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Research paper

Lysosomal localization of Japanese medaka (*Oryzias latipes*) Neu1 sialidase and its highly conserved enzymatic profiles with human

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ABSTRACT

Desialylation in the lysosome is a crucial step for glycoprotein degradation. The abnormality of lysosomal desialylation by NEU1 sialidase is involved in diseases of mammals such as sialidosis and galactosialidosis. Mammalian Neu1 sialidase is also localized at plasma membrane where it regulates several signaling pathways through glycoprotein desialylation. In fish, on the other hand, the mechanism of desialylation in the lysosome and functions of Neu1 sialidase are still unclear. Here, to understand the significance of fish Neu1 sialidase, *neu1* gene was cloned from medaka brain and the profiles of its polypeptides were analyzed. Open reading frame of medaka *neu1* consisted 1,182 bp and the similarity of its deduced amino acids with human NEU1 was 57%. As this recombinant polypeptide did not show significant sialidase activity, medaka cathepsin A, known in mammals as protective protein activating Neu1, was cloned and then co-expressed with medaka Neu1 to examine whether medaka cathepsin A activates Neu1 activity. As a result, Neu1/cathepsin A showed a drastic increase of sialidase activity toward MU-NANA. Major substrate of medaka Neu1 was 3-sialyllactose and its optimal pH was 4.0. With immunofluorescence analysis, signal of overexpressed medaka Neu1 was found to coincide with Lysotracker signals (organelle marker of lysosome) and co-localized with medaka cathepsin A in fish hepatic Hepa-T1 cells. Furthermore, part of medaka Neu1 was also detected at plasma membrane. Medaka Neu1 possessed signal peptide sequence at N-terminal and incomplete lysosomal targeting sequence at C-terminus. Medaka *neu1* gene was ubiquitously expressed in various medaka tissues, and its expression level was significantly higher than other sialidase genes such as *neu3a*, *neu3b* and *neu4*. The present study revealed the profiles of fish Neu1 sialidase and indicated its high conservation with human NEU1 for the first time, suggesting the presence of similar desialylation system in the medaka lysosome to human. Moreover, the present study showed the possibility of medaka as a model animal of human NEU1 sialidase.

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1. Introduction

Sialic acid is a key molecule for regulation of cell life, cell-cell recognition and virus infection. Sialidases catalyze the removal of sialic acids from non-reducing ends of glycoconjugates. Four mammalian sialidases, abbreviated to Neu1, Neu2, Neu3 and Neu4, have been cloned and characterized so far (Miyagi et al., 2012). These enzymes show different enzymatic properties including substrate specificity, optimal pH and subcellular localization. Among them, mammalian Neu1 is localized at the lysosome and is ubiquitously expressed in various tissues. Major substrates of mammalian Neu1 are glycopeptides and oligosaccharides and its optimal pH is 4.6, suitable for lysosomal enzyme. As well known, lysosome is a crucial vehicle for phagocytosis, autophagy, lysosomal exocytosis and plasma membrane repair, lysosomal cell death, cholesterol homeostasis, major histocompatibility (MHC) class II-dependent antigen presentation and exosome release (Saftig and Klumperman, 2009). Mammalian Neu1 possesses important functions in degradation of complex N-glycans in lysosome. N-glycans derived from glycoprotein are sequentially degraded by glycosidases, such as sialidase, in a specific order (Winchester, 2005). In lysosome, mammalian Neu1 sialidase forms a complex with protective protein (cathepsin A) and β-galactosidase. Cathepsin A is necessary for the activation of Neu1 sialidase by the correct compartmentalization and catalytic activation in lysosomes, and prevents Neu1 premature oligomerization (Bonten et al., 2009). Abnormal degradations of glycoconjugates in the lysosome are involved in many diseases. For instance, NEU1 genetic deficit causes sialidosis, an inborn genetic disorder caused by abnormal lysosomal storage (Seyrantepe et al., 2003). Recent study also revealed that deficiency of Neu1 affects amyloid precursor levels and amyloid-β

Abbreviations: HA, hemagglutinin; MU-NANA, methylumbelliferyl-N-acetyl neuraminic acid; ORF, open reading frame; rpl-7, ribosomal protein 7.

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secretion (Annunziata et al., 2013). Furthermore, many lysosomal proteins are known to translocate to plasma membrane and secreted extracellularly. Similar to other lysosomal proteins, mammalian Neu1 is known to be translocated to plasma membrane and/or to extracellular space by exocytosis, followed by regulation of signaling of integrin β4 (Uemura et al., 2009), Toll-like receptor (Amith et al., 2009), insulin receptor (Alghamdi et al., 2014) and CD44 (Katoh et al., 2010). Neu1 is targeted to cell surface in Neu1/cathepsin A/β-galactosidase complex, regulating elastin fiber assembly (Hinek et al., 2006; Duca et al., 2007). Neu1-null mice show abnormal elastic fiber developments (Starcher et al., 2008). Recently, it is reported that secreted NEU1 sialidase desialyzes cell surface polysialic acid and regulates neurotrophin signaling (Sumida et al., 2015).

Different from mammals, fish lysosomal functions are still unclear. Some reports show the significance of fish lysosomal catabolism system. N-linked glycans degradation by lysosomal 6-mannosidase is essential for zebrafish embryogenesis (Fan et al., 2010). Amino acid starvation are known to activate autophagy-lysosomal system in zebrafish ZE cells (Yabu et al., 2012). Salmon anemia infection also activates autophagy-lysosomal system in cells of Atlantic salmon (Schiøtz et al., 2010). Although desialylation would be the essential step for the degradation of sugar chains of glycoconjugates in fish lysosomal catabolism, enzymatic profiles and biological functions of fish lysosomal sialidases have remained obscure. According to the *in silico* genomic research, *neu1* sialidase gene is found to be widely conserved from Protista to Mammalia (Giacopuzzi et al., 2012), yet, only few studies about fish Neu1 sialidase have been reported until now. Only zebrafish *neu1* has been cloned and its sialidase activity was partially evaluated (Manzoni et al., 2007). However, its sialidase activity was relatively low compared with other reported sialidases. The optimal pH of zebrafish Neu1 is at 2.6, which is quite acidic and different from that of human NEU1 (optimal pH at 4.6). Furthermore, other profiles such as subcellular localization and substrate specifies were not described. To elucidate the significance of fish Neu1 sialidase, more detailed studies about Neu1 properties are necessary in other fish species.

We have focused on Japanese medaka (*Oryzias latipes*) to clarify the biological significance of fish sialidases because of easy handling for gene-knockdown and developmental observation, and numerous inbred strain of medaka have already been established. Recently, we have reported that four putative sialidases, *neu1*, *neu3a*, *neu3b* and *neu4*, are present in medaka genome. Cloning and characterization of medaka Neu3a and Neu3b revealed that Neu3a is highly conserved with human NEU3, but not with zebrafish, and that Neu3b is a unique cytosolic sialidase found in limited fish order (Shiozaki et al., 2013). Neu4 was found as lysosomal sialidase and its major substrate was oligosaccharides (Shiozaki et al., 2014). This Neu4 sialidase, however, may not be the major lysosomal sialidase in medaka because its gene expression level is relatively lower than Neu3 sialidases (Shiozaki et al., 2014). Furthermore, medaka liver shows sialidase activity which desialyzes sialyllactose in spite of low *neu4* expression, suggesting that other sialidase, possibly Neu1, would be a major sialidase for sialo-oligosaccharides degradation.

To clarify this hypothesis, the identification and characterization of medaka Neu1 is necessary. Until now, medaka Neu1 sialidase had not been cloned and characterized. Putative and partial medaka *neu1* open reading frame (ORF) sequences, predicted by automated computational analysis are deposited in NCBI and Ensemble database as XM_004073764 and ENSORLG00000002057, respectively. Using this prediction and medaka genome browser, we previously confirmed this putative gene as *neu1* by synteny and phylogenetic tree analysis. We also designed specific primers and estimated the alteration of putative *neu1* mRNA level during medaka embryogenesis by real-time PCR, which indicated the up-regulation of *neu1* gene in early and late stage in the development (Shiozaki et al., 2014). To obtain Neu1 functions in medaka embryogenesis, the information of Neu1 profiles are essential.

Here, to investigate biological significance of medaka Neu1, we identified and cloned medaka *neu1* cDNA. Next, using recombinant Neu1 polypeptide, its enzymatic properties were estimated to compare the conservation toward mammals and zebrafish. Subcellular localization of medaka Neu1 was investigated by indirect immunofluorescence, followed by the assessment of *neu1* mRNA level in medaka tissues.

2. Materials and methods

2.1. Animal

Inbred medaka (Hd-rR II strain) was obtained from Natural Institute for Basic Biology (Okazaki, Japan). Medaka was fed live brine shrimp under 14/10 h light–dark cycle at 23 °C. Excised tissues were immediately frozen by dried ice and kept at −80 °C until use.

2.2. Molecular cloning of medaka neu1 and ctsa gene

Total RNA was extracted from medaka brain using Sepazol-RNA I Super G (nacalai tesque, Japan). Synthesis of cDNA was carried out by ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). For the amplification of *neu1* and *ctsa* (gene name of cathepsin A), primers were designed according to the predicted nucleotide sequence of sialidase-1-like (XM_004073764) and lysosomal protective protein (XM_011474968) in NCBI database (http://www.ncbi.nlm.nih.gov/), respectively. In addition, genomic information of *neu1* and *ctsa* gene were analyzed by UTGB medaka genome browser (http://utgenome.org/medaka/) as described previously (Shiozaki et al., 2013 Shiozaki et al., 2014). PCR amplification of ORF region of the genes encoding *neu1* and *ctsa* was carried out using KOD-plus ver.2 (TOYOBO) with specific primers (Table 1). PCR was carried out as follows: pre-denature at 94 °C for 2 min, denature at 98 °C for 10 s and the extension at 68 °C for 90 s for 30 cycles. Each PCR product was subcloned into pBluescript (Agilent Technologies, CA) with restriction enzyme treatments. The nucleotide sequences of PCR products were analyzed by ABI3130xl Genetic Analyzer (Life Technologies, MA). Exon/intron boundary in medaka *neu1* was investigated using Ensembl Genome Browser (http://asia.ensembl.org/index.html).

For bioinformatics analysis, glycosylation sites in deduced amino acid sequences of Neu1 were predicted using NetNGlyc 1.0 server (N-glycosylation, http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 server (O-glycosylation, http://www.cbs.dtu.dk/services/NetOGlyc/). The signal peptide cleavage site was analyzed using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Key residues in catalytic sites of medaka Neu1 was predicted according to Giacopuzzi et al. (2012).

2.3. Cell culture

Human embryonic kidney HEK293T cells and tilapia hepatic Hepa-T1 cells were obtained from RIKEN CELL BANK (Osaka, Japan). HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) with 5% CO_2 at 37 °C and Hepa-T1 cells were in E-RDF medium containing 15% FBS without CO_2 supplementation at 28 °C. For the transfection of *neu1* and *ctsa*, each cDNAs in pBluescript obtained above were subcloned into pcDNA3.1 plasmid (Life Technologies). Expression plasmids were introduced into cultured cells using X-treme Gene HP DNA (Roche, Swiss) according to manufacturer's instructions.

2.4. Western blotting

To detect recombinant Neu1 and cathepsin A polypeptides by Western blotting, cDNA for N-terminal or C-terminal hemagglutinin (HA) tagged-Neu1 and C-terminal FLAG tagged-cathepsin A were constructed using PCR primers shown in Table 1. Amplification with

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