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Grandparental stem cells in leech segmentation: Differences in CDC42 expression are correlated with an alternating pattern of blast cell fates

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ABSTRACT

Embryonic segmentation in clitellate annelids (oligochaetes and leeches) is a cell lineage-driven process. Embryos of these worms generate a posterior growth zone consisting of 5 bilateral pairs of identified segmentation stem cells (teloblasts), each of which produces a column of segmental founder cells (blast cells). Each blast cell generates a lineage-specific clone via a stereotyped sequence of cell divisions, which are typically unequal both in terms of the relative size of the sister cells and in the progeny to which they give rise. In two of the five teloblast lineages, including the ventralmost, primary neurogenic (N) lineage, the blast cells adopt two different fates, designated nf and ns, in exact alternation within the blast cell column; this is termed a grandparental stem cell lineage. To lay groundwork for investigating unequal divisions in the leech *Helobdella*, we have surveyed the *Helobdella robusta* genome for genes encoding orthologs of the Rho family GTPases, including the *rho*, *rac* and *cdc42* sub-families, which are known to be involved in multiple processes involving cell polarization in other systems. We find that, in contrast to most other known systems the *Helobdella* genome contains two *cdc42* orthologs, one of which is expressed at higher levels in the ns blast cells than in nf blast cells. We also demonstrate that the asymmetric divisions of the primary nf and ns blast cells are regulated by the polarized distribution of the activated form of the Cdc42 protein, rather than by the overall level of expression. Our results provide the first molecular insights into the mechanisms of the grandparental stem cell lineages, a novel, yet evolutionarily ancient stem cell division pattern. Our results also provide an example in which asymmetries in the distribution of Cdc42 activity, rather than in the overall levels of Cdc42 protein, are important regulating unequal divisions in animal cells.

Introduction

In the embryos of clitellate annelids, including glossiphoniid leeches of the species *Helobdella*, segmental mesoderm and ectoderm arise by determinate lineages from a posterior growth zone (PGZ) consisting of five bilateral pairs of segmentation stem cells, the M, N, O/P, O/P and Q teloblasts (Fig. 1A). Columns of segmental founder cells called primary blast cells arise by the stem cell divisions of the teloblasts and coalesce, first ipsilaterally into left and right germinal bands and then along the ventral midline into a germinal plate. Developmental events in the segmentally iterated blast cell clones are indexed with reference to the clonal age (cl.ag.) at which they occur, i.e., the time elapsed since the birth of the primary blast cell that founded the clone in question. Morphologically recognizable segments arise from the extensive interdigitation of spatially stereotyped clones, the composition of which varies according to the teloblast of origin (Weisblat and Shankland, 1985).

An intriguing feature of teloblastic segmentation processes seen in clitellates is that the N and Q teloblast lineages exhibit a grandparental mode of stem cell divisions. The concept of grandparental stem cell divisions originates in the analogy between cell lineage and human genealogical diagrams (Fig. 1B; Chalfie et al., 1981). In these lineages, the primary blast cells within each column adapt two distinct fates in exact alternation, as evidenced by the distribution and composition of the clones to which they give rise, starting with the timing and differential asymmetry of their first mitoses.

In the primary neurogenic (N) lineage, primary blast cells designated as nf divide at about 40 h cl.ag., the anterior daughter (nf.a) in the 2-cell clone is significantly larger than the posterior daughter (nf.p), and the definitive clone consists largely of neurons in the posterior lateral portion of one segmental ganglion and the anterior edge of the ganglion just behind it. In contrast, the intervening cells, designated ns, divide at about 44 h cl.ag., the anterior daughter (ns.a) in the 2-cell clone is only slightly larger than the posterior daughter (ns.p), and the clone consists largely of

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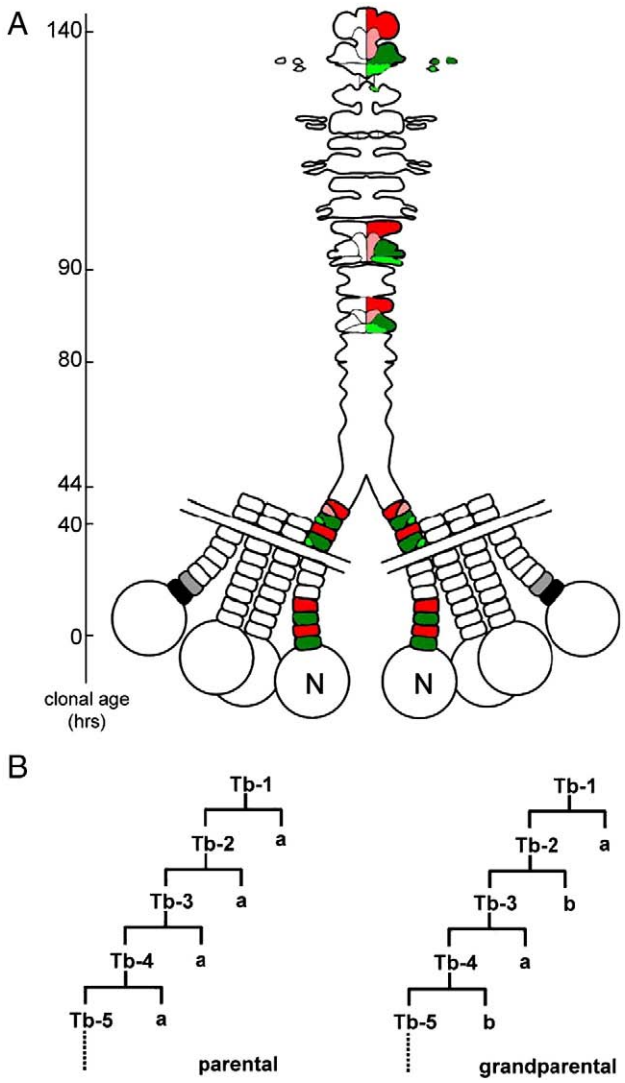

Fig. 1. Grandparental stem cell divisions in leech segmentation. (A) Asymmetric cell divisions in N teloblast lineage. Schematic depiction of cell lineage from the N teloblasts to segmental ganglia in *Helobdella*. The posterior growth zone comprises 4 pairs of ectodermal teloblasts (N is labeled) and 1 pair of mesodermal teloblasts (not shown). Each teloblast undergoes highly asymmetric divisions every 1.5 h, forming columns of segmental founder cells (primary blast cells) that coalesce first ipsilaterally and then along the ventral midline into the germinal plate, from which segmental tissues differentiate (only the ganglia are shown). Developmental events in primary blast cell clones can be timed in terms of "clonal age" (cl.ag., in hours on the scale at left), i.e., the time since the birth of the primary blast cell that founded the clone in question. In the N lineage, the primary blast cells comprise two types, ns (red) and nf (green) in exact alternation; ns and nf cells differ in terms of the timing (ns, 44 h cl.ag.; nf, 40 h cl.ag.) and extent (see Fig. 6) of their asymmetric, obliquely longitudinal first mitoses. These divisions yield ns.a/ns.p (red/pink) and nf.a/nf.p (green/light green) daughter cell pairs, which eventually contribute distinct sets of neurons to the differentiated ganglia (140 h cl.ag.). Anterior is up in all figures if not otherwise noted. (B) Cell lineage diagrams contrasting parental and grandparental stem cell divisions in the teloblast lineages. Each stem cell division gives a new generation of teloblast (Tb-x) and a segmental founder cell (a or b). In standard (parental) divisions, each generation of teloblast gives rise to the same type of blast cell; in grandparental divisions, odd numbered generations give rise to one type of blast cell (a) and even numbered generation give rise to another (b).

neurons in the medial and anterior lateral portion of a segmental ganglion (Fig. 1A; Zackson, 1984; Bissen and Weisblat, 1987).

The overall size differences between the nf and ns daughter cell pairs are accompanied by differences in nuclear size (Zackson, 1984). More recent work, involving 3-D reconstructions of pairs of nuclei prepared from stacks of confocal images, revealed that the nuclear volume ratios of the daughter cell pairs resulting from the mitosis of nf and ns blast cells are clearly distinct and show little variance (Zhang and Weisblat, 2005). The tightly regulated asymmetry of the nf and ns mitoses entails first a rotation of the mitotic apparatus and then its rearward shift relative to the cell cortex during anaphase. The rearward shift of the mitotic apparatus is greater in nf cells than in ns cells, which accounts for the differential asymmetry in the nf and ns divisions (Zhang and Weisblat, 2005).

In the budding yeast *Saccharomyces cerevisiae*, Cdc42, a Rho family small GTPase, is preferentially localized to and activated at the cell cortex at the prospective bud site (Ziman et al., 1993; Toenjes et al., 1999; Richman et al., 2002). This asymmetric distribution of Cdc42 activity is required for the formation of two daughter cells of different sizes and fates (Toenjes et al., 1999). Thus, the work presented here was initiated to test for the involvement of a *cdc42*-class gene in the unequal mitoses of nf and ns blast cells. Along with Rac- and Rho-class proteins, Cdc42 and its homologs make up the Rho family of the Ras superfamily of small GTPases (Takai et al, 2001; Boureux et al., 2007). As such, Cdc42 cycles between an activated GTP-bound state and an inactivated GDP-bound state, and has been implicated in the regulation of cell polarity and unequal cell divisions in yeast and multicellular organisms (reviewed in Etienne-Manneville and Hall, 2002). We sought to characterize the *Helobdella cdc42* gene to ask if differences in its expression, localization and/or activity control the differentially asymmetric mitoses in ns and nf blast cells.

We find two *cdc42* homologs in *Helobdella*, one of which is expressed at higher levels in the ns than in nf blast cells, as judged by *in situ* hybridization and by staining for CDC42-like immunoreactivity. This difference provides an early molecular marker for the alternating nf and ns fates in *Helobdella*. The difference in transcript levels is evident at very early clonal ages, many hours before the n blast cells undertake their first mitoses and before the appearance of CDC42-like immunoreactivity. Over-expressing the wildtype Cdc42 homolog itself did not affect the asymmetry of the n blast cell mitoses. Instead, we show that the asymmetric divisions of these cells are regulated by the polarized distribution of the activated form of the protein, rather than by the overall level of expression. These results provide the first molecular insights into the mechanisms of a novel, yet evolutionarily ancient stem cell division pattern and also provide an example of asymmetrically distributed Cdc42 activity in asymmetrically dividing animal cells.

Materials and methods

Embryos

Embryos were obtained from an as yet unnamed *Helobdella* species collected in Austin (Texas, USA) and provisionally referred to here as *Helobdella* sp. (Austin) (*Hau*; M. Shankland, personal communication). *Hau* is closely related to *Helobdella robusta* (*Hro*; Shankland et al., 1992; Bely and Weisblat, 2006), and until recently (Bely and Weisblat, 2006) has been regarded as a variant of that species (Zhang and Weisblat, 2005; Agee et al., 2006; Ren and Weisblat, 2006; Seaver and Shankland, 2000, 2001; Kuo and Shankland, 2004). Embryos of *Hau* were used in this work because this species is more readily cultured in the laboratory. Developmental progress is indicated according to a staging system applicable to all glossiphoniid leeches (Weisblat and Huang, 2001) or, for greater precision, in terms of the time after zygote deposition (AZD).

Molecular sequence analysis

Sequences for Rac/CDC42 small GTPases in human (Hsa), *Drosophila melanogaster* (Dme) and *Caenorhabditis elegans* (Cel) were recovered from NCBI database and were confirmed by reciprocal BLAST searches.