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A role of D domain-related proteins in differentiation and migration of embryonic cells in *Xenopus laevis*

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ABSTRACT

We have characterized a cDNA clone, *rdd* (*repeated D domain-like*), that encodes for a secretory protein consisting of repeated domains of cysteine-rich sequence. Whole-mount *in situ* hybridization analysis revealed that *rdd2*, *rdd3* and *rdd4* are transiently expressed in the ventral and lateral mesoderm and the overlying ectoderm at the late gastrula and tailbud stages. Morpholino oligonucleotide (MO) was used to inhibit the translation of endogenous *rdd3* and *rdd4*, and we found that the circulation of red blood cells completely disappears in the MO-injected tadpoles. Histological analysis showed that formation of the ventral aorta, dorsal aorta and posterior cardinal vein in the trunk region was severely disorganized in these animals. Injection of MO affected the expression of *α-globin*, a terminal differentiation marker of red blood cells, but did not affect the expression of *scl*, *flk-1* or *tie-2*, suggesting that angiopoietic and hematopoietic precursor cells differentiate normally in the rdd-depleted embryo. The transplantation of labeled tissues followed by tracing of the donor cells revealed a role of rdds in migration of the embryonic angioblasts and myeloid cells. These observations first demonstrate the role of the novel cysteine-rich proteins in migration of the embryonic cells.

1. Introduction

Differentiation of ventral blood islands (VBI) in amphibian embryo is regulated primarily by bone morphogenetic protein (BMP), a member of the TGF-β superfamily (Dale et al., 1992; Jones et al., 1992). BMP signaling activates essential genes that are related to hematopoietic and angiopoietic lineages (Walmsley et al., 1994; Maéno et al., 1996; Mead et al., 1998; Iraha et al., 2002). Among downstream factors of BMP signaling, stem cell leukemia (SCL), a basic helix-loop-helix transcription factor, is a key factor involved in the blood island formation and it starts to express as early as the neurula stage (st. 15–16) in the VBI mesoderm (Mead et al., 1998). Another transcription factor, GATA-2, is a direct target of the BMP signal (Friedle and Knochel, 2002) and is expressed in mesoderm and ectoderm cells in a broad area of the *Xenopus* embryo at the neurula stage (Walmsley et al., 1994). Furthermore, GATA-2 binds the enhancer sequence of *scl* and induces the expression of *scl* in mice (Gottgens et al., 2002), indicating that GATA-2 has a central role in the development of the VBI.

Studies on amphibian embryos have also shown that tissue–tissue interaction is important for blood cell differentiation. For example, the ventral marginal zone (VMZ) at the early gastrula stage contains blood cell precursor cells, but they need interaction with animal pole tissue to differentiate into erythrocytes and leukocytes (Maéno et al., 1992, 1994a;

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Miyanaga et al., 1998). Exogastrula embryos induced in a high-salt medium lose the interaction of mesoderm and ectoderm, and such embryos lack the differentiation of erythrocytes (Kikkawa et al., 2001). These findings suggest the existence of a factor(s) in ectodermal cells that is necessary for the differentiation of blood cells in the ventral mesoderm (Maéno, 2003), but the nature of this factor(s) is unknown.

In the present study, therefore, we predicted the existence of uncharacterized factors that are expressed in ventral tissues at the early neurula stage and involved in the development of blood cells and blood vessel cells. We made a subtracted cDNA library from early neurula embryos and isolated several cDNA clones that were expressed in the ventral region of the neurula embryo. Three related clones, referred to as *rdd* (*repeated D domain-like*) 2–4, were characterized in the present study because *rdd* transcripts were localized to the ventral and lateral regions of the neurula embryo. Analysis of the nucleotide sequences showed that *rdd2*, 3 and 4 code for predicted secretory proteins consisting of repeated cysteine-rich domains. Inhibition of endogenous expression of rdd3 and rdd4 by using antisense Morpholino oligonucleotides revealed that these proteins are essential for the differentiation of blood vessels and blood cells in early embryogenesis.

2. Results

2.1. *Isolation of cDNA related to the differentiation of lateral and ventral tissues*

It has been shown in amphibian embryos that BMP-4 is an essential factor for morphogenesis of lateral and ventral tissues and for initiation of the hematopoietic program (Mead et al., 1998; Maéno et al., 1994a, b; Walmsley et al., 2002). Expression of BMP-4 in the prospective ventral region of the embryos peaks at the gastrula stage (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994) and decreases at the neurula stage. Therefore, we predicted the existence of unknown factors that positively regulate the formation of ventral tissues at neurula stage. In order to obtain candidate cDNA clones for such factors, we made cDNAs from early neurula embryos that had been injected with *BMP-4* RNA. The cDNAs were then hybridized with driver RNA extracted from early gastrula embryos. Excess *BMP-4* RNA was also added to remove cDNA derived from the injected *BMP-4*. Further screening was performed by whole-mount *in situ* hybridization analysis to select cDNAs that were expressed in the ventral but not the dorsal part of the embryo at neurula stage. In 48 randomly selected clones, 8 clones were preferentially expressed on ventral side of the embryos (S-Fig. 1). The nucleotide sequences of these 8 clones were analyzed, and it was found that the sequence of one cDNA clone completely matched the sequence of *rdd2* (AF465786). Three other sequences related to *rdd2* (*rdd1*, AF465785; *rdd3*, AF465787; *rdd4*, AF465788) were also identified in public databases. These cDNAs were originally isolated by yeast-based selection to identify secretory proteins (Sun et al., 1999).

Sequence analysis revealed that each cDNA encodes for a protein containing a putative signal sequence followed by repeats of a cysteine-rich domain (Fig. 1). These repeats are distantly related to the D domains found in von Willebrand factor (vWF) (26–30% identical, 39–50% similar to the D domain of vWF) and other secreted proteins, such as mucin and zonadhesin (Verweij et al., 1986; van de Bovenkamp et al., 1998; Hardy and Garbers, 1995; Gao and Garbers, 1998). Rdd proteins are unusual in that, while other D domain proteins contain additional unrelated domains, rdds are composed only of D-like (DL) domains. Comparison of the amino acid sequences of rdd3 and rdd4 shows that they are highly conserved (95–100% identical in whole coding region). Compared to rdd3, rdd4 has an extra DL domain (DL2) and a few sequence differences in the 5′ UTR (Fig. 1A). Although no genomic data for these clones are available, it is possible that these two cDNAs represent different splice products of the same gene.

2.2. *Expression of rdd2 and rdd4 in embryogenesis*

We performed Northern blot analysis to examine the expression patterns of *rdd2*, *rdd3* and *rdd4* in *Xenopus* embryogenesis (Fig. 2A). The *rdd2* probe revealed a single band of approximately 0.7 kb in size, and the *rdd4* probe revealed two bands of 0.9 kb and 1.1 kb in size. Since *rdd3* and *rdd4* shared the nucleotide sequence in a great degree, it was pertinent to predict that the 0.9 kb-band corresponds to *rdd3* transcript and that the 1.1 kb-band corresponds to *rdd4* transcript. Expression of these messages was not detectable at the early gastrula stage (st. 10), but it increased during early neurula stages and peaked at the early tailbud stage. To examine the spatial pattern of *rdd* expression in embryos, whole-mount *in situ* hybridization was performed using *rdd2* and *rdd4* antisense riboprobes (Fig. 2B–P). Expression of *rdd2* and *rdd4* (and *rdd3*) was not observed in embryos until the early gastrula stage (Fig. 2B and J). No maternal message was detected at all in *in situ* hybridization or RT-PCR analysis (data not shown). Weak expression of *rdd2* and *rdd4* was first visible in the prospective ventral marginal zone at late gastrula stage (st. 12, Fig. 2C and K), and expression became stronger in the same region by neurula stage (st. 13, Figs. 2D and 3L; st. 15, Fig. 2E and M). At early tailbud stage, the highest level of expression was seen in the lateral and ventral trunk region (st. 19, Fig. 2F and N; st. 23, Fig. 2G and O). At this stage, no expression was observed in the somites or more dorsal tissues, nor was transcripts detected in the head or tail region (Fig. 2F, G, N and O). The expression level decreased through tailbud stages, and weak expression was still observed in the lateral plate mesoderm (pronephric region) at the late tailbud stage (st. 32, Fig. 2H and P). Dissection of an early tailbud embryo after whole-mount staining showed the expression of *rdd2* in both the mesoderm and ectoderm (Fig. 2I). We examined the expression of *rdd1* by *in situ* hybridization analysis and found that *rdd1* is strongly expressed in dorsal ectoderm including the central nervous system at the neurula stage (data not shown). This result suggested a distinct role of rdd1 in embryogenesis and we discontinued the experiments on rdd1 since this protein may not be involved in the blood and vascular cell differentiation.

In order to examine whether the expression of *rdd2–4* is regulated by BMP-4 signaling, *BMP-4* RNA or *tBR* RNA was injected into early embryonic cells and the expression of *rdd2*