Zebrafish Angiotensin II Receptor-like 1a (agtrl1a) is expressed in migrating hypoblast, vasculature, and in multiple embryonic epithelia

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Abstract

The human gene AGTRL1 is an angiotensin II receptor-like gene expressed in vasculature, which acts as the receptor for the small peptide APELIN, and a co-receptor for Human Immunodeficiency Virus. Mammalian AGTRL1 has been shown to modulate cardiac contractility, venous and arterial dilation, and endothelial cell migration in vitro, but no role in the development of the vasculature, or other tissues, has been described. We report the identification and expression of the zebrafish ortholog of the human gene AGTRL1. Zebrafish agtrl1a is first expressed before epiboly in dorsal precursors. During epiboly it is expressed in the enveloping layer, yolk syncytial layer and migrating mesendoderm. During segmentation stages, expression is observed in epithelial structures such as adaxial cells, border cells of the newly formed somites, developing lens, otic vesicles and venous vasculature.

Keywords: Zebrafish; Epiboly; Cell migration; G protein-coupled receptor; Somitogenesis; Delta/Notch; Gene expression; Veins; Vasculature; Embryogenesis; Angiotensin receptor

1. Results and discussion

G protein-coupled receptor proteins (GPCRs) are multiple-pass transmembrane domain proteins involved in the signal transduction of many major developmental pathways (Strosberg, 1996; Malbon, 2005). Angiotensin receptor proteins are GPCRs that bind short polypeptide ligands (Angiotensins) and have been intensively studied due to their role in regulation of blood pressure (Thomas and Mendelsohn, 2003). Angiotensin receptors define a vertebrate subfamily of the GPCRs that includes Angiotensin II receptor-like 1 (AGTRL1), also known as APJ or Mr (Devic et al., 1996; Devic et al., 1999; O’Dowd et al., 1993), and Angiotensin II type 1 and 2 receptors (AGTR1, AGTR2). Control of cell migration is a feature of many angiotensin receptor subfamily members, for example endothelial cells (ECs, Benndorf et al., 2003), vascular smooth muscle cells (VSMCs, Chassagne et al., 2002) and neurons (Cote et al., 1999). In addition, the zebrafish chemokine SDF-1 and its angiotensin subfamily receptor odysseus/Cxcr4b are involved in the directional migration of primordial germ cells (Doitsidou et al., 2002; Knaut et al., 2003) and angiotensin II acts through AGTR1 to stimulate migration of rat VSMCs (Jing et al., 2002). Angiotensin receptors are also involved in vascular smooth muscle cell proliferation during development (Sayeski and Ali, 2003).

AGTRL1 binds the short polypeptide ligand Apelin (Tatemoto et al., 1998) and acts as a co-receptor for human and simian HIV strains (Choe et al., 1998; Edinger et al., 1998). Recently, a role for Apelin/AGTRL1 has emerged in control of cardiovascular function (Chen et al., 2003). Apelin is known to be a potent stimulator of cardiac contractil-
ity (Szokodi et al., 2002), an arterial and venous dilator (Cheng et al., 2003) and can stimulate gastric cell differentiation in vitro (Wang et al., 2004). In mouse, Agtrl1 has been shown to be an early marker of vascular development (Devic et al., 1999; Saint-Geniez et al., 2003; Saint-Geniez et al., 2002). However, targeted mutation of Agtrl1 in mice had no observable effect on embryonic development or histology of various tissues that were examined (Ishida et al., 2004), and so potential embryonic functions for AGTRL1 remain unclear. In this paper we report the discovery and developmental expression of the zebrafish ortholog of human AGTRL1 in a screen for genes expressed during somitogenesis.

1.1. Isolation of the zebrafish ortholog of human AGTRL1

In a whole mount in situ transcript hybridization screen for genes involved in somitogenesis and neurogenesis (Tamme et al., 2001), we discovered a cDNA clone (BR131) of a gene with sequence similarity to the Angiotensin Receptor-like subfamily of the GPCRs, expressed at high levels in the epithelia separating newly formed somites and at lower levels in other epithelial structures (Figs. 2, 3). We isolated a cDNA clone containing the entire open reading frame from a 9 to 16 h post fertilisation (hpf) library and compared its putative peptide sequence with those of other Angiotensin Receptor-like genes (Fig. 1A). Phylogenetic analysis revealed that the zebrafish gene is orthologous to the human gene AGTRL1 (Fig. 1B; see Section 2). Recent data base searches have revealed another zebrafish AGTRL1 ortholog, therefore we have named the gene studied in this manuscript zebrafish agtrl1a (encoding the putative protein Agtrl1a).

1.2. agtrl1a expression during blastula and gastrula stages

To define the tissues in which agtrl1a is expressed during embryonic development we performed whole mount in situ transcript hybridisation on embryos throughout the first 24 h post fertilisation (hpf). No maternal agtrl1a expression was detected prior to MBT. Zygotic agtrl1a expression was first detected at the oblong stage (3.7 hpf) in a radially asymmetric domain in the deep cells (Fig. 2A,A’), persisting to dome stage (4.3 hpf, Figs. 2E,E’); this expression domain was absent in Maternal-Zygotic one-eyed pinhead (MZoep) embryos, indicating a dependence on Nodal signalling (Gritsman et al., 1999); (data not shown). agtrl1a expression is located dorsally, as shown by double staining with probes for transcripts of agtr1a and for the dorsal marker chordin (chd, Schulte-Mmerker et al., 1997); (Figs. 2C and D). At dome stage (4.3 hpf), agtrl1a was also expressed in a superficial layer of cells, likely the enveloping layer (EVL) (Fig. 2E,E’). Starting at germ-ring stage (5.7 hpf), agtrl1a transcripts accumulated in the margin (arrowheads), and could be observed in flattened ring shapes adjacent to the yolk over the animal pole (arrows; Fig. 2F,F’). To help distinguish these cell types, thin sections were cut and examined, and were consistent with expression in the EVL, yolk syncytial layer (YSL) and the marginal hypoblast (Fig. 2G1-3).

During epibolic gastrulation (6–10 hpf), agtrl1a was expressed in a dispersed hypoblastic cell population, the majority of which appeared to migrate animaly and dorsally away from the margin (Figs. 2H–K’). These cells were absent in MZoep embryos, indicating a mesendodermal identity, but the larger flattened, predominantly animal pole staining pattern remained, confirming this as YSL expression (Fig. 2I’). Some of the agtr1a-positive hypoblastic cells accumulated at the dorsal midline (arrow), and others appeared to form adaxial precursors (arrowheads) or contribute to the prechordal plate (asterisks; Fig. 2J’ and K’). The arrangement and number of these cells was not dramatically perturbed in either spadetail (spt) or casanova (cas) mutant embryos (data not shown), indicating that they are not entirely of anterior trunk paraxial mesoderm, or endodermal fate (Dickmeis et al., 2001; Griffin et al., 1998; Kikuchi et al., 2001). Expression of agtr1a at the 2 somite stage was consistent with cephalic mesendodermal identity (Fig. 2L). Thus, migratory cells are located in regions that normally contribute to head mesenchyme, pharyngal endoderm, vasculature and myeloid blood lineages. We conclude that expression of agtr1a in migrating cells is a feature conserved with other angiotensin receptor subfamily members.

1.3. agtrl1a expression during segmentation and pharyngula stages

In segmentation and pharyngula stage embryos, agtr1a was expressed in a range of epithelial tissues (Fig. 3). At 14 hpf, agtr1a expression was evident in the otic vesicle (Figs. 3A and C) and in the epithelium covering the retina, being maintained as these cells invaginated to form the lens (Figs. 3A, B, and D). We observed agtr1a expression in the tailfin primordium before 24 hpf (Fig. 3C). agtr1a was also expressed in vascular primordia and then in forming vessels such as the middle cerebral vein (Fig. 3E) and primary caudal vein (Figs. 3F and G). We did not observe expression in the dorsal aorta, suggesting that agtr1a expression may be restricted to venous vasculature. Thus, vascular expression is evolutionarily conserved for vertebrate AGTRL1 genes. In addition to cranial vasculature, clusters of agtr1a-positive cells were observed in the pharyngeal region at 24 hpf (Fig. 3H). To test whether these cells might be the endoderm of the pharyngeal pouches, we examined expression in oep and in cas, where pharyngeal expression of nkx2.5 is absent due to failure of endodermal differentiation (Alexander et al., 1999; Schier et al., 1997). We find that agtr1a expression was absent in both oep and cas mutants at 24 hpf, specifically in the pharyngeal region and presumptive mouth (Figs. 3I,J, asterisks).
1.4. *agtr1a* expression and regulation during somitogenesis

During somitogenesis, *agtr1a* was expressed in the posterior presomitic mesoderms (PSM) and lateral tail bud, adaxial cells, and in stripes in 3–5 of the most recently formed somitic epithelia (Figs. 3A and C, Fig. 4). In addition, *agtr1a* shows variability in the anterior PSM where new somites are forming, with either one or two strong stripes of expression (Figs. 4A and B). To establish the part of a somite in which *agtr1a* is expressed we stained embryos simultaneously for *agtr1a* and *myod* (Weinberg et al., 1996) or *dld* (Dornseifer et al., 1997) expression. The stripes of *agtr1a* are complementary to those of *myod* (Fig. 4A and B) and overlap those of *dld* (Fig. 4C). Note, however, that *agtr1a* expression overlaps that of *myod* in adaxial cells (Figs. 4A and B). Thus, *agtr1a* is expressed in the anterior half of newly formed or forming somites. To test whether the variable
PSM expression observed reflects dynamic changes like her1 cyclic gene transcription (Holley et al., 2000; Sawada et al., 2000), we stained embryos simultaneously for agrt1a and her1. agrt1a expression domains in the PSM were always static in comparison to the wavefronts of her1 expression, indicating that agrt1a is not expressed cyclically (Fig. 3D). Combined, these data indicate that cells that will form the anterior epithelial border of a somite begin to express agrt1a approximately an hour before the morphological appearance of the furrow.

A number of mutations are known to affect the formation of somites (van Eeden et al., 1996). Mutations affecting Notch signalling (e.g. beamter bealdeletac) (Julich et al., 2005), deadly seven (desnotch1a) (Holley et al., 2002), mind bomb (mib) (Itoh et al., 2003), and after eight (aexldeletad) (Holley et al., 2000) lead to a loss of coordination
of cyclic gene expression in the cells of the PSM (Jiang et al., 2000; Oates and Ho, 2002), while mutation of the fused somites (fss/tbx24) gene results in loss of any division of paraxial mesoderm into somites (Nikaido et al., 2002). To understand the regulation of agril1a in the paraxial mesoderm, we compared agril1a expression in mutant backgrounds to wild-type (Figs. 4E–I). Reduced “salt and pepper” expression of agril1a was observed in the somitic regions of mutant embryos with defective Notch signalling (Figs. 4F–H) while no expression could be seen in this region in the fss/tbx24 mutant (Fig. 4I). agril1a expression in adaxial cells was not affected in any of the above mutant backgrounds. Thus, agril1a expression is downstream of mechanisms controlling the patterning of paraxial mesoderm into segments.
2. Experimental procedures

2.1. Cloning of agtr1a cDNA

Clone BR131 was isolated in a whole mount in situ transcript hybridisation screen for genes involved in somitogenesis and neurogenesis (Tamme et al., 2001). Primers BaRa1 (5'-ACTACAGTAGACG ACAC TGGG-3' and 5'-TCTTCAGCACATGAAAAGGCG-3') were designed from BR131 sequence and used to screen -bacteriophage sub-libraries (Lardelli, 2002) generated from a 9 to 16 hpf library kindly donated by D. Grunwald (University of Utah, Salt Lake City). A cDNA clone containing the entire open reading frame of agtr1a was subsequently isolated and sequence submitted to GenBank with the Accession No. DQ983235.

2.2. Phylogenetic analysis

AGTRL-related DNA sequences (accession numbers below) were aligned using ClustalW. Bayesian analysis was conducted using the MRBAYES v3.1.2 program with Danio rerio rhodopsin as an outgroup (Huelsbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Markov Chain Monte Carlo convergence was conducted essentially as described (Larget and Simon, 1999) using a General Time-Reversible model with invariable sites and gamma distribution values included (GTR + I + G; Tamura and Nei, 1993; Yang, 1993). We ran four simultaneous MCMC chains for 40000 generations five times to generate five distinct data sets, each with distinct random seeds. Trees were sampled every 40 generations, and a total of 751 trees were saved for each data set. The data sets were summarized and statistically analysed independently and in conjunction to confirm consistency between runs. The combined dataset summary was used to determine the most probable tree topology, branch lengths, and to calculate final Bayesian posterior probabilities. All MCMC analysis was performed using MRBAYES v3.1.2. For our phylogenetic analysis we have used the agtr1a sequence previously deposited by others (see accession number BC056308) but note that we believe this to contain the entire open reading frame (see Fig. 1A), agtr1b (BC097125), C. auratus-somatostatin-r (AF252879), H. sapiens-somatostatin-r2 (BC019610), M. musculus-G-protein-coupled-r1 (BC032934), H. sapiens-G-protein-coupled-r1 (BC067833), X. laevis-mesenchyme-associated-serpentine-r (XLXMSRGEN), M. musculus-angiotensin-rilike1 (BC039224), M. musculus-angiotensin-r1 (BC036175), X. laevis-angiotensin-II-r (S73274), H. sapiens-b-chemokine-r-CCR4 (AB023889), M. musculus-chemokine-r (MMU15208), M. musculus-il8-r-beta (BC051677), H. sapiens-chemokine-orphan-r1 (BC033661), D. rerio-rhodopsin (NM_131084), H. sapiens-AGTRL1 (NM_005161).

2.3. In situ transcript hybridization on whole-mount zebrafish embryos

Embryos were raised at 28.5°C and staged as previously described (Kimmel et al., 1995). In situ transcript hybridisation was performed as described (Tamme et al., 2001) using single-stranded RNA probes labelled with digoxigenin-UTP or FITC-UTP (Roche Ltd, Basel, Switzerland). Riboprobe synthesis was conducted directly from cDNA clones in the Bluescript SK vector (Stratagene) or were synthesised used T7 RNA polymerase after PCR amplification of the template with M13 and M13R primers.

2.4. Sectioning of embryos

6 hpf embryos were prepared routinely for paraffin embedding after in situ hybridisation against agtr1a. 5 μm thick sections were cut with a rotary microtome.
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References


