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Identification and developmental expression of the *ets* gene family in the sea urchin (*Strongylocentrotus purpuratus*)

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Abstract

A systematic search in the available scaffolds of the *Strongylocentrotus purpuratus* genome has revealed that this sea urchin has 11 members of the *ets* gene family. A phylogenetic analysis of these genes showed that almost all vertebrate *ets* subfamilies, with the exception of one, so far found only in mammals, are each represented by one orthologous sea urchin gene. The temporal and spatial expression of the identified ETS factors was also analyzed during embryogenesis. Five *ets* genes (*Sp-Ets1/2*, *Sp-Tel*, *Sp-Pea*, *Sp-Ets4*, *Sp-Erf*) are also maternally expressed. Three genes (*Sp-Elk*, *Sp-Elf*, *Sp-Erf*) are ubiquitously expressed during embryogenesis, while two others (*Sp-Gabp*, *Sp-Pu.1*) are not transcribed until late larval stages. Remarkably, five of the nine sea urchin *ets* genes expressed during embryogenesis are exclusively (*Sp-Ets1/2*, *Sp-Erg*, *Sp-Ese*) or additionally (*Sp-Tel*, *Sp-Pea*) expressed in mesenchyme cells and/or their progenitors. Functional analysis of *Sp-Ets1/2* has previously demonstrated an essential role of this gene in the specification of the skeletogenic mesenchyme lineage. The dynamic, and in some cases overlapping and/or unique, developmental expression pattern of the latter five genes suggests a complex, non-redundant function for ETS factors in sea urchin mesenchyme formation and differentiation.

Keywords: *ets* gene family; Sea urchin; Mesenchyme; *Strongylocentrotus purpuratus*

Introduction

The *ets* gene family of transcription factors is noted for its wide distribution among metazoans (Degnan et al., 1993). Genes belonging to this family have been identified in a variety of animals, including sponges and ctenophores, but not in plants, fungi, yeast or any protozoan (Laudet et al., 1993). There are 27 paralogous *ets* genes in *Homo* (Hollenhorst et al., 2004), 8 in *Drosophila* (Hsu and Schulz, 2000), 14 in *Ciona* (Leveugle et al., 2004; Yagi et al., 2003) and 10 in *Caenorhabdtis* (Hart et al., 2000). Three main features characterize this family and allow for sub-classification. These are: (i) sequence homology of highly conserved domains such as the ETS-(DNA-binding-) domain, shared by all members, and the pointed (PNT) domain, which is conserved in a subset of family members and also found in various receptors, protein kinases and adaptor proteins (Graves and Petersen, 1998; Sharrocks et al., 1997); (ii) the widespread and diverse interaction with co-regulatory partner proteins, which is a consequence of domain conservation and often reflected in similar functions observed within members belonging to the same subfamily (Li et al., 2000); (iii) the ability to act as a nuclear target for signal-transduction pathways, in particular those mediated by MAP kinases, which bind to specific docking domains and target conserved phosphor-acceptor motifs found in a subset of family members (reviewed in Yordy and Muise-Helmericks, 2000).

Functional analyses of these genes have revealed essential, non-redundant roles in many different developmental processes including cell proliferation, apoptosis, differentiation, migration, transformation and hematopoiesis (for a recent review see Sharrocks, 2001).

Genes belonging to only two members of this family have so far functionally been characterized in sea urchin. These are the *Sp-Ets4* gene, identified as a regulator of the mechanism that establishes the animal–vegetal axis of the sea urchin embryo

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(Wei et al., 1999a), and three homologues of the human *ETS-1* gene, the founder member of the ETS domain family that was first discovered from the E24 avian leukemia virus as the *v-ets* (*e twenty-six*) oncogene (Karim et al., 1990). For these sea urchin genes, an important role in the process of micromere specification and skeletogenic cell differentiation has been recently uncovered in three different species (Kurokawa et al., 1999; P. Oliveri, personal communication; Rottinger et al., 2004). The central function exhibited by these sea urchin *ets* genes, together with the high degree of conservation of ETS domains displayed by *ets* family members, which is often reflected in overlapping binding specificities (Sharrocks et al., 1997), persuaded us to investigate this gene family systematically in the sea urchin. Moreover, the availability of the *Strongylocentrotus purpuratus* genome sequence (released by Human Genome Sequencing Center at Baylor College of Medicine) and the phylogenetic position of sea urchins as non-chordate deuterostomes offered the opportunity of re-evaluating the evolution of this important gene family with particular regard to the emergence of vertebrates. Here we report the isolation, domain structure, phylogenetic analyses and expression profiling during embryonic development of eleven *ets* genes identified in the *S. purpuratus* genome. Given the well documented amenability of the sea urchin embryo for experimental analysis of gene regulatory networks (Davidson et al., 2002; Oliveri and Davidson, 2004), this work may provide the essential framework for investigations into the complex molecular interactions and multiple biological roles of ETS proteins *in vivo*.

Materials and methods

Animals

Adult *S. purpuratus* were obtained from Pat Leahy (Kerchoff Marine Laboratory, California Institute of Technology, USA). Spawning was induced by vigorous shaking of animals or by intracoelomic injection of 0.5 M KCl. Embryos were cultured at 15°C in Millipore filtered Mediterranean seawater (MFSW) diluted 9:1 in deionized H_2O.

Gene search and phylogenetic analyses

S. purpuratus Ets proteins were identified with BLAST searches against the traces, contigs, scaffolds and Glean3 database at the HGSC, Baylor College of Medicine site: http://www.hgsc.bcm.tmc.edu/blast/?organism=Spurpuratus. Identified Glean3 predictions were manually annotated and validated where possible with known ESTs or by PCR amplification and sequencing. A phylogenetic tree comparing sea urchin *ets* genes and homologues from multiple species was constructed in order to name accurately the newly identified genes. Orthologous sequences were obtained by database searches using BLASTP and TBLASTX (http://www.ncbi.nlm.nih.gov/BLAST/). Protein domains were identified using SMART (Letunic et al., 2006; Schultz et al., 1998) (http://smart.embl-heidelberg.de/) and Pfam (Bateman et al., 2004) (http://www.sanger.ac.uk/Software/Pfam/) databases. Accession numbers of all the sequences are listed in Table S1. Multiple sequence alignment of ETS domains was generated by CLUSTAL X 1.83 (Thompson et al., 1994) then manually optimized with GeneDoc (http://www.psc.edu/biomed/genedoc). Phylogenetic reconstruction was carried out using the neighbor-joining method, and bootstrap values determined by 1000 replicates. The tree was also generated using maximum parsimony methods with bootstrap replicates of 1000. The final output of the phylogenetic tree was obtained using Treeview software version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Isolation of sea urchin ets cDNAs

The cDNAs corresponding to the complete coding sequences of *Sp-Elk*, *Sp-Erg* and *Sp-Elf* were obtained by screening arrayed cDNA libraries from *S. purpuratus* 20 h and 40 h embryos (Cameron et al., 2000) following an established protocol (Rast et al., 2000). For the *ets* genes, and where necessary, PCR and 3′ or 5′ RACE (Invitrogen, Carlsbad, CA) of *S. purpuratus* cDNA were performed to confirm transcript sequence of predicted genes. Isolated cDNAs were fully sequenced using an Automated Capillary Electrophoresis Sequencer *3730 DNA Analyzer* (Applied Biosystems, Foster City, CA). The list and reference numbers of all *ets* cDNAs are reported in Table S2 of Supplementary material.

Quantitative PCR (QPCR)

Total RNA was extracted from embryos at various stages (egg, 8, 10, 15, 18, 21, 24, 33, 40, 45, 48, 52, 72 h post fertilization, hpf) using Eurozol reagent (Euroclone, Celbio, Milan, Italy). Residual DNA was digested with DNase I using a DNA-free kit (Ambion, Austin, TX). First-strand cDNA was synthesized in a 50 μl reaction from 1 μg of total RNA using random hexamers and the TaqMan Reverse transcription Kit (Applied Biosystems). Specific primer sets (Table S3) for each gene were designed using the Primer3 program (Rozen and Skaletsky, 2000) (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). Primer sets were chosen to amplify products 100–200 bp in length. Blast searches were used to ensure that primers were specific for each individual *ets* gene. cDNA was diluted to a concentration of 1 embryo/μl. Reactions were performed in triplicate using the Chromo 4 real-time detector (BioRad, Hercules, CA) with SYBR Green chemistry (Applied Biosystems). Data for each gene were normalized against ubiquitin mRNA, which is known to be expressed at constant levels during the first 72 h of development (Nemer et al., 1991). Primer efficiencies (i.e., the amplification factor for each cycle) were found to exceed 1.9. Calculations from QPCR raw data used the formula $1.9^{\Delta Ct}$, where 1.9 is the multiplier for amplification per PCR cycle, and ΔCt is the cycle threshold difference with ubiquitin found for that sample. Absolute quantification of the number of transcripts was obtained by using SpZ12-1 as an internal standard. The number of SpZ12-1 transcripts in embryos of the relevant stages had been measured earlier by RNA titration (Wang et al., 1995).

Whole-mount in situ hybridization (WMISH)

Fragments of *Sp-Elk*, *Sp-Ese*, *Sp-Erf*, *Sp-Ets*, *Sp-Gabp*, *Sp-Pea* and *Sp-Tel* were amplified from cDNA templates by PCR using specific primers (Table S3, Supplementary material). PCR products were purified and cloned into pCRII-TOPO (Invitrogen) according to the manufacturer's instructions and the identity of inserts confirmed by sequencing. For *Sp-Erg*, a fragment of 450 bp was subcloned into $pBSK^+$ (Stratagene, La Jolla, CA) using *Kpn*I and *Eco*RI sites. For *Sp-Elf*, a 1856 bp long fragment was derived from the cDNA clone 1K4 (20 h library). The position of each probe with reference to the corresponding ETS protein sequence is depicted with a blue line in Fig. 1B.

Whole-mount *in situ* hybridization was performed as described by Minokawa et al. (2004). The accuracy of whole-mount *in situ* hybridization data was confirmed by control experiments using sense probes (not shown). Both antisense- and sense-digoxigenin-labeled RNA probes were obtained using a DIG-RNA labeling kit (Roche, Indianapolis, IN), following the manufacturer's instructions and using 1 μg of linearized plasmids. RNA probes were purified using Mini Quick Spin RNA Columns (Roche). Following staining, embryos were mounted in glycerol and analyzed using a Zeiss Axio Imager M1 microscope operating in DIC mode.

Results

Identification, domain structure and phylogenetic analysis of sea urchin ets genes

The completion of the sequencing of the *S. purpuratus* genome and the release of the first draft genome assembly by