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Bone morphogenetic protein/retinoic acid inducible neural-specific protein (brinp) expression during *Danio rerio* development

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ABSTRACT

Prototype Membrane Attack Complex/Perforin (MACPF) superfamily proteins such as complement and perforin play crucial roles in immune defense where they drive lytic pore formation. However, it is evident that other MACPF family members are important in the central nervous system. For example, three bone morphogenetic protein/retinoic acid inducible neural-specific proteins (Brinp1, Brinp2 and Brinp3) are present in developing and mature mammalian neurons, but their molecular function is unknown. In this study we have identified and cloned full-length orthologues of all three human *brinps* from *Danio rerio* (zebrafish). Zebrafish and human *brinps* show very high sequence conservation, and the chromosomal loci are syntenic. We also identified two additional *brinp3* paralogues at a separate locus in the zebrafish genome. The spatiotemporal expression of all five zebrafish *brinps* was determined by RT-PCR and whole mount RNA *in situ* hybridisation. Each *brinp* is expressed broadly in the developing nervous system at early stages (24 hours post fertilisation), but localises to specific structures in older embryos (48–72 hpf), as has been reported in mice. The conserved structures and spatiotemporal expression patterns of *brinps* reported in this study suggest that zebrafish will be useful for generating loss of function phenotypes to assist in determining the molecular role of these proteins.

The Membrane Attack Complex/Perforin (MACPF) superfamily includes vertebrate pore forming immune defense proteins that lyse microbes and infected – or otherwise compromised – eukaryotic cells (reviewed in Kondos et al., 2010; Law et al., 2010). However, it is clear that not all MACPF proteins are lytic, and it has become increasingly evident that some members function outside of the immune system (Rosado et al., 2007). For example, *Drosophila torso-like* (*tsl*) is essential for terminal patterning in larvae and for proper growth (Martin et al., 1994), *astrotactins* (*astns*) are reported to direct neural migration in the murine brain (Zheng et al., 1996), and bone morphogenetic protein/retinoic acid inducible neural-specific proteins (Brinps) are associated with rodent neural development (Kawano et al., 2004).

In mammals Brinps are expressed in developing and adult brain. Specifically, Brinp1 is broadly expressed in the brain of early mouse embryos (E9.5), and is highly expressed in the cerebral cortex, cerebellum, hippocampus and olfactory bulb in adult mice. Brinp2 is weakly detected throughout embryogenesis, but is found in the cerebellum and hippocampus in adults with low levels in the cerebral cortex. Similar to Brinp2, low levels of Brinp3 are detected in the cerebral cortex, but higher levels are seen in the cerebellum, olfactory bulb, cerebral cortex, diencephalon, midbrain and cerebellum (Kawano et al., 2004). Each member of this MACPF sub-family contains the signature MACPF domain, but the remainder of the protein has no significant sequence or domain similarity to any other structurally or functionally characterised protein. It has been proposed that human Brinp1 is a tumour suppressor as it is lost in some bladder cancers and astrocytomas (Habuchi et al., 1998; Wright et al., 2004), and has the ability to cause cell cycle arrest in cells overexpressing the protein (Nishiyama et al., 2001). Recent *in vivo* studies on a newly-generated *Brinp1* knockout mouse line have focused on neuroanatomy and behavior of adult mice. The absence of Brinp1 causes increased neurogenesis in the subgranular zone of the dentate gyrus. Knockout mice also display increased locomotor activity, reduced anxiety-like behavior, poor social interaction, and impaired working memory (Kobayashi et al., 2014). No functional roles for Brinp2 or Brinp3 have been examined and described to date.

The structure and function of Brinp homologues in non-mammalian vertebrates have not been studied, but could provide insights into their function. Here we have identified zebrafish

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orthologues of the three mammalian Brinps, and two Brinp3 paralogues, via EST and synteny analysis. RT-PCR and whole mount *in situ* hybridisation show differential and developmentally regulated neural expression of the *brinp* genes, consistent with patterns seen in the mouse. These findings suggest zebrafish will provide a useful model for examining *brinp* function in the developing embryo.

1. Results and discussion

1.1. Identification and cloning of zebrafish brinps

Annotation of genome assemblies of zebrafish (Zv9) and fugu (FUGU4) predicted the existence of five *brinp* genes in zebrafish: *brinp1*, *brinp2* and three paralogues of *brinp3*. To verify expression, these sequences were first used to probe zebrafish ESTs. We identified, obtained and sequenced a full-length EST for *brinp1*. For *brinp2* we identified multiple incomplete oligo dT-primed ESTs, the longest encoding the last 300 amino acids of the predicted gene, confirming expression. We then designed an antisense primer in the 3′ UTR paired with a sense primer designed from the predicted 5′ UTR sequence of *brinp2*. A *brinp2* cDNA containing the complete coding region was amplified by RT-PCR using mRNA from adult head, cloned and sequenced.

A full length EST for *brinp3a* was identified, obtained, and sequenced. Multiple partial ESTs for *brinp3b* were identified, verifying expression. To obtain a *brinp3b* cDNA, primers annealing in the 5′ and 3′ UTRs were designed from the predicted gene sequence and used to amplify the coding region from adult head cDNA. For *brinp3c*, a single partial EST (containing at least two exons) was identified, obtained and sequenced. Results from sequencing this EST confirm *brinp3c* is expressed, however repeated attempts to amplify a full-length coding region using 5′ and 3′ UTR primers were unsuccessful. For subsequent bioinformatic analysis the NCBI predicted coding sequence was used.

1.2. The Brinp amino acid sequence and domain structure is highly conserved in vertebrates

Predicted amino acid sequences of the zebrafish Brinps were aligned with reference GenBank sequences from human and mouse using a ClustalW alignment algorithm. Results from this alignment showed that the zebrafish and mammalian proteins are highly conserved with >90% similarity for Brinp1, > 80% similarity for Brinp2 and approximately 80% similarity for Brinp3 (Table 1).

Brinps have been documented to contain a MACPF domain, indicating a potential role in membrane interaction or pore formation, however outside of this domain Brinps have no sequence homology to any other known protein. We noted two additional features within the protein sequence, a cysteine rich region (CRR) and a putative insulin-like growth factor binding protein domain (IGFBP). Although the function of these regions in *brinps* is unknown, the number and positions of cysteines are exceptionally well conserved across all Brinps in mammals and fish (Fig. 1, asterisks). It is likely that these region(s) fold into a disulfide-linked domain, which may promote protein–protein interactions.

Table 1
Percentage amino acid identity and similarity (in parentheses) of human and zebrafish Brinps. Protein Blast (NCBI) was used to align pairs of BRINP sequences. Sequences were aligned from the conserved WLL/WLI position near the N-termini of the protein.

	% Identity (similarity) to human		
	hBRINP1	hBRINP2	hBRINP3
H. sapiens			
BRINP1	100	53 (70)	52 (69)
BRINP2	53 (70)	100	71 (84)
BRINP3	52 (69)	71 (84)	100
D. rerio			
Brinp1	84 (93)	53 (71)	53 (71)
Brinp2	51 (71)	65 (81)	67 (80)
Brinp3a	50 (67)	61 (76)	77 (88)
Brinp3b	50 (70)	63 (80)	69 (85)
Brinp3c	52 (70)	68 (81)	64 (77)

1.3. Syntenic relationships of brinps

The current human (Grch38) and zebrafish (Zv9) genome assemblies were used to examine the loci and neighbours of the *brinp1*, *brinp2* and *brinp3* genes. Schematics of chromosomal locations of zebrafish and human *brinps* are shown in Fig. 2. Results of this analysis indicated that the locus arrangements of *brinp* genes are highly conserved. In both humans and zebrafish *brinp1* is flanked at the 5′ end by the neural MACPF protein, *astrotactin 2* (*astn2*) and protease, *pappalysin 1* (*pappa1*) (Fig. 2A). *brinp2* is flanked at the 5′ end by *astrotactin 1 (astn1)* and *pappalysin 2 (pappa2)*. In mammals *BRINP2* and *BRINP3* are on the same chromosome 25 Mbp apart. In zebrafish, *brinp3c* is located on the same chromosome as *brinp2*, whereas the *brinp3a* and *brinp3b* are present on a different chromosome (Fig. 2B). This strongly suggests that *brinp3c* is the orthologue of mammalian Brinp3.

1.4. Spatiotemporal expression of brinp1 during zebrafish development

In adult rats, *Brinps* are highly expressed in neural tissues such as the cerebrum, cerebellum and spinal cord. However, during rodent development *Brinps* are broadly expressed throughout the brain, and weakly detected in some non-neuronal tissues (Kawano et al., 2004). To relate the rodent *Brinp* expression pattern to zebrafish, we determined the temporal and spatial expression of zebrafish *brinps* by reverse transcription PCR (RT-PCR) in embryos and various adult tissues. In addition, we performed whole mount RNA *in situ* hybridisation (WISH) during embryonic development. Results from RT-PCR analysis revealed that *brinp1* mRNA is present at the 1-cell stage indicating maternal deposition. Expression is maintained throughout development and is observed in both the adult head and body (Fig. 3). To determine which adult tissues express *brinp1*, RNA was extracted from adult brain, eyes, heart, gill, gut, muscle and skin. Following cDNA synthesis and PCR on tissue libraries, *brinp1* was detected in the brain, eyes, heart, gill and muscle. Smaller PCR products were detected in heart and muscle. We sequenced this product and found *brinp1* is alternatively spliced from exon 2 to exon 8 in these particular tissues.

WISH analysis demonstrated that *brinp1* is ubiquitously expressed in embryos from 6 hours post fertilisation (hpf; shield stage) to 16 hpf (Fig. 4A–C), and is broadly expressed in the brain at 24 hpf (Fig. 4D) and muscle (Fig. 4Di). Cross-sections of 24 hpf embryos confirm expression in the muscle (4F). By 48 hpf *brinp1* mRNA is restricted to specific regions of the brain such as the midbrain, mid-hindbrain boundary and hindbrain (Fig. 4G). At this stage *brinp1* is expressed in various non-neural tissues such as the primordial pectoral fin buds, neuromasts and the median fin fold (Fig. 4G–I). At 72 hpf, the same expression pattern is observed (Fig. 4J–L). Similar to zebrafish *brinp1*, the mouse *Brinp1* orthologue exhibits ubiquitous expression in early development (E9.5) and specific expression in areas of the brain such as the olfactory bulb, cerebellum and cerebral cortex (Kawano et al., 2004). Such similarities in expression patterns in both species suggest a conserved function in the brain.