation period (12 somites, data not shown).

In later-stage embryos, expression of both receptors ceased to be uniform and became progressively more restricted to the anterior region of the embryo. By late segmentation (18 HPF), staining was intense and even throughout the anterior region of the embryo but was much weaker along the trunk and tail (Fig. 4k–l). During the early pharyngula stage (24 HPF), staining became concentrated in the anterior regions of the embryo, including the telencephalon, retina, tectum, midbrain and hindbrain (Fig. 5a,b). Although expression was detected along the spinal cord and somites at this stage, it was less intense and more diffuse than the anterior expression. By 36, 48 and 72 HPF, expression of both receptors was restricted to the anterior region of the developing embryo, covering the forebrain, midbrain, hindbrain and retina (Fig. 5c–h).

In 48-HPF embryos, an unusual staining pattern was observed for the *CD-MPR*. This consisted of two intensely stained stripes in the brain region (Fig. 5e, inset). In an attempt to identify the stained regions, we performed double in situ staining using a probe specific for *Shh* (*sonic hedgehog*), which marks periventricular cells in the diencephalon at this stage (Krauss et al. 1993). The *CD-MPR* stain was dorsal to the *Shh*-stained region (data not shown). The identity of the *CD-MPR*-expressing regions is still unclear, but the larger stripe (stripe 2) may represent the pretectum, and the narrower stripe (stripe 1) is slightly caudal to the zona limitans intrathalamica. Further analysis with additional informative markers will be required to clarify this.

Fig. 4 Expression of zebrafish MPRs during early embryonic development. In situ hybridization was performed using *CD-MPR*- or *CI-MPR*-specific antisense riboprobes. The embryonic stages used were one-cell zygote (a, b), cleavage period (c, d), blastula (e, f), gastrula (g–j) and segmentation period (k, l). Embryos are shown in lateral view, photographed at $\times 30$ magnification

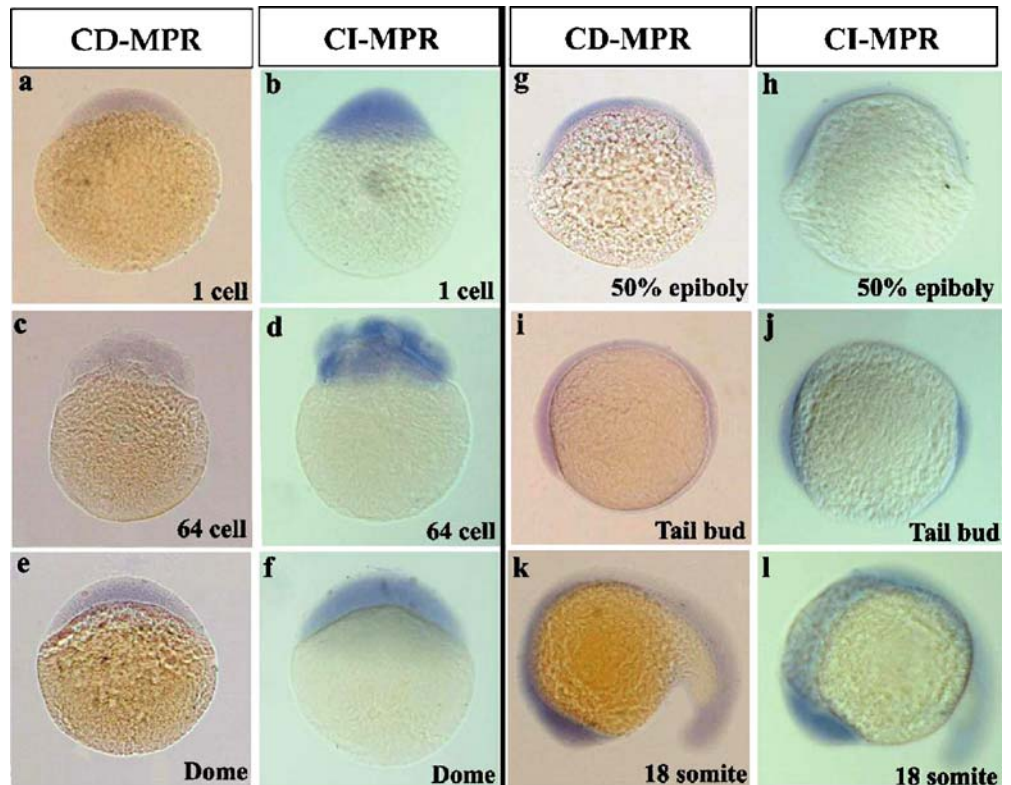
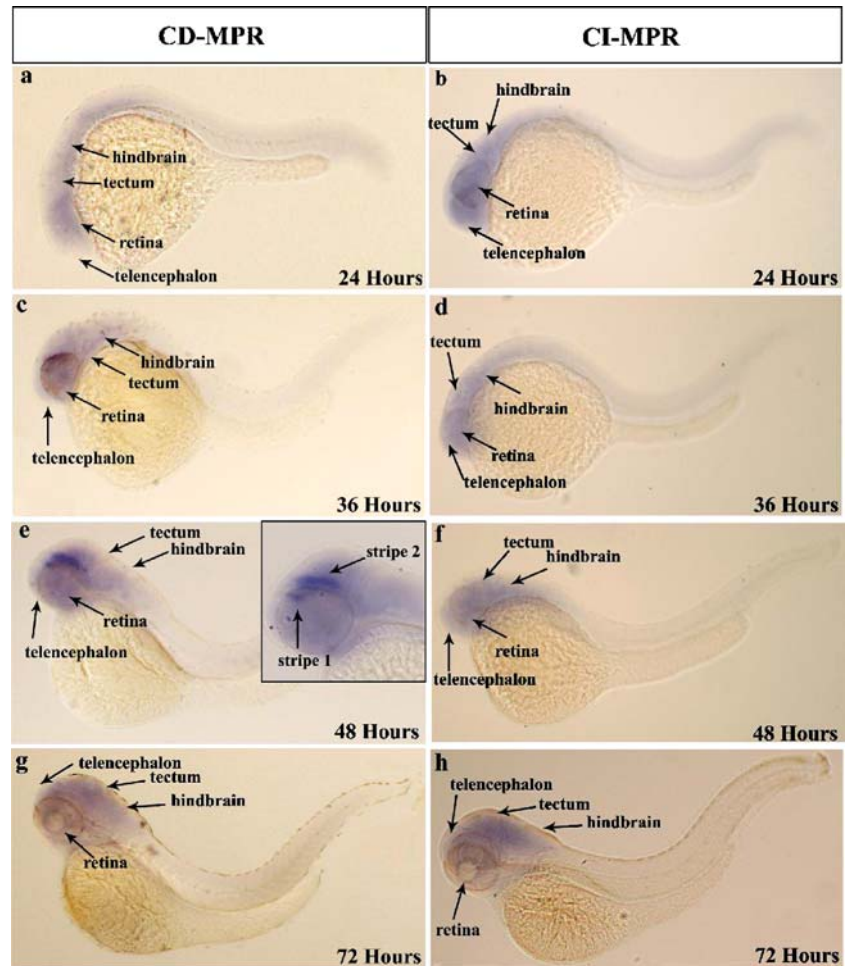


Fig. 5 Expression of zebrafish *MPRs* during late embryonic development. In situ hybridization was performed using *CD-MPR*- or *CI-MPR*-specific antisense riboprobes. Embryos are in the pharyngula period at 24 (a, b) and 36 HPF (c, d), and in the hatching period at 48 (e, f) and 72 HPF (g, h). Embryos are shown in lateral view, photographed at $\times 30$ magnification



Potential conservation of MPR function in neuronal development

Endosomes and lysosomes regulate the activity of critical signaling molecules in development and may play a role in morphogen generation (Piddini and Vincent 2003). The widespread expression of both zebrafish *MPRs* in very early embryos, followed by more restricted expression in later stages, suggests time- and tissue-specific functions for both receptors, with particular indication of potential roles in neural development. MPR expression patterns in chickens and mice suggest that such roles may be conserved. Thus, the *CD-MPR* is expressed uniformly in very early chicken embryos, and this uniformity is lost at later stages, when expression becomes most prominent in neuronal tissues (Matzner et al. 1996). Expression of the *CD-MPR* is also developmentally regulated in rat brain (Romano et al. 2005). *CI-MPR* expression is prominent in developing neural tissue of chickens (Matzner et al. 1996). In early rodent embryos the *CI-MPR* is widely expressed and, although the heart, dorsal aorta and somites are the major sites of expression in post-implantation mouse embryos, *CI-MPR* expression is also readily detectable in the brain (Matzner et al. 1992; Lerchner and Barlow 1997). Zebrafish embryos are more accessible than chicken or mice embryos, and the ability to manipulate gene expres-

sion in zebrafish, using loss-of-function and gain-of-function approaches, makes it possible to test our hypothesis of a role for *MPRs* in neural development.

Conclusion

Zebrafish *MPRs* are structurally similar to the mammalian and chicken orthologs, indicating that the duplications of the common ancestral gene that are proposed to have given rise to extant *CI-MPR* and *CD-MPR* (Kornfeld 1992) must have occurred prior to the evolution of bony fish. Zebrafish *MPRs* possess all the residues that are important in mammals for M6P recognition and for intracellular trafficking pathways, suggesting that targeting of lysosomal enzymes by *MPRs* is an ancient pathway that was a feature of the common ancestor of modern vertebrates. Further studies in non-vertebrate chordates and other invertebrates should clarify the origin of these two genes and their functional relationship to *Drosophila* LERP, as well as the origin of the multi-functional *CI-MPR*. Our study establishes *Danio rerio* as a valuable model vertebrate for functional studies that will complement the use of more traditional, but genetically less tractable, mammalian species. The comparative approach should help to uncover the individual contributions of the two receptors to normal physiology and to pathological processes.

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