Comparative Expression Pattern of Two Vestigial-Like 2 Genes in Zebrafish

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Abstract The vestigial Vg gene, initially characterized in Drosophila, encodes a transcription co-factor which is crucial for wings development. Vg binds via its Sd interaction domain (SID) to the Scalloped (Sd) transcription factor and its vertebrate homolog Tef1. Previous studies identified several vertebrate genes sharing high homology with Drosophila Vg SID such as Vgl (for Vg-like), TONDU (also known as Vgl1) or Vito-1 (also known as Vgl2). In order to investigate the role of vestigial-like 2 (vgll2) in zebrafish muscle development, we managed to clone and characterize two zebrafish vgll2 homolog genes, Vgll2a and Vgll2b. Alignment data showed high sequence homology of Vgll2a to vertebrates Vgll2 sequences. In situ hybridization showed that the two investigated Vgll2 genes had a similar expression pattern: they were first detected in adaxial cells (11 hpf), than expanded laterally in somites and at the end of segmentation, both genes were expressed in additional structures including head muscles and fin buds. In addition, different expression patterns of the two genes were observed. Vgll2a was expressed in branchial arches precursor streams, derived gill muscles and hypothalamic precursors. Vgll2b was expressed in notochord at 14 hpf and regressed following notochord maturation at 18hpf. Furthermore, the genetic regulation of vgll2 genes was analysed using smu mutants and data revealed that both genes are regulated via the Hedgehog signaling pathway.

Keywords Zebrafish; Daniorerio; Vgll2, Vgll, Vestigial, Somite, Myogenesis, Skeletal Muscles, Jaws Muscles, Branchial Arches, Gill Muscles, Notochord, Hypothalamus

1. Introduction

Invertebrates and vertebrates vestigial (vg) and vestigial-like (vgl) genes are involved in embryonic patterning and cell fate determination.

In Drosophila, the nuclear protein Vestigial (Vg) is a cofactor of Scalloped (Sd) and plays a central role in the development and patterning of wings [1,2]. Several works reported that Vg is also involved in the specification of a subset of flight muscle. Indeed, Vg is expressed in myoblast that will contribute to indirect flight muscle and controls the expression of Cut in the direct flight muscle [3,4]. In vertebrates, the expression of Tef1, the homologue of Sd, is not restricted to a specific tissue however it controls the expression of several genes during development in skeletal muscle and placenta through binding to a M-CAT element in regulatory regions of these genes [5-8]. This has lead to the hypothesis that tissue-specificity of Tef-1 control is due to interaction of Tef1 with tissue-specific cofactor(s). Four genes have been identified in mouse which contains a sequence motif highly related to the Sd/Tef1 Interaction Domain (SID) of Vg. These genes have been named vgll1, vgll2, vgll3 and vgll4. vgll1 and vgll3 are expressed in placenta [9] while vgll2, also known as VITO1, is expressed in developing somites and muscle, in branchial arches and in limb buds [6,10,11]. The fourth gene, vgll4 has been cloned that has a widespread expression but is the only vertebratesvgllgene expressed in heart muscle [12]. Functional studies have revealed that Vgll2 and Vgll4 interact with Tef1 through their SID domain and with Myocyte enhancer factor 2 (Mef2) [10-12]. Vgll4 modulates Tef1 activity in vitro [12] and Vgll2 activates muscular differentiation and is translocated in the nucleus during this process in vitro [10,13]. Thus Vgll2 appears to be the skeletal muscle specific cofactor of Tef-1 in vertebrates. In addition, a recent report has described the expression of Vgll2 in the skeletal myogenic lineages of the chicken embryo under the control of myogenic factors [14].

In order to characterise genes involved in the control of muscle development, we have cloned vgll2 in zebrafish. Here, we report the cloning and expression of two zebrafishvgll2 genes. Both genes share high homology with mammalian Vgll2 and are expressed in body, head and fin bud muscles during embryonic development. In addition to this expression in muscle, vgll2a is also expressed in
2. Material & Methods

2.1. Zebrafish Breeding

Fish were obtain from a local pet shop and were breed following classical condition with a 14h/10h day/night period. Embryos were obtained from pair mating and developmental stages determined according to Kimmel [27].

2.2. Molecular Biology

PCR primers were deduced from sequence of zebrafish ESTs and genomic fragments sharing homology with mouse Vgll2 identified in databases Ensembl[28]and WashU-Zebrafish Genome Resources Project [29]. mRNA were extracted from embryos of mixed developmental stages in the segmentation period using and Trizol reagent (Life Technologies). RT-PCR were performed with 1µg of total RNA using ExpandRT (Roche) followed by PCR reaction (Qbiogene). PCR fragments were cloned in pGEM-T vector (Promega). Sequences were performed using BigDye v1.1 fluorescent sequencing kit from Applied Biosystems.

Alignments were performed with the ClustalX program [30] with default settings. Alignment was decorated with Boxshade software [31]. Vgll2a and vgll2b names have been approved by Zebrafish gene name nomenclature committee.

2.3. In Situ Hybridisation

Antisense DIG probes were synthesised with appropriate RNA polymerase (Roche). In situ hybridisations were performed as previously described [24] and signal revealed with NBT/BCIP (Roche).

Gene’s expression in wild-type and smu mutants has been quantified using ImageJ. Image was converted to greyscale after background subtraction and then expression intensity quantified in somites using the Fire LUT (Schröter et al., 2012). Statistical significance of relative expression was evaluated using a Mann-Whitney non-parametric test (Prism 6.0, Graphpad) [32].

3. Results and Discussion

3.1. Molecular Characterisation of Two Vgll2 Genes in Zebrafish

We identified two putative vgll2-related sequences in Ensembl and ESTs WashU databases. We deduced primers from these sequences and used them to clone corresponding cDNAs by RT-PCR. Both cDNAs encode a protein containing a SID and sharing high homology with vertebrates Vgll2 (Fig. 1A). The two zebrafish Vgll2s share a rather weak identity (39%). One deduced peptide appears to be much closer to vertebrate Vgll2 and was named Vgll2a. Vgll2a shares 64 to 72 % identity with vertebrates Vgll2. The second gene was named vgll2b, the deduced peptide (Vgll2b) shares 39 to 43 % identity with vertebrate Vgll2. When comparison is restricted to the putative SID domain, conservation of zebrafish Vgll2 is much higher with vertebrate Vgll (92 to 100%) and with Drosophila Vg, 67% and 71% for Vgll2a and Vgll2b respectively (Fig. 1B). Vgll2a and Vgll2b SIDs share 96% identity. Sequence homology with other Vgll is limited to the SID (not shown).
From RT-PCR experiments, we obtained two cDNAs for **vgll2a** differing only by their 3’ extremities. Comparison with genomic sequence information from Ensembl database reveals that they are obtained after alternate splicing of exon 4 and 5 (Fig 1C). The peptide deduced from exon 5 shows high homology with the C-terminus of Xenopus and human Vgll2 (Fig. 1A). When exon 4 is used, exon 5 is included in the 3’ untranslated region and both cDNAs use the same polyadenylation site. Vgll2b gene structure is simpler with only 3 exons, however, no stop codon has been found within the open reading frame. Comparison of cDNA and genomic sequences suggests that the oligo-dT oligonucleotide used for reverse transcription has primed in a A-rich region of the cDNA. Presented sequence is thus partial. The sequence of a vgll2b EST suggests that an alternate transcript with a readthrough of genomic sequence located after exon 2 may exist. We searched for such an alternate splicing by 3’ RACE strategy and identified such a cDNA. 

**In situ** hybridizations performed with probes covering vgll2b exon 1 and 2, vgll2b exon 3 and all three exons (see Fig. 1C) reveal the same expression pattern indicating that if this readthrough transcript has a physiological significance, it is not associated with a specific tissue expression (not shown). It is to note that even if gene organization of vgll2a and vgll2b is similar, a first short exon and a second exon containing the SID, splicing sites are not conserved between these genes (Fig. 1C).

### 3.2. Expression during Development

We have analysed spatial and temporal expression pattern of zebrafish vgll2a and vgll2b by whole mount **in situ** hybridisation during embryonic development up to 72 hpf using labelled antisense riboprobes. Both vgll2 genes are first detectable at the 4-somite stage (~11.5 hpf) in somites, in adaxial cells lining the notochord (Fig. 2A, B). This expression pattern extends as new somites are formed (Fig. 2C, D) and is similar to that of myogenin and of all three mef2 genes identified in zebrafish albeit vgll2 genes expression timing is closer to mef2D[15,16]. Expression of vgll2 in cardiac precursors has never been detected. This observation is similar to the one made in developing mouse embryos and contrast with mef2A and mef2C expression which are detectable in this structure by 14 hpf[15]. As somites mature, expression extends laterally in somites from 15 hpf (Fig. 2E -H). Expression in whole somite is still observed at 24 hpf and decreases by 36 hpf. By 72 hpf both vgll2 genes are expressed in fin buds mesenchyme (Fig. 2K, L).

In somites, vgll2 genes expression pattern is similar to the one of myogenin and mef2 myogenic factors [17,18]. This is in agreement with the involvement of Vgll2 in the control of muscle differentiation program suggested in mouse [10]. This expression is also similar to the recent study in xenopus embryos, where Vgll2 is expressed in the skeletal muscle lineage downstream of myogenic factors [19].

At the 10-somite stage (14 hpf) vgll2b begins to be expressed in anterior part of the notochord (Fig. 2D) and rapidly in the entire notochord (Fig. 2F,H). From 18hpf, expression regresses in the anterior aspect of notochord (Fig. 2I). This expression remains in the tip of the tail until 24 hpf (Fig. 2J). This dynamic expression appears to be a novel function acquired by vgll2b since Vgll2 expression has never been detected in axial structure in mouse during development. We have never detected vgll2a expression in notochord.

From the 18-somite stage, vgll2 genes are also expressed in several developing head structures. Vgll2a is expressed in branchial arches precursor’s streams from 18-somite stage (Fig. 2G) and in eye muscle at later stages (Fig. 3C-F). From 24 hpf, vgll2a is also expressed in hypothalamus precursors (Fig. 3A). By 36 hpf, vgll2a expression in hypothalamus is more precisely seen in pre-optic hypothalamus and in migrating neuro-hypophysis precursors (Fig. 3C). This pattern is similar to the one of xenopus Vgll2 where it occurs in the branchial arches and the stomodeal-hypophyseal anlage [19]. Expression in these territories persists until 72 hpf (Fig. 3D-F). This striking expression reminds the expression of another myogenic factor, myf5, in mouse hypothalamus [20]. From the 18-somite stage, vgll2b is expressed in bilateral clusters in the head (Fig. 2H, 3G). At 48 hpf, expression is detected in several head muscle precursors (Fig. 3H). At 72 hpf, these muscles can clearly be identified as jaw muscles and eye muscles (Fig. 3I). It is to note that in head, vgll2 genes are expressed in mutually
exclusive subsets of muscle suggesting they acquired specialized functions. A recent report has detected zebrafish vgll2a expression in the pharyngeal endoderm and ectoderm surrounding the neural crest derived mesenchyme of the pharyngeal arches [21].

3.3. Genetic Control of vgll2 Expression

To identify genetic pathways that could be involved in the control of vgll2 genes expression. We analyzed the expression of vgll2 genes in acerbellar(ace) and slow muscle omitted (smu) mutants. Acmutation results in the production of an inactive Fgf8 [22] and an altered fast myogenesis[23,24]. Expression of vgll2 genes appears unchanged in ace mutants indicating they are not regulated by Fgf8 (not shown). Smoothened is part of the Hedgehog (Hh) receptor complex and is mutated in smu mutant [25,26], this mutation produces a blockade of all Hh signalling and, among other phenotypes, in a suppression of myoDexpression in adaxial cells and then of slow muscle development [25]. We analyzed the expression of vgll2 genes at the 15-somite stage, when these genes are both expressed in adaxial and somitic muscle progenitors. In both cases, expression in adaxial cells is suppressed in smuembryos (Fig 4). Strikingly, vgll2b is also suppressed in somites (Fig. 4D). The mechanism underlying this regulation of vgll2b in fast muscle precursors by Hh signalling remains unknown. Expression of vgll2b is not affected in notochord of smu embryos (Fig. 4D). Relative expression of vgll2a and vgll2b has been assessed in somites of smu mutants. Expression of vgll2a in smu embryos and wild-type were similar (relative expression 91.7 ± 26.3 %; p=0.4; n=10). Expression of vgll2b in somites of smu mutants was significantly lower compared to expression in wild-type embryos (relative expression 27.9 ± 17.1 %; p<0.01; n=10).

4. Conclusion

In summary, we have identified two zebrafish vgll2 orthologous. These genes are expressed in trunk and head muscles precursors during development. Expression in trunk muscle follows myogeninand mef2 genes expression timing. In the head, vgll2 genes are expressed in exclusive muscle, vgll2a being expressed in gill and a subset of eye muscles while vgll2b is expressed in jaw and other eye muscles. In addition, vgll2 genes are expressed in non-muscle structures, vgll2a in ventral hypothalamus and vgll2b in notochord. Expression in such structures has never been described in mouse and reveals new functions acquired by these factors during evolution which deserve additional investigation. Finally, we have shown that in trunk muscle both vgll2 genes are regulated by Hh signalling, but again differentially, vgll2a is controlled by Hh in adaxial cells as are myoD and myogenin, while vgll2b requires Hh signal to be expressed in both adaxial cells and somites.

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