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Matrix metalloproteinase-2 and -9 in the cerebellum of teleost fish: Functional implications for adult neurogenesis

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ABSTRACT

Matrix metalloproteinases (MMPs) are a family of highly conserved zinc-dependent proteases involved in both development and pathogenesis. The present study examines the role of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in adult neurogenesis, using the corpus cerebelli, a subdivision of the cerebellum, of knifefish (*Apteronotus leptorhynchus*) as a model system. Transcripts of five isoforms of these gelatinases were identified in the central nervous system of this species. Sequence similarity analysis and homology modeling indicated that functionally and structurally critical elements were highly conserved in knifefish gelatinases. Immunohistochemical staining revealed a differential distribution of MMP-2 and MMP-9 at both the cellular and subcellular level. MMP-2 expression was found mainly in Sox2-immunopositive stem/progenitor cells, both quiescent and mitotically active; and was localized in both the cytoplasmic compartment and the nucleus. By contrast, MMP-9 immunoreactivity was absent in neurogenic niches and displayed a more homogenous distribution, with low to moderate intensity levels, in the molecular and granular layers. MMP-9 expression appeared to be restricted to the extracellular space. *In situ* zymography indicated that gelatinase activity matched the cellular and subcellular distributions of the two MMPs. The observed patterns of gelatinase activity and expression support the hypothesis that MMP-2 is primarily involved in regulation of the activity of stem/progenitor cells that give rise to new granule neurons, whereas MMP-9 facilitates migration of the progeny of these cells by proteolysis of extracellular matrix proteins.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases, comprising 25 members in vertebrates (for review, see Sternlicht and Werb, 2001). Together with their intrinsic inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), these proteolytic enzymes are crucially involved in the remodeling of the pericellular environment by degrading protein constituents of the extracellular matrix. In addition, they activate or inactivate numerous cell signaling pathways by cleaving and releasing various guidance and adhesion molecules, receptors, growth factors, and cytokines (for reviews, see Nagase et al., 2006; Sternlicht and Werb, 2001; Visse and Nagase, 2003).

MMPs have been implicated in a number of pathophysiological processes associated with malignant, inflammatory, and degenerative diseases (for review, see Klein and Bischoff, 2011). In pathological conditions affecting the central nervous system (CNS), the expression of several MMPs increases as the result of contributions from peripheral macrophages that enter the CNS, microglia intrinsic to the CNS, as well as neural and endothelial cells. This leads to detrimental effects, particularly in early stages after injury and in association with a number of degenerative diseases, such as Alzheimer's and Parkinson (for reviews, see Rosenberg, 2009; Yong et al., 2001).

Studies of embryonic or early postnatal development and of regeneration after CNS injury have shown that, in addition to the detrimental effects, specific MMPs can also make significant beneficial contributions to cellular processes underlying neural plasticity and tissue repair (for review, see Yong, 2005). This functional duality makes the development of drug therapies that target the activity of MMPs a complex challenge. Evidently, the safe and effective use of inhibitors of MMPs requires a thorough understanding of the role of these proteolytic enzymes in the healthy CNS. Yet, surprisingly little is known about their expression and function in the adult brain or spinal cord.

In light of the involvement of MMPs in cellular processes related to neural plasticity during embryonic and early postnatal development, it is conceivable that such functions persist into adulthood in areas of the CNS that exhibit a high degree of neural plasticity. To test this hypothesis, in the present investigation we have examined the possible involvement of MMPs in adult neurogenesis, using the cerebellum of teleost fish as a well-characterized model system. Previous investigations have demonstrated that this part of the teleost brain is distinguished by extensive generation of new neurons during adulthood

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(Ekström et al., 2001; Grandel et al., 2006; Teles et al., 2012; Zupanc et al., 1996, 2005; Zupanc and Horschke, 1995), greatly surpassing the neurogenic potential of the mammalian brain. The results of our study indicate that the expression and activity pattern of two members of the MMP family, gelatinase A (MMP-2) and gelatinase B (MMP-9), is consistent with their differential involvement in the generation and subsequent migration of new granule neurons in the adult cerebellum.

2. Results

2.1. Identification of gelatinases in the knifefish CNS

An exhaustive search of the annotated knifefish CNS transcriptome yielded 8 candidate MMP-2 transcripts and 30 candidate MMP-9 transcripts, provisionally identified through their similarity to homologues from other teleost species (primarily *Astyanax mexicanus* and *Danio rerio*, species with close phylogenetic relationship to *Apteronotus leptorhynchus*). Sequence similarity (BLAST percent identity) ranged from 45% to 93% (median: 78%), whereas coverage (BLAST percent of target length aligned) ranged from as low as 2% to more than 99% (median: 27%), indicating that many of these candidate transcripts were likely fragments. Indeed, investigation of the deBruijn graphs constructed during the assembly process (data not shown), complemented by examination of possible open reading frames, confirmed this notion.

All MMP-2 candidate transcripts represented alternate and/or partial paths on the same deBruijn graph. The two longest paths shared 7 nodes (1308 nucleotides) and then diverged, continuing separately for 3 and, respectively, 2 additional nodes, yielding two alternate MMP-2 transcripts with total lengths of 1876 and 1710 nucleotides. Both isoforms contained stop codons in the first non-shared node but lacked a start codon, and hence are incomplete at the N-terminus. The translated protein sequences reached lengths of 468 and 463 amino acids, respectively, for the two isoforms. Interestingly, the shorter isoform had the stop codon located closely after the end of the shared sequence, indicating that it was generated by a frameshift mutation.

Nine of the MMP-9 candidate transcripts represented short and/or isolated graph paths, and had lengths ranging from 445 to 1361 nucleotides (median: 556). These candidates also yielded low coverages of the MMP-9 sequences matched during the automated annotation process, ranging between 2% and 12% (median: 7%). Therefore, they were classified as fragments and discarded. All of the remaining 21 candidate transcripts were based on the same deBruijn assembly graph, and, as above, a significant proportion of them represented partial paths. The longest path in the graph extended for 12 nodes, yielding a sequence of 2971 nucleotides. The 8th and/or 10th nodes on the path (146 and 144 nucleotides, respectively) could be skipped, resulting in three alternate sequences with total lengths of 2825, 2827, and 2681 nucleotides. All four transcripts contained complete open reading frames, with the start codon located in the initial node, and stop codons found in the 8th node, in the 10th node if the 8th was skipped, or in the 11th node if both the 8th and the 10th were skipped. Translation of the open reading frames yielded three unique MMP-9 protein isoforms, totaling 607, 672, and 677 amino acids, respectively. Interestingly, the longest protein isoform was based on the shortest transcript isoform, indicating that the longer transcript isoforms include inserts that introduce frameshifts.

2.2. Molecular characterization of gelatinases in the teleost CNS

Alignment of the longer knifefish MMP-2 isoform against reference zebrafish and human MMP-2 sequences indicated that the available protein sequence fragment lacked the signal peptide and propeptide, as well as the initial part of the catalytic domain. Other MMP-2 regions, including the fibronectin domain characteristic of gelatinases, the zinc-binding subdomain, and the C-terminal hemopexin domain, were present and appeared to be highly conserved. Also highly conserved were the active site motif within the catalytic domain, and the nuclear localization sequence within the hemopexin domain (Fig. 1). Sequence similarity analysis using BLAST revealed that the knifefish MMP-2 fragment matched 71.4% of the zebrafish MMP-2, with 86.1% of the aligned residues being identical in the two proteins and 93.6% having positive alignment scores. Comparison of the knifefish MMP-2 protein with its human homologue yielded somewhat lower identity and positive alignment scores, of 74.4% and 86.3%, respectively, at 71.5% coverage of the subject protein. Similar BLAST scores – 74.6% identity and 85.6% positive alignments – were obtained when contrasting the zebrafish and human MMP-2 proteins. Overall, the two fish MMP-2 proteins showed roughly the same level of similarity to the human homologue, and a higher degree of identity between themselves, in good agreement with the phylogenetic relationships between these species. Similarity with the human MMP-2 protein appeared lowest within the signal sequence (data for zebrafish only), as well as in the hinge region, which is 4 residues shorter in both fish proteins (Fig. 1).

Alignment of the longest knifefish MMP-9 protein isoform against reference zebrafish and human MMP-9 protein sequences indicated that the available knifefish sequence is indeed complete and similar to its homologues (Fig. 2). BLAST analysis revealed a 76.0% sequence identity score and a total of 86.6% positive alignments between knifefish and zebrafish MMP-9 proteins, a 56.3% identity (71.2% positive alignments) between knifefish and human MMP-9 proteins, as well as a 58.0% identity (73.7% positive alignments) between the zebrafish and human MMP-9 proteins. While these results parallel the within-class/between-class trend also observed for MMP-2, it appears that MMP-9 is not conserved as highly across taxa. Sequence similarity was rather low in the signal peptide and propeptide regions, and especially so in the hinge region, which is significantly shorter in the two fish proteins than in their human homologue.

Transcriptome analysis revealed the presence of additional, shorter isoforms of both MMP-2 and MMP-9 in the knifefish CNS. These isoforms featured a truncated or altered hemopexin domain, with the third and/or fourth blades of this complex being affected. The fourth hemopexin blade is incomplete in the shorter MMP-2 isoform (Fig. 1) and completely missing in the shortest MMP-9 isoform, which also lacks parts of the third blade (Fig. 2). Crucially, both of these isoforms lack the cysteine residue involved in the disulfide bond between the first and fourth blades, likely compromising the structural integrity of this domain. The third MMP-9 isoform also features a shortened, heavily modified fourth hemopexin blade (Fig. 2).

Existing structural information on human MMP-2 and MMP-9 (Fig. 3A–A′) was used to predict the molecular structure of zebrafish and knifefish gelatinases through homology modeling. Local quality estimates typically ranged from 0.6 to 0.9, but were occasionally as low as 0.2 (on a 0 to 1 scale). Structural similarity between teleost and human MMP-2 was low at the N-terminus of the propeptide and the junction between the propeptide and the catalytic domain (Fig. 3B), in the second fibronectin insert (Fig. 3B, D), as well as in the hinge region and at multiple sites in the hemopexin domain, particularly the first and fourth blades (Fig. 3B, C, D). A similar pattern was observed for MMP-9, with an additional site of low similarity found within the first catalytic subdomain, and with no data on the hinge region since no empirically determined structure was available as reference at this time (Fig. 3B′, C′, E–E′). Notably, the shorter isoforms of MMP-2 and MMP-9 found in the knifefish CNS were predicted to have hemopexin domains with drastically altered structures (Fig. 3D, E–E′).

2.3. Distribution of gelatinase activity in the corpus cerebelli

For the analysis of MMP expression and gelatinase activity, we focused on the best-characterized of the three subdivisions of the knifefish cerebellum, the corpus cerebelli. In accordance with the Bauplan of the vertebrate cerebellum, this structure consists of an inner granular layer, a molecular layer, and a Purkinje cell layer located