Darmin is a novel secreted protein expressed during endoderm development in Xenopus

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Abstract

Endoderm development is an area of intense interest in developmental biology, but progress has been hampered by the lack of specific markers for differentiated endodermal cells. In an unbiased secretion cloning screen of Xenopus gastrula embryos we isolated a novel gene, designated Darmin. Darmin encodes a secreted protein of 56 kDa containing a peptidase M20 domain characteristic of the glutamate carboxypeptidase group of zinc metalloproteases. We also identified homologous Darmin genes in other eukaryotes and in prokaryotes suggesting that Darmin is the founding member of a family of evolutionarily conserved proteins. Xenopus Darmin showed zygotic expression in the early endoderm and later became restricted to the midgut. By secretion cloning of Xenopus cleavage-stage embryos we isolated another novel protein, designated Darmin-related (Darmin-r) due to its sequence similarity with Darmin. Darmin-r was maternally expressed and showed at later stages expression in the lens and pronephric glomus. The endoderm-specific expression of Darmin makes this gene a useful marker for the study of endoderm development.

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1. Results and discussion

1.1. Darmin is a novel secreted protein of the glutamate carboxypeptidase family

In metazoans, the endoderm constitutes the innermost germ layer and differentiates into the epithelium of the digestive tract and associated organs (Wells and Melton, 1999; Stainier, 2002; Shivdasani, 2002). Molecular studies in Xenopus have identified multiple factors implicated in endoderm formation. These include transcription factors, such as VegT, Mix1, Mixer, Xsox17, and XGATA5 (Henry and Melton, 1998; Lemaire et al., 1998; Hudson et al., 1997; Zhang and King, 1998; Weber et al., 2000), the RNA-binding molecule Bicaudal-C (Wessely and De Robertis, 2000), and secreted proteins like Nodal-related TGF-β ligands, Chordin, Noggin, and Cerberus (Henry et al., 1996; Sasai et al., 1996; Bouwmeester et al., 1996). Despite intense interest, our knowledge of how the endoderm develops is still limited. This is partly due to a lack of genes uniquely expressed in this germ layer. Therefore, early markers specific for differentiated endoderm are needed. We have recently reported a method, designated secretion cloning, that allows the direct identification of secreted proteins (Pera and De Robertis, 2000). Using an expression cDNA library from Xenopus midgastrula embryos, we identified a novel gene, which we describe here and call Darmin (‘Darm’ means gut in German; GenBank accession number AY166869). It encodes a secreted metalloproteinase that is exclusively expressed in the endoderm and early midgut.

Xenopus Darmin was first identified as a 56 kDa protein in the supernatant of transfected human 293T cells after metabolic labeling with 35S-methionine and 35S-cysteine (Fig. 1A). The full-length Darmin cDNA clone encoded a 489 amino acid protein containing a putative hydrophobic signal peptide at the N-terminus and a predicted peptidase M20 domain (pfam 01546; NCBI Conserved Domain Search; Figs. 1B,D). M20 domains are found in glutamate carboxypeptidases (Rawlings and Barrett, 1995; Sherwood and Melton, 1998). These proteins belong to the group of zinc metallopeptidases that hydrolyze peptide bonds and produce amino acids as products.
depend on zinc for their activity. Glutamate carboxypeptidases release C-terminal glutamate residues from many N-acyl moieties, including peptidyl, aminoacyl, benzoyl, benzoyloxy carbonyl, folyl, and pteroyl groups.

Xenopus Darmin is most closely related to a human hypothetical protein called glutamate carboxypeptidase-like protein 2 (hGCP2, Figs. 1C,D). The two proteins share 56% amino acid identity. hGCP2 has an N-terminal signal...
peptide, which is predicted to be cleaved at the same position as Darmin (indicated by the arrowhead in Fig. 1D). Other hypothetical proteins related to Darmin include human glutamate carboxypeptidase (hGCP, 55% amino acid identity) and homologous hypothetical proteins in mouse, Drosophila melanogaster, Caenorhabditis elegans, Schizosaccharomyces pombe, and Bacillus halodurans (55, 55, 48, 42 and 32% amino acid identity, respectively). All these sequences contain a predicted peptidase M20 domain. Darmin is weakly homologous to human N-acetylated-α-linked acidic dipeptidase (NAALADase; 19% amino acid identity; Rawlings and Barrett, 1997). NAALADase is a well-studied glutamate carboxypeptidase that hydrolyzes the neurotransmitter N-acetyl-aspartyl-glutamate into N-acetyl-aspartate and glutamate. However, in contrast to Darmin, NAALADase is a transmembrane protein and belongs to the M28 peptidase family of catalytic zinc metallopeptidases. We conclude that Darmin is a member of a novel family of evolutionarily conserved glutamate carboxypeptidases.

1.2. Expression of Xenopus Darmin during early development

The expression pattern of Darmin was determined by whole-mount in situ hybridization in Xenopus embryos (Fig. 2). We did not detect maternal transcripts at the four-cell stage (Fig. 2A). At the onset of gastrulation, Darmin transcripts appeared on the dorsal side of the vegetal cell mass (Fig. 2B). Nuclear staining was evident in yolky endoderm precursors that lined the invaginating dorsal mesoderm in Spemann's organizer (Fig. 2C). As gastrulation proceeded, Darmin expression soon expanded throughout the endoderm (Fig. 2D). In late gastrula embryos, Darmin strongly demarcated the endoderm, but excluded the anterior region fated to become the foregut (Fig. 2E, see arrow indicating the liver diverticulum). In late neurula embryos, Darmin was abundantly expressed in the ventral and lateral walls of the gut (Figs. 2F–H). By tail bud stage, the gut was densely stained (Fig. 2I). Expression in the intestinal tract persisted until the swimming tadpole stage (Figs. 2J,K). We performed in situ hybridization on guts dissected from 4 to 7 day-old embryos as described in Chalmers and Slack (2000), but did not detect any Darmin transcripts at this stage (data not shown). We conclude that Darmin is a zygotic gene exclusively expressed in the endoderm during the early phases of gut development.

1.3. Darmin is a midgut-specific marker

We compared the expression of Darmin to that of Endoderm in (Edd; Figs. 3A,B). Edd is a pan-endodermal gene marker that encodes a secreted proteinase inhibitor of the α2-macroglobulin family (Sasai et al., 1996). In tail bud embryos, Edd demarcated the entire gut (Fig. 3A), whereas Darmin transcripts were excluded from the fore- and hindgut (Fig. 3B). This confirms that Darmin marks the midgut.

1.4. Expression of Xenopus Darmin-r

By secretion cloning using a Xenopus 64-cell stage cDNA library, we identified another novel protein, which we refer to as Darmin-related (Darmin-r). Like Darmin, this protein had an apparent molecular weight of 56 kDa (Fig. 1A). Partial sequencing of the Darmin-r cDNA (GenBank accession number BU993920) revealed considerable homology to Darmin (data not shown). Whole-mount in situ hybridization revealed a very different expression pattern for Darmin-r in Xenopus embryos (Figs. 3C,D). Darmin-r was not expressed in the endoderm. High levels of maternal Darmin-r transcripts were abundant at the four-cell stage (Fig. 3C). Zygotic Darmin-r expression was not detectable until the tail bud stage (data not shown). By stage 32, Darmin-r was weakly expressed in the lens and the pronephric glomus (Fig. 3D). To investigate the activity of Darmin and Darmin-r, we microinjected mRNA into early Xenopus embryos, but did not observe any obvious phenotypic effects (data not shown).

1.5. Conclusions

We have isolated two novel members of the glutamate carboxypeptidase family of zinc metallopeptases. Darmin and Darmin-r belong to the glutamate carboxypeptidase family of zinc metallopeptases.
was transiently expressed in the endoderm and developing midgut at the time of yolk resorption. Future experiments will address whether proteinase inhibitors such as Edd regulate the catalytic activity of Darmin. Due to its specific and strong expression in the early midgut, Darmin provides a useful marker for the study of endoderm development in vertebrate embryos.

2. Experimental procedures

Embryos were staged according to Nieuwkoop and Faber (1994). An amplified cDNA library from Xenopus UV-ventralized stage 11 embryos in pCDNA3 (kind gift of Dr Leonard Zon, Harvard Medical School, Boston) and an unamplified library from Xenopus stage 6 embryos in pCS2
were used. Secretion cloning was performed as described by Pera and De Robertis (2000). Briefly, 293T cells were transfected with pools of cDNA clones and 1.5 days later labeled with 35S-methionine and 35S-cysteine under serum-free conditions. Secreted proteins were identified by SDS-PAGE and autoradiography, and positive clones individualized in a second transfection step by sib-selection. For antisense RNA, pCDNA3-Darmin and pCS2-Darmin-r were linearized with BamH I and transcribed with SP6 and T7 RNA polymerase, respectively. The probe for Edd was synthesized as described (Sasai et al., 1996). Whole-mount in situ hybridizations were performed as described (http://www.hhmi.ucla.edu/derobertis/).

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