SpTie1/2 is expressed in coelomocytes, axial organ and embryos of the sea urchin Strongylocentrotus purpuratus, and is an orthologue of vertebrate Tie1 and Tie2

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1. Introduction

Echinoderms are frequently used as the evolutionary outgroup in studies comparing deuterostome relationships and the evolution of vertebrate characteristics. Sea urchins, as members of the echinoderm phylum, are easily obtained, housed, handled, spawned and otherwise manipulated, and are frequently used in investigations of early development (Davidson, 2006; Sodergren et al., 2006a,b). The echinoderms, as the sister phylum to the chordates, are an important basal group for making evolutionary inferences about the immune system in deuterostomes (reviewed in Rast and Messier-Solek, 2008). Adult echinoderms differ significantly in their structure from chordates, in that they possess a unique water vascular system and rely on a large open coelomic cavity rather than a closed circulatory system with a heart and blood vessels (Hyman, 1955). Yet, there are aspects of their immune system that show similarities to innate immunity in vertebrates including complement homologues, Toll-like receptors, NACHT/NOD-like receptors, and a variety of lectins (Smith et al., 1999, 2001, 2006; Hibino et al., 2006; Rast et al., 2006). The cells in the coelomic cavity, the coelomocytes, are a mixture of different morphologically identifiable types of which the majority resemble phagocytic cells of vertebrates in their participation in innate immune functions such as phagocytosis, encapsulation, and cellular clotting in response to injury (Smith et al., 2006; Johnson, 1969; Smith and Davidson, 1994; Gross et al., 1999). By investigating conserved aspects of immune function in the immune cells of both vertebrates and echinoderms, we may be able infer the origins and evolution of the basic, underlying immunity that is present in animals within the deuterostome lineage.

Abbreviations: Ig, immunoglobulin; RTK, receptor tyrosine kinase; Tie, tyrosine kinase with Ig and EGF domains; TEK, tunica interna endothelial cell kinase; Ang, angiopoietin; EST, expressed sequence tag; FGFR, fibroblast growth factor receptor; GFR, growth factor receptor; SMART, simple modular architecture research tool; P3E3, phosphoinositide 3-kinase; LPS, lipopolysaccharide; wCF, whole coelomic fluid; CMFSW-EI, calcium- and magnesium-free sea water with EDTA and imidazole; Ct, cycle threshold; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetic acid; SSC, sodium chloride and sodium citrate; BSA, bovine serum albumin; nt, nucleotide(s); UTR, untranslated region; EGF, epidermal growth factor; FNIII, fibronectin type III; qPCR, quantitative PCR; hpf, hours post-fertilization; HSCs, hematopoietic stem cells; LTR-HSCs, long-term repopulating HSCs; MAPK, mitogen-activated protein kinase; WGS, whole genome shotgun; BCM, Baylor College of Medicine; SNP, single nucleotide polymorphisms.

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A full length cDNA sequence expressed in coelomocytes shows significant sequence match to vertebrate Tie1 and Tie2/TEK. Vertebrate Tie2/TEK is the receptor for the angiopoietins and plays an important role in angiogenesis and hematopoiesis, whereas Tie1 regulates the activity of Tie2. The deduced sequence of the SpTie1/2 protein has a similar order and organization of domains to the homologous vertebrate proteins including a highly conserved receptor tyrosine kinase domain in the cytoplasmic tail. The N terminus of the ectodomain has one immunoglobulin (Ig)-Tie2 domain, followed by an Ig domain, four epidermal growth factor domains, a second Ig domain, and three fibronectin type III domains. The SpTie1/2 gene is expressed in coelomocytes and the axial organ, whereas other organs do not show significant expression. The timing of embryonic expression corresponds with the differentiation of blastocoelar cells, the embryonic and larval immune cells. Searches of the sea urchin genome show several gene models encoding putative ligands and signaling proteins that might interact with SpTie1/2. We speculate that SpTie1/2 may be involved in the proliferation of sea urchin immune cells in both adults and embryos.

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A hallmark of the vertebrate immune system is cellular interactions mediated through ligand interactions with receptors displayed on the cell surface. One of the major families of receptors involved in regulation of cellular activity and proliferation in chordates is that of the receptor tyrosine kinases (RTKs) (Schlessinger, 2000; Fantl et al., 1993; Simon, 2000). Upon binding ligands, these receptors dimerize and induce cross-phosphorylation of specific tyrosine residues in the cytoplasmic tail. The phosphotyrosines initiate a signaling cascade that causes a set of responses specific to the cell and to the receptor, with the main targets being regulation of transcription and modulation of the cytoskeleton. A recent inventory of RTKs encoded in the genome of the sea urchin, Strongylocentrotus purpuratus, has shown that most families of vertebrate RTKs are also represented in sea urchins (Sodergren et al., 2006b). Although many RTK families in vertebrate genomes have multiple members, sea urchins typically have a single orthologue for all but two of these families (Lapraz et al., 2006). Similar results have been reported for amphioxus (D’Aniello et al., 2008).

A specific RTK family, consisting of two vertebrate genes, Tie1 and Tie2/TEK, encode proteins with a unique arrangement of extracellular domains, and function in hematopoietic and blood vessel forming tissues (Dumont et al., 1992; Maisonnepierre et al., 1993; Partanen et al., 1992; Sato et al., 1993; Martin et al., 2008). These two receptors seem to be present universally in vertebrates, where they are expressed in the cell lineage that forms the endothelium of blood vessels (Dumont et al., 1994a,b; Sato et al., 1995, reviewed by Jones et al., 2001) and develops into the stem cells for hematopoiesis (Hsu et al., 2000; Tachibana et al., 2005; Takakura et al., 1998; Batard et al., 1996; Hashiyama et al., 1996). Tie1 and Tie2 are expressed in endothelial cells (Schnur and Risau, 1993), particularly in normal cells undergoing neovascularization (Korhonen et al., 1995) and in metastatic melanoma endothelial cells (Kaipainen et al., 1994). The Tie receptors also are expressed in early stages of hematopoiesis (Batard et al., 1996; Iwama et al., 1993) and appear to be necessary for proliferation of blood cells in adult bone marrow, but do not function in embryonic or fetal hematopoietic tissues (Puri and Bernstein, 2003). The angiopoietin (Ang) family of ligands, including Ang1 (Davis et al., 1996) and Ang4 (Jones et al., 2001) interact with Tie2 and activate tyrosine phosphorylation activity. Ang2 is usually a negative regulator of the tyrosine kinase function (Jones et al., 2001; Huang et al., 1999), but sometimes enhances the downstream signaling activity of Ang1 (Huang et al., 1999). No ligand has been positively identified for sea urchin Tie1, but Ang1 and Ang3/4 promote its phosphorylation in cells co-transfected with both Tie1 and Tie2 genes (Yuan et al., 2007). The critical role of Ang1 and Tie2 in non-fetal hematopoiesis appears to be maintenance of a quiescent, anti-apoptotic population of hematopoietic stem cells that are protected from stresses associated with active proliferation and adhere to osteoblasts in the bone marrow (Arai et al., 2004).

During an initial expressed sequence tag (EST) study of coelomocytes from the purple sea urchin, S. purpuratus, EST059 matched to the cytoplasmic domain of an RTK (accession number R61943) (Smith et al., 1996). BLAST matches suggested that the sequence fragment encoded a growth factor receptor or Tie receptor. The full length cDNA sequence, Sp059, corresponded to a single gene model identified from the S. purpuratus genome that encodes a member of the Tie family designated SpTie1/2 (Sodergren et al., 2006b; Hibino et al., 2006; Bradham et al., 2006). The combination of the suite of extracellular domains, the highly conserved cytoplasmic domain, plus a phylogenetic analysis of the tyrosine kinase domain from Tie1, Tie2 and other RTK growth factor sequences suggested that sea urchin SpTie1/2 is an ancestral Tie1 and Tie2 homologue, in agreement with gene annotations (Sodergren et al., 2006b; Hibino et al., 2006; Lapraz et al., 2006; Bradham et al., 2006). The SpTie1/2 gene is highly expressed in coelomocytes, axial organ, and embryos, suggesting an involvement in immune cell proliferation in adults, in addition to a time in development when embryonic secondary mesenchyme cells differentiate into blastocoelar cells, which are the immune cells of embryos and larvae (Smith et al., 2006; Tamboline and Burke, 1992; Silva, 2000; Furukawa et al., 2009).

2. Methods

2.1. Care and treatment of sea urchins and embryo cultures

Sea urchins, S. purpuratus, were supplied by Marinus Scientific Inc. (Long Beach, CA), the Southern California Sea Urchin Company (Corona Del Mar, CA) or Westwind Sealab Supplies (Victoria, British Columbia, Canada) and maintained as previously described in a closed aquarium at 14 °C with filtered sea water sterilized with UV light (Gross et al., 2000; Shah et al., 2003). Immune activation was induced in adult sea urchins by injections of lipopolysaccharide (LPS) as described (Terwilliger et al., 2007; Smith et al., 1995). Gametes were collected after KCl injection to induce spawning and fertilized embryos were cultured in 0.45 μM filtered Instant Ocean in suspension flasks at 15 °C.

2.2. Immunoefficient and immunologically activated sea urchins

Immunoefficient sea urchins were maintained without manipulation according to Nair et al. (2005). Immunoefficient animals were injected with LPS (2 μg/ml of coelomic fluid) (Sigma–Aldrich, St. Louis, MO) as described (Terwilliger et al., 2007; Smith et al., 1995) to generate immunologically activated animals. Whole coelomic fluid (wCF) samples were collected before challenge with LPS and 24 h after challenge, and tissues were collected from animals that had not been challenged or were collected 15 days after challenge. Animal #8 was considered non-immunoefficient because samples were taken within 24 h of shipment.

2.3. cDNA libraries and screening

Clones for sequencing were obtained by screening two coelomocyte cDNA libraries. The first was a conventional, random primed, directionally cloned λExCell cDNA library that was constructed from mRNA collected from 18 sea urchins, and excised and stored as a plasmid library (Smith et al., 1996; Al-Sharif et al., 1998). The second was an arrayed cDNA library constructed from mRNA collected from non-activated coelomocytes from six sea urchins and cloned into pSPORT (Cameron et al., 2000; Rast et al., 2000). The libraries were screened with riboprobes (see below) as described (Al-Sharif et al., 1998; Muller and Smith, 2004).

2.4. Cycle sequencing

Cycle sequencing was performed on the pSPORT clones using the Big Dye kit (Perkin-Elmer/Applied Biosystems, Foster City, CA) with Sp6 and T7 primers plus internal primers (Operon Technologies, Huntsville, AL) (Table 1) and analyzed on a single capillary 373A automated sequencer (Applied Biosystems, Foster City, CA) or by the Nucleic Acid Core Facility of the Medical College of Wisconsin. The overlapping sequences were aligned using the DNAsis sequence analysis program (Hitachi, Yokohama, Japan). The consensus sequence was generated by comparing the cDNA sequences and whole genome shotgun (WGS) trace sequences from the sea urchin genome available at the NCBI website (http://www.ncbi.nlm.nih.gov/genome/seg/BlastGen/BlastGen.cgi?taxid=7668). The final Sp059 cDNA sequence was submitted to GenBank with accession number GQ979611.
2.5. Assembly of the gene sequence from gene models; determination of structural domains for the encoded protein

The genomic scaffolds containing the SpTie1/2 gene were identified by BLAST searches against the sea urchin genome (v2.1) at NCBI, and the Baylor College of Medicine (BCM) Spurpuratus BAC plus WGS Assembly (v2.0, June 15, 2006 [http://www.hgsc.bcm.tmc.edu/blat/blat.cgi?organism=Spurpuratus]). Exons were identified by aligning the cDNA to the genomic sequence, and verified by the presence of GT-AG splice signals located at the ends of the introns. The Sp059 cDNA sequence was used to search GenBank by BLASTx to identify homologues from other species. Structural domains in the deduced protein were determined with the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/) in the normal mode.

2.6. Sequence alignments and phylogenetic analyses

The SpTie1/2 protein sequence was used in tBLASTx searches of GenBank to identify the most similar sequences in vertebrates and invertebrates for phylogenetic analysis. The tyrosine kinase domain was employed in an alignment in ClustalW using the default parameters (Thompson et al., 1994) with subsequent manual editing in BioEdit (Hall, 1999). A minimum evolution tree with 1000 bootstrap iterations was constructed in MEGA4 (Kumar et al., 2004). Other phylogenetic methods were used to confirm the results (see the legend of Fig. 8).

The sea urchin genome (v2.0) was searched for the Ang homologue by BLASTp using four vertebrate angiopoietin sequences (Thompson et al., 2006). Each sample, with 10^5 to 10^7 coelomocytes per ml, was centrifuged at 10,000 × g for 1–2 min at 4 °C and the cell pellet was overlain with RNAiLter® (Ambion Diagnostics, Austin, TX). Tissue samples (pharynx, gut, gonad, esophagus, and axial organ) were obtained by dissection and transferred immediately to RNAiLter®. Total RNA was extracted from coelomocytes and tissue samples using the RNeasy® Micro or Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s directions. Flanking DNAase (Promega, Madison, WI) to digest any contaminating genomic DNA. RNA (0.75–1 μg) was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s directions.

Embryos were collected at the several different time points and total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA), treated with DNase using the DNA-free kit (Ambion Diagnostics, Austin, TX). RNA (2 μg) was reverse transcribed into cDNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems) in 100 μl reactions.

2.7. Total RNA isolation

wCF samples were withdrawn from adult sea urchins and diluted (1:3) into calcium–magnesium-free sea water with 70 mM EDTA and 50 mM imidazole pH 7.4 (CMFS-W-E) (Terwilliger et al., 2006). Each sample, with 10^8 to 10^7 coelomocytes per ml, was centrifuged at 10,000 × g for 1–2 min at 4 °C and the cell pellet was overlain with RNAiLter® (Ambion Diagnostics, Austin, TX). Tissue samples (pharynx, gut, gonad, esophagus, and axial organ) were obtained by dissection and transferred immediately to RNAiLter®. Total RNA was extracted from coelomocytes and tissue samples using the RNeasy® Micro or Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s directions. Flanking DNAase (Promega, Madison, WI) to digest any contaminating genomic DNA. RNA (0.75–1 μg) was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s directions.

Primers used in qPCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Target cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpTie1/2F</td>
<td>CAGTCAGAGCTCGTAAAGATG</td>
<td>Coelomocyte</td>
</tr>
<tr>
<td>SpTie1/2R</td>
<td>CACACGTGCTGCTGGTGC</td>
<td>Coelomocyte</td>
</tr>
<tr>
<td>Tie1-2 QPCRFR</td>
<td>CATTCCCTACCTCTGGAAGTG</td>
<td>Embryo</td>
</tr>
<tr>
<td>Tie1-2 QPCRFR</td>
<td>TGAATTCCTGCTCTCTGGT</td>
<td>Embryo</td>
</tr>
<tr>
<td>SpL8rev</td>
<td>CAAACGACCTGACAGGAGAGGA</td>
<td>Coelomocyte</td>
</tr>
<tr>
<td>SpL8for</td>
<td>ACCGATCTGAGTCTGGCGAGT</td>
<td>Coelomocyte</td>
</tr>
<tr>
<td>18S-F</td>
<td>CGAGTTGCTAGTCGCTGAG</td>
<td>Embryo</td>
</tr>
<tr>
<td>18S-R</td>
<td>CTCCTCAGGTATCTGCTTTA</td>
<td>Embryo</td>
</tr>
<tr>
<td>Sp6</td>
<td>GATTAGGCTACACTATAG</td>
<td>Sequencing</td>
</tr>
<tr>
<td>T7</td>
<td>TAATAGCAGCTAAGATG</td>
<td>Sequencing</td>
</tr>
</tbody>
</table>
The libraries from which the Sp059 cDNA clones were isolated were constructed from coelomocyte RNA pooled from 18 sea urchins (Smith et al., 1996; Al-Sharif et al., 1998) and a second that was constructed from six sea urchins (Cameron et al., 2000; Rast et al., 2000). Consequently, as many as 48 alleles may have been used to assemble the cDNA sequence plus the two alleles from the genome. More than half of the single nucleotide polymorphisms (SNPs) (125 of 239 nt) in the Sp059 sequence encoded nonsynonymous changes, including 67 that changed the type of amino acid (Fig. S1). This represented a polymorphism frequency of 5.5% in the cDNA sequence and 2.9% in the protein sequence.

3.2. Gene copy number and the SpTie1/2 gene model

Several vertebrate genomes have been investigated for the presence of Tie homologues, and two genes are consistently present; Tie1 and Tie2/TEK (Jones et al., 2001). However, during the process of annotating the sea urchin genome, one gene model, SPU_024044 was reported as encoding SpTie1/2 (Sodergren et al., 2006b; Hibino et al., 2006). This sequence was missing some of the exons that matched to the cDNA sequence, while a second gene model SPU_026748, was missing other exons. The two gene models were used to generate a corrected SPU_024044 gene model that included all exons encoding sequences in the cDNA. The two gene models were likely the result of mis-assembly of a single gene. Based on comparisons to the cDNA sequence, SpTie1/2 gene model had 27 exons, which was significantly more than the average sea urchin gene with 8.3 exons (Sodergren et al., 2006b). Sizes and characteristics of the introns differed among genome builds and therefore are not described. In agreement with the single gene model, a genome blot of three individual sea urchins showed two or three bands for each digest, which was consistent with a single copy gene for SpTie1/2 (Fig. 1).

3.3. The deduced SpTie1/2 protein

The deduced SpTie1/2 amino acid sequence was used to search the non-redundant sequences in GenBank using BLAST, and the best match was to the Tie proteins in vertebrates (Jones et al., 2001; Peters et al., 2004). Domain analysis of the deduced protein using SMART showed that there were two hydrophobic regions in SpTie1/2; a N terminal signal sequence of 19 amino acids and a transmembrane region of 21 amino acids (Figs. S1, 2). The extracellular region of the protein had three types of domains, immunoglobulin (Ig), epithelial growth factor (EGF) and fibronectin.

Fig. 1. SpTie1/2 is a single copy gene. Genomic DNA from sperm collected from three sea urchins was digested to completion with three restriction enzymes, electrophoresed and blotted. The filter was analyzed with a riboprobe generated from a cDNA template pBsc-M18a (see legend of Fig. 2 for details). Each lane shows two or three bands, consistent with a single copy, heterozygous locus. Standards are indicated to the right.
type III (FNIII) domains, that were in the same order as those in vertebrate Tie1 and Tie2/TEK proteins (Sato et al., 1993). The first two Ig domains were separated from the third by three EGF domains and one EGF-like domain, which were followed by four FNIII domains (Fig. 2). SpTie1/2 was similar to vertebrate Tie2 in having three Ig, but differed in having four EGF and four FNIII domains, while the vertebrate proteins have only two or three EGF domains and three FNIII domains. The second and third Ig domains in SpTie1/2 had conserved N-linked glycosylation sites at amino acid 481 (NVTG) (Fig. S1). The tyrosine kinase domain in the cytoplasmic tail contained a typical catalytic site, ATP-binding site, and activation loop showing a high degree of sequence similarity between the sea urchin and vertebrate sequences (Fig. 3). Overall, the types, numbers and relative organization of the encoded domains in addition to BLAST results indicated that the sea urchin sequence was a Tie homologue.

3.4. Exons in SpTie1/2 correlate with the protein domains

The SpTie1/2 gene model was composed of 27 exons and a mean length was 158 nt, a range of 37–306 nt, and a median length of 132 nt. Many of the exons in the SpTie1/2 gene model encoded separate domains in the protein (Figs. S1, 2). The signal sequence (exon 1), the three Ig domains (exons 2, 3, 10), the EGF domains (exons 4, 5, 6, 7), the first and fourth FNIII domains (exons 11, 16), the transmembrane region (exon 18), and the C terminal cytoplasmic tail (exon 27) were all encoded by single exons. The remaining
domains in SpTie1/2 were encoded by two or more exons, including the second and third FNIII domains (exons 12 and 13, 14 and 15) and the tyrosine kinase domain (exons 19-26; Figs. S1, 3). Exons 8, 9, and 17 did not encode discernable domains for a total of 27 exons in SpTie1/2 compared to 22 exons present in human Tie genes (Fig. 2).

The tyrosine kinase domain in the cytoplasmic region was the most highly conserved region of the Tie sequences. The domain was encoded by eight exons for the sea urchin, which is the same for the vertebrate genes (Figs. 2 and 3). As in the human Tie genes, the functional regions of the tyrosine kinase domain were encoded by separate exons; the ATP-binding site in exon 21, the catalytic site in exon 22, and the activation loop in exons 22 and 23 (Fig. 3). Positions of all seven introns within the tyrosine kinase domain of SpTie1/2 were identical to those in the human Tie genes, as was the length of all exons except exon 21 for SpTie1/2, which had an insertion that encoded an additional six amino acids. The intron position pattern for the Tie genes is unique and different from other RTK families, which have different numbers of exons and at least one intron position that is different (e.g., D’Aniello et al., 2008) (Fig. 3). Consequently, conservation of the positions of the introns within SpTie1/2 and the vertebrate genes indicates homology. A similar result was found for the tyrosine kinase domains in the BtTie homologues in Branchiostoma floridae (D’Aniello et al., 2008), however the types, numbers and organization of the ectodomains for these two proteins were not well conserved.

When the boundaries of the other SpTie1/2 domains and exons were compared to the human Tie sequences, a number of additional similarities and some differences were noted (Fig. 2). Each of the Ig domains in the vertebrate Tie proteins was encoded by two exons, while the Ig domains in SpTie1/2 were encoded by single exons. There were two or three EGF domains in the human Tie sequences, whereas SpTie1/2 had four. SpTie1/2 had four FNIII domains, while both human sequences had three, and each was encoded by one or two exons. Phylogenetic analysis of the FNIII domains from human Tie1, Tie2 and SpTie1/2 showed that the first two FNIII domains from SpTie1/2 consistently clustered together, suggesting that they were either the result of exon duplication and were very similar, or were both very different from the third and fourth FNIII domains in SpTie1/2 and the vertebrate sequences (results not shown). Overall, comparison of exons encoding domains suggested that the sea urchin Tie gene has undergone some diversification in the ectodomain. However, the conservation of the exons and the intron positions within the tyrosine kinase domain lent strong inference to the notion that SpTie1/2 is a Tie homologue.

3.5. SpTie1/2 is expressed in coelomocytes, axial organ and embryos

To understand the gene expression patterns of SpTie1/2, quantitative PCR (qPCR) was used to analyze the Sp059 mRNA content in coelomocytes, as well as tissue samples from axial organ, gut, gonad, pharynx, and esophagus. Relative SpTie1/2 gene expression was determined by calculating the ratio of Sp059 starting quantity with respect to SpL8 (ribosomal L8 homologue) starting quantity. Results indicated that the highest SpTie1/2 gene expression was in the axial organ, followed by the coelomocytes, and was lowest in the other organs (P < 0.05; Fig. 4). The expression in axial organ was significantly higher than in coelomocytes (P < 0.05) and the increase in axial organ was correlated with immune challenge (P < 0.05). No association was observed between SpTie1/2 gene expression and immune challenge in coelomocytes or in the other organs. These results confirmed previous results for SpTie1/2 gene expression in coelomocytes (Smith et al., 1996) and demonstrated that it was also expressed in the axial organ.

Expression of SpTie1/2 was also investigated during development of sea urchin embryos from unfertilized egg to pluteus (51.5 h post-fertilization (hpf)). Results from qPCR analysis showed that expression was very low during early development and became detectable at 27 hpf (early gastrula) (Fig. 5). Expression peaked at 36 hpf (late gastrula) and continued to be expressed, but at slightly lower levels until the pluteus stage. The onset of expression coincided with the differentiation of secondary mesenchyme cells into blastocoelar cells, the immune cells in the sea urchin embryo (Smith et al., 2006; Hibino et al., 2006; Tamboline and Burke, 1992; Silva, 1998).
2000; Furukawa et al., 2009) and suggested a potential role for SpTie1/2 protein in the sea urchin larval analogous to that of Tie2 in mouse hematopoiesis (Takakura et al., 1998).

3.6. Phylogenetic relationships among Tie homologues

When the SpTie1/2 gene model was first identified in the sea urchin genome it was reported to encode proteins similar to both vertebrate Tie1 and Tie2 proteins, and therefore was called SpTie1/2 (Sodergren et al., 2006b; Hibino et al., 2006). To illustrate the phylogenetic relationships among many of the Tie sequences that have become available more recently, Tie-like sequences were collected from amphioxus, Branchiostoma floridae, a solitary tunicate, Ciona intestinalis, Hydra, Hydra magnipapillata, the starlet anemone, Nematostella vectensis, honey bee, Apis mellifera and mosquito, Anopheles gambiae. Several fibroblast growth factor receptors (FGFRs) were added to the analysis, and in some cases were used as the outgroup because they were consistently present in BLAST results as the most closely allied sequence to the Tie family. The tyrosine kinase domain was selected for phylogenetic analysis because it was most highly conserved and most uniform in size among the various domains, which enabled a high quality alignment and robust phylogenetic trees. Several different phylogenetic approaches were used to generate trees and all produced very similar results with respect to the relationship of the SpTie1/2 protein to the human Tie1 and Tie2 sequences (a representative tree is shown in Fig. 6). The vertebrate Tie sequences fell into two clades, Tie1 and Tie2/TEK with SpTie1/2 positioned at the base of these two clades along with B. floridiae Tie1 and B. floridiae GFR. Most of the sequences from the invertebrates clustered with the FGFR sequences including the B. floridiae GFR and Tie1 sequences suggesting that the B. floridiae Tie1 sequence may not be a Tie homologue. Overall, the SpTie1/2 sequence appears equally similar to vertebrate Tie1 and Tie2/TEK in agreement with a preliminary phylogenetic tree of RTKs identified from the sea urchin genome (Lapraz et al., 2006).

Similar analyses were performed for the Ig-Tie2_1 domain and the second Ig domain at the N terminal end of the proteins (see Fig. 2), which, in mammalian Tie proteins, are involved with ligand binding based on crystal structure (Barton et al., 2006). The resulting trees showed that each SpTie1/2 Ig domain clustered at the base of the Tie1 and Tie2/TEK clades for the respective Ig domain (data not shown). The phylogenetic position of SpTie1/2 at the base of the Tie1 and Tie2 clades in analyses for both the Ig and tyrosine kinase domains suggested that SpTie1/2 may be descended from a single ancestral Tie gene that was present in an ancestral deuterostome prior to duplication in the chordates. The phylogenetic results also suggested that there is only a single Tie homologue in B. floridiae, and that the duplication of the Tie genes occurred in the higher chordates.

3.7. A putative ligand for SpTie1/2

The ligand for vertebrate Tie receptors is Ang, which is a secreted glycoprotein of about 55 kD consisting of a signal sequence and a single fibrinogen domain (Davis et al., 1996). To determine whether a sea urchin Ang homologue was present in the genome (v2.0), four vertebrate Ang sequences, human Ang1 (NP_001137.2), human Ang4 (NP_001138), Danio rerio Ang1 (NP_571888), and Danio rerio Ang2 (NP_571889.1), were used in BLAST searches. The sea urchin fibrinogen domains from candidate Ang-like homologues (n = 30) were identified using SMART, aligned with the vertebrate Ang fibrinogen sequences (n = 14), and used in a variety of phylogenetic analyses. The resulting trees consistently showed that the putative sea urchin Ang-like sequences clustered separately from the vertebrate Ang (types 1, 2 and 4) sequences (data not shown). Although many of the deduced fibrinogen domains from the sea urchin gene models had the Ca2+ binding motif, the amino acids important for binding angiopoietin to Tie receptors in vertebrates (Barton et al., 2006) were not conserved in the deduced sea urchin proteins (data not shown). Therefore, identification of the gene model that encodes the activating ligand for SpTie1/2 will require functional analyses of the encoded proteins.

3.8. Putative downstream signaling proteins

Vertebrate Tie2 proteins have three tyrosines (Y1101, Y1107, and Y1112) located near the C terminus of the cytoplasmic tail that are phosphorylated to enable interaction with SH2 domain proteins such as Grb2 and the p85 subunit of PI3K (Huang et al., 2000; Furukawa et al., 2009). The ligand for vertebrate Tie receptors is Ang, which is a secreted glycoprotein consisting of a signal sequence and a single fibrinogen domain. The tyrosine kinase domain was selected for phylogenetic analysis because it was most highly conserved and most uniform in size among the various domains, which enabled a high quality alignment and robust phylogenetic trees.
Fig. 7. Alignment of predicted amino acid sequence of sea urchin growth factor receptor-bound protein (Grb)-like sequences with human Grb2. Sea urchin sequences were identified using BLASTp with the human Grb2 sequence to search the *S. purpuratus* genome (v2.1). Dots indicate identities to the human sequence. Amino acids with similar characteristics are indicated by lower case. Asterisks above the alignment denote amino acids that function in the phosphotyrosine binding pocket. The SH2 and SH3 domains are indicated with single and double underlining, respectively. SpGrb2A-like (XP_786023.2 or XP_001175588.1) and SpGrb2B-like (XP_001180827.1 or XP_788430.1) are similar to human Grb2. SpGFR-bound-like (XP_001180717 and XP_001181623) is similar to human Grb2. SpGrb3-like (XP_001180777) is similar to human Grb3.

1995) to initiate signaling. The SpTie1/2 sequence only had a single tyrosine conserved at the C terminus of the cytoplasmic tail (the equivalent Y1101 position; see Fig. 3), which in vertebrates interacts with Grb2 and the p85 subunit of PI3K. Consequently, the sea urchin genome was searched for homologues of Grb2 and p85 using the human Grb2 sequence (CAG29359) and 12 gene models were identified that encode proteins similar to human Grb2. The four gene models with the best matches based on e-values were aligned with human Grb2 (Fig. 7). All of these proteins had SH2 and SH3 domains, and three or four of the conserved basic amino acids expected for a phosphotyrosine binding motif (Inohara et al., 2005). Additional genome searches using human p85 (AK302049) identi-
fied a sea urchin gene model (XP_793526; SPU_015590) encoding a protein with p85 similarity. Alignment of the relevant region of the sea urchin protein against human p85 showed conservation of the four basic amino acids that form the phosphotyrosine binding pocket (Fig. 8). Thus, there were several likely candidates for Grb2-like adapter proteins and a p85 protein encoded in the sea urchin genome that may interact with the cytoplasmic tail of SpTie1/2 to initiate signaling.

4. Discussion

The data presented here confirm and extend the initial reports of a Tie homologue in the purple sea urchin (Hibino et al., 2006; Smith et al., 1996; Bradham et al., 2006). The Sp059 cDNA sequence encodes an integral membrane protein of the RTK family. The characteristics of the tyrosine kinase domain, the types, numbers and arrangements of extracellular domains in the deduced protein, the exon structure of the gene, and the intron positions between the exons encoding in the tyrosine kinase domain all group SpTie1/2 within the Tie family, which is the only known protein family to have this unique combination of extracellular domains (Dumont et al., 1992; Maisonnierre et al., 1993; Partanen et al., 1992; Sato et al., 1993; Jones et al., 2001; Schnurch and Risau, 1993; MacDonald et al., 2006). The tyrosine kinase domain, with a highly conserved catalytic site, nucleotide binding site, and activation loop, is typical of RTK proteins (Kissau et al., 2003) and vertebrate Tie2 in particular (Shewchuk et al., 2000).

Although SpTie1/2 is a single copy gene, multiple alleles are present in the population (as represented by the various cDNAs that were sequenced) and show 5.5% sequence variation (239 of 4296 nt) that is similar to the 4–5% overall rate of polymorphism that were sequenced) and show 5.5% sequence variation (239 of 4296 nt) that is similar to the 4–5% overall rate of polymorphism estimated for the sea urchin population (Britten et al., 1978). The SNPs and amino acid polymorphisms are not evenly distributed throughout the coding region and are less abundant in functionally important regions of the protein. The conserved regions include the tyrosine kinase domain and the region of the ectodomain including the three Ig domains and an EGF domain that putatively bind the ligand (Table 2). In vertebrate proteins, the Ig and EGF domains in the ectodomain form a compact globular structure that present the second Ig domain outward from the cell surface for binding the Ang proteins (Barton et al., 2006; MacDonald et al., 2006). The stalk of the protein, which is composed of the four FNIII domains, functions to extend the Ang binding site away from the cell surface. The stalk is the most variable region of the sequence with the most SNPs and amino acid polymorphisms. Overall, the distribution of SNPs suggests that the most important functions of SpTie1/2 are carried out by the tyrosine kinase domain and the ligand binding region, which is the same for the vertebrate Tie proteins.

4.1. Exons and structural motifs

The SpTie1/2 gene differs from the vertebrate Tie genes in having more exons (27 vs. 22 for the vertebrate Tie genes) that correlate with additional extracellular domains (extra Ig-like, EGF and FNIII domains), plus two exons that are absent from the vertebrate Tie genes but that are not associated with a recognizable domain (Figs. S1, 2). Conversely, the number and approximate sizes of exons, and the positions of the exons is highly conserved within the catalytic tyrosine kinase domain among vertebrate Tie genes and SpTie1/2. This consistency contrasts with the differences that exist among different RTK families in which the number of exons that encode the catalytic region range from five to ten based on SMART analysis of protein sequences. Even the FGFRs, which are the most closely related to Tie family on the basis of similarity searches, are missing one of the introns found in the vertebrate Tie genes and in SpTie1/2 (e.g., D’Aniello et al., 2008). Thus SpTie1/2 is closely allied with the vertebrate Tie genes.

4.2. Possible function of the encoded protein

There are three lines of evidence to consider when speculating on the function of SpTie1/2: (i) the structure of the protein itself, (ii) the function of orthologues in other organisms, and (iii) the pattern of expression within the tissues of both the adult and developing sea urchin. In post-embryonic vertebrates, hematopoiesis in the bone marrow requires periodic division of a self-renewing but relatively quiescent population of stem cells called the long-term repopulating hematopoietic stem cells (LTR-HSCs), which express Tie2 and require the activity of the functional protein (Hsu et al., 2000). The Tie proteins are not required for fetal hematopoiesis or for the initial production of adult HSCs (Puri and Bernstein, 2003) but are involved in maintaining the LTR-HSCs in a quiescent state that promotes their long-term survival (Arai et al., 2004). Tie1 is not required for the function or survival of HSCs (Partanen et al., 1992; Rodewald and Sato, 1996). Although equivalent cells to LTR-HSCs are not known in echinoderms and the site(s) of coelomocyte proliferation are also unknown, the sea star Asterias rubens responds to LPS and concanavalin A with proliferation of coelomocytes, the epithelial lining of the coelomic cavity, the TieEdemman body, and, most notably, the axial organ (Holm et al., 2008). The function of the axial organ in the sea urchin is poorly understood, but it is known to contain phagocytic cells, as well as cells that are identical to several classes of coelomocytes: phagocytes, red sphere cells, and vibratile cells (Bachmann et al., 1980). Millott (1966) reported that the axial organ rapidly releases cells into the haemal system in response to injury. Furthermore, ultrastructural analysis suggests that the axial organ functions as an excretory organ and as a site for the destruction of senescent coelomocytes (Bachmann et al., 1980) or foreign cells (Millott, 1966). The presence of coelomocytes in the axial organ, whether it is their proliferative site of origin or whether they are sequestered there for recycling may be the underlying basis for elevated SpTie1/2 expression. In embryos, it is noteworthy that SpTie1/2 expression correlates with ingestion and differentiation of secondary mesenchyme cells into blastocoelear cells (Kinnander and Gustafson, 1960; Hardin and McClay, 1990) and pigment cells (Gustafson and Wolfpert, 1967; Gibson and Burke, 1985; Ruffins and Ettensohn, 1993), which are competent to carry out immune functions shortly after ingestion (Smith et al., 2006; Hibino et al., 2006; Silva, 2000; Furukawa et al., 2009). Results from both adult and embryonic gene expression studies suggests that SpTie1/2 may be involved in immune cell proliferation.

The function of the vertebrate Tie proteins in angiogenesis has been studied extensively because of the importance of this process in tumor formation and tissue repair (reviewed by Martin et al., 2008; Peters et al., 2004). There is good evidence that endothelial and hematopoietic cells develop from a single type of progenitor cell (Huang et al., 1999; reviewed by Schatteman et al., 2006), and Tie proteins are expressed in the endothelial cells of developing blood vessels (Dumont et al., 1992, 1994a,b; Korhonen et al., 1992) and in hematopoietic cells of the fetal liver (Hsu et al., 2000) and bone marrow (Batard et al., 1996; Hashiyama et al., 1996). Neither Tie1 nor Tie2 are required for initial formation of blood vessels in the vertebrate embryo (vasculogenesis), but both are required for

<table>
<thead>
<tr>
<th>Domain or region</th>
<th>% of ORF</th>
<th>% of SNPs</th>
<th>% of amino acid polymorphisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectodomain: 3 Ig, 4 EGF</td>
<td>38.6</td>
<td>28.1</td>
<td>31.4</td>
</tr>
<tr>
<td>Stalk region: 4 FNIII</td>
<td>26.6</td>
<td>54.8</td>
<td>55.6</td>
</tr>
<tr>
<td>Tyrosine kinase domain</td>
<td>19.3</td>
<td>10.4</td>
<td>5.0</td>
</tr>
</tbody>
</table>
angiogenesis in the adult (Puri et al., 1999), including Tie2 involvement in blood vessel sprouting (Jones et al., 2003; Kim et al., 2000; Takakura et al., 2000). Thus, the roles of the vertebrate Tie proteins in angiogenesis, are to influence the interaction of the endothelium with the underlying connective tissue (Suri et al., 1996), i.e., to regulate the associations of existing cells rather than to regulate cell proliferation. Although sea urchins do not have blood vessels, they do have tubes that function in the water vascular system, which use water hydraulics to move and extend the tube feet for locomotion. Although the water vascular system is a permanent system in the adult, we speculate that the SpTie1/2 gene may be expressed during late development (a time point that was not evaluated) and function in remodeling the tubes of the water vascular system during metamorphosis. The adult rudiment grows in the larva and upon metamorphosis, everts to form a juvenile sea urchin with five tube feet (Smith et al., 2008). The water vascular system begins to develop in the adult rudiment and likely undergoes remodeling, reorganization and/or expansion during metamorphosis resulting in a pentamersous, sessile, juvenile sea urchin. If SpTie1/2 plays a role in tube remodeling that is similar to the vertebrate Tie protein functions in blood vessel formation, the tubes of the water vascular system are a possible candidate. Future work on the developing water vascular system in the adult rudiment and the newly metamorphosed juvenile sea urchin will be required to demonstrate a correlation with SpTie1/2 expression. Results from both adult and embryonic gene expression studies suggest that SpTie1/2 may be involved in immune cell survival and/or proliferation based on expression in the axial organ.

4.3. Possible downstream adaptor proteins

Upon ligand binding, the Tie proteins initiate signaling cascades by phosphorylating the cytoplasmic tail so that associated adaptor proteins can bind. There are three tyrosines (Y1101, Y1107, and Y1112) located near the C terminus of the mouse Tie2 protein that, when phosphorylated, enable interaction with adaptor proteins with SH2 domains (Huang et al., 1995). The first tyrosine, Y1101, binds to the SH2 adaptor proteins Grb2 and Grb7 (Jones et al., 1999), while the third tyrosine, Y1112, binds SHP2 adaptors (Peters et al., 2004; Huang et al., 1995). Phosphorylation of Y1101 also promotes binding to the p85 subunit of PI3K (Kontos et al., 1998), which activates the PI3K/Akt pathway (Kim et al., 2000; Jones et al., 1999) promoting cell survival and enhanced cell migration (Peters et al., 2004; Jones et al., 1999; Kontos et al., 1998). Binding of Grb2 can activate the mitogen-activated protein kinase (MAPK) pathway leading to morphogenetic changes in vertebrate endothelial cells (Peters et al., 2004). Vertebrate Tie proteins also interact with the p85 subunit of PI3K, and the activation of Tie2 leads to an inhibition of apoptosis and an increase in cell survival, as well as changes in mobility through effects on the cytoskeleton (Kontos et al., 1998). The equivalent Y1101 position at the C terminus of the SpTie1/2 cytoplasmic tail is the only tyrosine in a conserved position for phosphorylation. Because SpTie1/2 only has one of the three possible functional tyrosines, it may have restricted activities compared to the vertebrate homologues. Phosphorylated Y1101 in SpTie1/2 may bind the homologues of Grb2 and/or p85 to act in a manner analogous to vertebrate Tie2. This infers that SpTie1/2 function in coelomocytes may be involved with the control of the cytoskeletal transformations and changes in adhesion or mobility that have been observed for coelomocytes in contact with foreign substances or cells (Eds, 1985). Because SpTie1/2 only has one of the three possible functional tyrosines, it may have restricted activities compared to the vertebrate homologues.

Unlike Tie2, vertebrate Tie1 has no known ligands, although its activity is influenced by Ang analogues (Saharinen et al., 2005). Tie1 is postulated to regulate the activity of Tie2 by binding to its extra-cellular domain and interfering with activation by Ang1 (Marron et al., 2007, 2000). Although the two vertebrate Tie proteins are known to interact, the nature of this interaction is still incompletely understood. Tie1 may function in angiogenesis as an agonist to Tie2 activation. It is worth noting that Tie1 from several vertebrates has the consensus sequence YYNN at the binding site for p85 (Y1100/1101), whereas the Tie2 proteins have the sequence YYNT (Fig. 3). This would indicate that phosphorylated Tie1 should bind to p85 more strongly than Tie2 and might activate the PI3K/Akt pathway. The equivalent putative Ang binding site in SpTie1/2 is YYNL, which would seem to be more like the Tie1 sequence because of the hydrophobic amino acid in the fourth position. However, because sea urchin SpTie1/2 appears to be a single copy gene, the role of SpTie1/2 may be more similar to the vertebrate Tie2 and perhaps function in hematopoiesis of coelomocytes in the axial organ and the differentiation of blastocoelar and pigment cells during early embryonic development. The hematopoietic role might be ancestral, while angiogenesis function may have evolved after Tie gene duplication in vertebrates.

We have confirmed the expression of the SpTie1/2 gene in the coelomocytes and have identified expression in the axial organ and in the gastrula and post-gastrulating embryo. Our evidence indicates that this gene follows the evolutionary pattern found in many RTKs, in that sea urchins have a single gene whereas vertebrates have two (or more) paralogues (Lapraz et al., 2006). Based on sequence comparisons, domain organization and combination, intron locations, and gene expression in the adult and embryonic sea urchin, we suggest that SpTie1/2 might be acting to regulate coelomocyte mobility and/or proliferation. Elevated expression in the axial organ suggests that it may be a source of coelomocytes and that further investigations of this organ are warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2010.03.010.

References


