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P16 is an essential regulator of skeletogenesis in the sea urchin embryo

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Abstract

The primary mesenchyme cells (PMCs) of the sea urchin embryo undergo a dramatic sequence of morphogenetic behaviors that culminates in the formation of the larval endoskeleton. Recent studies have identified components of a gene regulatory network that underlies PMC specification and differentiation. In previous work, we identified novel gene products expressed specifically by PMCs (Illies, M.R., Peeler, M.T., Dechtiaruk, A.M., Ettensohn, C.A., 2002. Identification and developmental expression of new biomineralization proteins in the sea urchin, *Strongylocentrotus purpuratus*. Dev. Genes Evol. 212, 419–431). Here, we show that one of these gene products, P16, plays an essential role in skeletogenesis. P16 is not required for PMC specification, ingression, migration, or fusion, but is essential for skeletal rod elongation. We have compared the predicted sequences of P16 from two species and show that this small, acidic protein is highly conserved in both structure and function. The predicted amino acid sequence of P16 and the subcellular localization of a GFP-tagged form of the protein suggest that P16 is enriched in the plasma membrane. It may function to receive signals required for skeletogenesis or may play a more direct role in the deposition of biomineral. Finally, we place *P16* downstream of *Alx1* in the PMC gene network, thereby linking the network to a specific "effector" protein involved in biomineralization.

Keywords: Sea urchin embryo; Skeleton; Biomineralization; Morphogenesis; Primary mesenchyme cells; P16; Gene regulatory network

Introduction

The skeleton of the sea urchin embryo is a biologically formed mineral (Decker and Lennarz, 1988; Ettensohn et al., 1997; Wilt, 2002; Wilt et al., 2003). It consists of a branched network of magnesian calcite rods that contain small amounts of occluded spicule matrix proteins. The skeleton is deposited within the blastocoel cavity of the developing embryo by primary mesenchyme cells (PMCs), descendants of the micromeres of the 16-cell stage embryo. PMCs ingress into the blastocoel at the start of gastrulation and migrate on the basal surfaces of epithelial cells that line the blastocoel. The PMCs gradually become arranged in a characteristic ring pattern which consists of two ventrolateral clusters of cells linked by cellular chains on the oral (ventral) and aboral (dorsal) surfaces of the blastocoel wall. As the ring pattern forms, filopodial protrusions of the PMCs fuse, joining the cells in a syncytial network. The PMCs in each of the ventrolateral clusters secrete a single triradiate skeletal rudiment in response to local cues from overlying ectodermal cells. During later embryogenesis, the three arms of each skeletal rudiment elongate in a stereotypical fashion, giving rise to the branched, bilaterally symmetrical spicules of the pluteus larva. The spicules are deposited within a "privileged" space enshrouded by the fused filopodial processes of the PMCs. Local cues from overlying ectodermal cells play an important role in regulating gene expression, skeletal rod growth, and rod branching within the PMC syncytium (Ettensohn and Malinda, 1993; Guss and Ettensohn, 1997). The skeleton supports the distinctive angular shape of the pluteus and influences its orientation and swimming (Pennington and Strathmann, 1990).

Recent studies have revealed components of a gene regulatory network (GRN) that operates in the large micromere–PMC lineage (reviewed by Angerer and

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Angerer, 2003; Oliveri and Davidson, 2004). Upstream components of this network include several maternal proteins as well as early zygotic transcription factors. One key transcription factor is the Paired-class homeodomain protein, Alx1 (Ettensohn et al., 2003). Alx1 is expressed selectively in the large micromere lineage and is essential for PMC specification. Downstream of maternal factors and transcriptional regulators are gene products that directly control the morphogenetic behaviors of PMCs; viz., the ingression, migration, and fusion of the cells and their deposition of the biomineralized endoskeleton. A few of these molecules have been identified. Among the proteins involved in biomineralization are the spicule matrix proteins, a family of secreted proteins which are occluded within the spicules (Benson et al., 1987; George et al., 1991; Harkey et al., 1995; Illies et al., 2002; Lee et al., 1999). Antisense knockdown experiments have shown that the spicule matrix protein, SM50, is essential for normal rod elongation (Peled-Kamar et al., 2002).

To identify new molecules involved in various aspects of PMC morphogenesis, including biomineralization, we have carried out large-scale sequencing of an arrayed cDNA library generated from *Strongylocentrotus purpuratus* PMCs (Zhu et al., 2001). We are analyzing the developmental functions of gene products identified in the sequencing project by microinjecting morpholino antisense oligonucleotides (MOs) into fertilized eggs to block the translation of the corresponding mRNAs (Ettensohn et al., 2003; Sweet et al., 2002). In a previous study, we noted that highly abundant mRNAs represented in the PMC cDNA library fell into two major categories: (1) "housekeeping" proteins such as tubulins, cyclins, and histones, and (2) proteins thought to play a role in biomineralization (Illies et al., 2002). Examples of the latter category included spicule matrix proteins and PMC-specific cell surface proteins of the MSP130 family. Because most or all abundant housekeeping proteins are already in the public databases, we reasoned that any highly abundant sequences in the gene collection that contained relatively long open reading frames (>300 nt) that did not match known proteins by BLAST analysis were likely to encode new biomineralization-related proteins. One such gene product identified by this approach was *SpP16*, an mRNA later shown to be expressed exclusively by PMCs (Illies et al., 2002).

In the present study, we have used MOs to show that P16 plays an essential role in skeletogenesis and also report that *P16* is downstream of *Alx1* in the PMC gene regulatory network. P16 functions at a late stage of skeletogenesis, following PMC specification, ingression, migration, and fusion. The protein does not appear to be required for the initial formation of skeletal rudiments but is essential for their efficient elongation. We have compared the predicted sequences of P16 from *S. purpuratus* and *Lytechinus variegatus* and show that this small, acidic protein is highly conserved in both structure and function in the two species. The predicted amino acid sequence of P16 and the subcellular localization of a GFP-tagged form of the protein suggest that P16 is enriched in the plasma membrane. It may function to receive signals required for skeletogenesis or may play a more direct role in the deposition of biomineral.

Materials and methods

Embryo culture

Adult *S. purpuratus* were obtained from Charles Hollahan (tidalflux@yahoo.com). Adult *L. variegatus* were obtained from the Duke University Marine Laboratory (Beaufort, NC, USA) and Carolina Biological Supply (Burlington, NC, USA). Adults were induced to shed gametes by intracoelomic injection of 0.5 M KCl and fertilizations were carried out using standard methods (Foltz et al., 2004). Embryos were cultured in natural seawater collected at the Marine Biological Laboratory (Woods Hole, MA, USA). The rate of development was controlled by incubating embryos in temperature-controlled water baths.

Cloning of LvP16

Eight full-length cDNA clones encoding P16 from *S. purpuratus* (SpP16) were identified previously in a PMC cDNA sequencing project (Illies et al., 2002; Zhu et al., 2001). One of these clones, 13-F19, was used to screen *L. variegatus* mid-gastrula stage cDNA macroarray filters following the protocol of Rast et al. (2000). Two full-length cDNA clones, 26-J12 and 46-M8, were identified. The nucleotide sequences of the open reading frames of these two clones were identical except that clone 26-J12 contained a repeated hexanucleotide sequence encoding two additional amino acids (one glycine and one threonine residue) just downstream of the putative signal sequence. Because inclusion of the additional six base pairs resulted in a better alignment with the nucleotide and amino acid sequences of the eight full-length clones of SpP16, all of which were identical in this region, we chose to use the longer *L. variegatus* clone (26-J12) for all further experiments (Genbank accession number DQ058410).

Generation of GFP-tagged SpP16 and LvP16

The full-length open reading frames of SpP16 and LvP16 were amplified using PCR primers with *Bam*HI and *Cla*I sites added. The PCR fragments were cloned into CS2 + GFP vector and the resulting plasmids (SpP16.GFP and LvP16.GFP) were used to transform DH5α. SpP16.GFP and LvP16.GFP were linearized with *Not*I and capped mRNA was synthesized using the SP6 mMessage mMachine kit (Ambion Inc., Austin, TX).