SpCoel1: a Sea Urchin Profilin Gene Expressed Specifically in Coelomocytes in Response to Injury

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SpCoel1 is a single copy gene that is specifically expressed in most of the coelomocytes of the adult purple sea urchin, Strongylocentrotus purpuratus. The 4-kb transcript from this gene has a relatively short (426 nucleotide) open reading frame (ORF) with long 3' and 5' untranslated regions. The ORF encodes a protein that has strong amino acid sequence similarity to profilins from yeast to mammals. Transcript titrations of SpCoel1 show significant increases per coelomocyte in animals that have been physiologically challenged. Increases in transcript levels are of similar magnitudes between animals receiving different treatments, such as injuries from needle punctures or from injections of foreign cells. The evidence presented here implies a molecular mechanism by which this lower deuterostome defense system responds to external insult, viz that an external "injury signal" activates a signal transduction system, which in turn mediates the alterations in cytoskeletal state that are required for coelomocyte activation.

INTRODUCTION

Coelomocytes, the immune effector cells of echinoderms, carry out many functions that are also performed by the cellular effectors of the immediate nonspecific defense systems of mammals. Thus, coelomocytes are capable of phagocytosis, chemotaxis, and expression of cytotoxic agents on activation in response to injury or invasion by foreign cells. There are four morphologically distinct types of coelomocyte in the purple sea urchin, Strongylocentrotus purpuratus. The majority of coelomocytes, 66.3 ± 11.6% (mean ± SD), are phagocytes; vibratile cells account for 13.9 ± 9.8%, colorless spherule cells account for 5.1 ± 4.6%, and red spherule cells account for 14.8 ± 8.5% (our unpublished data). Specific functions of the various coelomocyte classes are poorly defined. The phagocytes and the red spherule cells are apparently those that respond to injury, as indicated by their accumulation at injury sites (Coffaro and Hinegardner, 1977; Höbaus, 1979). The coelomic fluid is itself bactericidal (Wordlaw and Unkles, 1978; Service and Wordlaw, 1985), and echinochrome-A, contained within the red spherule cells, is a bacteriocidal agent (Service and Wordlaw, 1984). These cells degranulate in response to bacteria (Johnson, 1969). Various substances, cells, and microbes injected into the coelom of echinoderms are quickly removed by coelomocytes (Reischl and Bang, 1971; Coffaro, 1978; Yui and Bayne, 1983). Clearance activities have also been observed in vitro where they are assigned to the phagocytic class of coelomocytes (Johnson, 1969; Messer and Wordlaw, 1979; Bertheussen, 1981). In addition, mixtures of adherent phagocytes from the same or different sea urchin species in culture display cytotoxic reactions (Bertheussen, 1979).

Coelomocytes have also been implicated in graft rejection. In sea urchins, as in other animals, surgically implanted body wall autografts heal in, whereas allografts and xenografts are rejected. An increase in the rejection rate of any secondarily implanted graft has been reported (Coffaro and Hinegardner, 1977). These results imply that the cellular rejection system in sea urchins can be generally enhanced by activation, but there is no evidence for recognition specificity as assayed by the effector functions that mediate rejection. Thus, a second set allograft is not rejected at a significantly faster rate than a third party allograft (Coffaro and Hinegardner, 1977). Nor do repeated injections into the coelom of a variety of substances, such as xenogeneic cells, proteins, viruses, and inert substances, result in detectable increases in clearance rates (Reischl and Bang, 1971; Coffaro, 1978; Yui and Bayne, 1983). The immune defense systems of sea urchins are thus entirely nonspecific. Coelomocyte responses to challenge entail rapid alterations in motility, induction of phagocytic and encapsulation functions, and release of cytotoxic factors by degranulation. Similar cytological transformations
have been observed when coelomocytes are removed from coelomic fluid (Edds, 1977, 1980; Edds et al., 1983). These responses involve reposition of actin filaments and require a rapid transformation of the entire cytoskeletal organization.

This communication concerns a gene, SpCoel1, that appears to be involved in a sensitive response of S. purpuratus coelomocytes to minor injury to the organism. The transcript of the SpCoel1 gene encodes a polypeptide that displays striking amino acid sequence similarity to profilin, a small protein that has both actin and phosphatidylinositol bisphosphate (PIP2) binding functions (Reichstein and Korn, 1979; Lassing and Lindberg, 1985; Goldschmidt-Clermont et al., 1991a). In mammalian cells, profilin appears to act at the intersection of signal transduction and cytoskeletal organization systems (Machensky et al., 1990). We show that in response to challenge SpCoel1 transcripts increase sharply and specifically in coelomocytes. The SpCoel1 gene product thus may be involved in the protective response mechanisms of coelomocytes.

MATERIALS AND METHODS

Source, Handling, and Housing of Sea Urchins

Sea urchins used for this study were collected near Corona del Mar, California, from subtidal regions down to 30 ft. They were housed at the Caltech Kerckhoff Marine Laboratory (Corona del Mar, CA) in running, chilled, aerated sea tables under a scheduled light/dark cycle and fed kelp on a biweekly basis (Lehay et al., 1978; Leagy, 1986).

RNA Isolation

Coelomocytes from adult sea urchins were drained from the animals after removal of the mouth structures, or “Aristotle’s Lantern.” The coelomic fluid was poured through sterile cheese cloth and mixed into 10 ml of cold Ca- and Mg-free sea water (CMPSW) (Humphreys, 1963) containing 30 mM EDTA, pH 8, salinity 32. The cells were pelleted and lysed by vortexing in 5 μl guanidinium thiocyanate containing 50 mM NaOAc, 50 mM EDTA, and 5% beta mercaptoethanol. Other adult tissues, including ovary, testis, and gut, were dissected out of the animal, minced with razor blades, rinsed in filtered sea water, and lysed by vortexing in the guanidinium solution. After lysing, 0.1 vol of 30% sodium lauryl sarcosine was added to each tissue sample, the DNA was sheared with a tight fitting dounce homogenizer, and the nucleic acids were isoprecipitated by centrifugation using a 5.7 M CsCl cushion containing 50 mM NaOAc and 50 mM EDTA. The pellets were washed in 70% ethanol, dried, resuspended in RNAse-free extract buffer (20 mM tris(hydroxymethyl) aminomethane [Tris], pH 8, 10 mM KCl, 3 mM MgCl2, 0.5% sodium dodecyl sulfate [SDS]), extracted with phenol/Sevag (1:1:1) (Sevag solution is 24 parts chloroform, 1 part isoamyl alcohol), precipitated with 2.5 volumes of absolute ethanol, and resuspended in diethyl pyrocarboneate-treated water. Poly(A)* RNA was selected on an oligo(dT) cellulose column.

cDNA Library Construction

RNA was isolated from “activated” coelomocytes. For coelomocyte activation protocols, see below. Blunt-ended double-stranded cDNA was synthesized from poly(A)* RNA according to the manufacturers instructions (RNA Synthesis System Plus kit, Amersham, Arlington Heights, IL). The cDNA was either ligated to EcoRI linkers, size selected on a Biogel A-50 column (Bio-Rad, Richmond, CA) and ligated into lambda gt 10 phage, or ligated to BstXI adapters (Invitrogen, San Diego, CA), size selected on a Select-5L spin column with a DNA fragment retention size of <271 base pairs (5', 3'), ligated into pTZ18R-B plasmid (Invitrogen), and transformed into DH1αF bacteria (Invitrogen). The libraries were amplified once. The average insert size for the phage library was 1.7 kb and for the pTZ18R-B plasmid library was 1.4 kb.

SpCoel1 Coding Region Subcloned into the pET Expression Vector

The coding region of SpCoel1 (minus the first 20 amino acids) located in the Bsc9b cDNA between the KpnI site and the 3' end of the clone was subcloned into the BamHI site of the pET-3a expression vector (Studier et al., 1990) using specially constructed BamHI to KpnI linkers (from the Caltech Microchemical Facility) that restored seven amino acids to the 5' end of the coding region. The construct was transformed into BL21(DE3) bacteria containing the pLYS plasmid that expressed a fusion protein of expected size. The fusion product included the first 11 amino acids of gene 10 and amino acid SpCoel1 sequence from amino acid 14 to the end of the sequence.

Rabbit Antiserum Against SpCoel1 Fusion Protein

The 15-kDa SpCoel1 fusion protein was isolated from the bacterial lysate by acrylamide gel purification. Two hundred micrograms were sonicated with Freund’s complete adjuvant and injected intramuscularly in both hind legs of two New Zealand white rabbits. Subsequent booster injections were done with Freund’s incomplete adjuvant subcutaneously on the rabbit’s back. The rabbits were bled from the ear vein 10 d after the last injection. Blood was allowed to clot overnight at 4°C, the serum was recovered after centrifugation, and stored at -70°C.

Density Centrifugation of Coelomocytes on Percoll Step Gradients

Coelomic fluid was removed from a sea urchin and diluted into an equal volume of cold CMPSW-EDTA. The diluted cells were overlaid on a Percoll (Pharmacia, Piscataway, NJ) step gradient. The gradients were formed by overlaying decreasing concentrations of Percoll (dialyzed against CMPSW-EDTA overnight) diluted in CMPSW-EDTA. The best range of Percoll concentrations for coelomocyte separations was 20%, 30%, 50%, 70%, and 100%. The gradients were spun at 118 x g at 4°C for 15 min without the brake. The layers of cells were unloaded from the top of the gradient.

RNA Blot Hybridizations

Hybridizations were carried out under standard conditions at 48°C with 50% deionized formamide, 5X SET (0.15 M NaCl, 30 mM Tris, pH 8, 2 mM EDTA), 1X Denhardt’s solution (1% Ficoll, 1% bovine serum albumin, 1% SDS, 1% polyvinyl pyrrolidone), 20 mM phosphate buffer, pH 6.8, 200 μg/ml sheared denatured salmon sperm or calf thymus DNA, 200 μg/ml yeast transfer RNA. Final washes were carried out at 68–72°C in 0.3X SSC (1X SSC = 0.15 M NaCl, 15 mM Na citrate, pH 7) with 1% SDS.

32P-rUTP-Labeled RNA Probes

Antisense RNA probes of known length from specific regions of the various cDNAs were generated on linearized templates with RNA polymerase T3 or T7 (New England Biolabs, Beverly, MA) incorporating 32P-rUTP (800 Ci/mmo, from Amersham or New England Nuclear, Boston, MA) to the specific activity of 1.2 × 109 counts/min/μg probe. Run-off transcripts were generated according to technical information from Promega Biotech (Madison, WI). After RNA synthesis, the DNA templates were removed by digestion with RNase-free RQI DNase (Promega). The remaining RNA probe
was extracted with phenol/Sevag and spun through a G-50 Sephadex (Pharmacia) 1-ml column to remove unincorporated nucleotides. The riboprobes were heated to 80°C for 4 min before being used.

**In Vitro Translation of SpCoelI**

Run-off strand RNA was produced from cDNA clone Bsc9b (see Figure 1). The 5' end of this clone is nt 491 of the full-length message. The transcript ended at the HindIII site, at nt 1911. This transcript includes the start and the following two stop codons (Figure 1). This message was added to wheat germ lysate (Amersham) with 25S-methionine (Amersham) and incubated according to manufacturers instructions. The proteins were separated on a 4% stacking and 15% running SDS-polyacrylamide gel, stained with Coomassie, destained, dried, and autoradiographed for 2 d, together with markers and controls lacking SpCoelI RNA.

**Probe Excess Transcript Titration**

Probe excess analysis of specific transcripts in varying amounts of total RNA from sea urchin tissues has been described in detail (Lee et al., 1986). Numbers of transcripts per cell were derived by the calculations of Lee et al. (1986). Briefly, transcripts per cell = ab/cde, where as follows: a. cpm/pg of total RNA (a is the slope of the linear regression line); b. 1.5 pg RNA per cell (2.8 ng RNA/1800 cells per 72 h embryo) (Gouw and Will, 1981; Cameron et al., 1989); c. cpm/pg of probe (specific activity of the probe); d. fraction of the probe represented in the message; and e. pg mass per transcript.

**Coelomocyte Activation**

Because sea urchins have been shown to increase their rejection rate of second set and third party allografts, we attempted to activate the coelocytes from which the cDNA library was constructed to increase the numbers of transcripts coding for gene products involved in the cellular defense responses of the animal. This was accomplished by injecting 100 μl of whole coelomic fluid (including proteins and cells) from a congeneric species Stronglyocentrotus franciscanus into the coelomic cavities of 16 adult S. purpuratus individuals. Injections were performed on days 1, 2, 3, 6, and 7; the cells were collected and pooled on day 9 and immediately processed for isolation of RNA.

Coelomocytes were also activated in a variety of other ways in the experiments in which SpCoelI transcripts were measured. Sea urchins were injected with 50 or 150 μl of coelomic fluid from S. franciscanus; 50 μl of their own coelomic fluid was withdrawn and reinjected without removing the needle from the peristomial membrane or they were injured with 1 or 10 needle holes in the peristomial membrane per day. These treatments were carried out on days 1, 2, 4, and 5.

**RESULTS**

**SpCoelI, a Coelomocyte-Specific cDNA Clone**

This study began with the isolation of cDNA clones from a lambda gt10 library prepared from activated S. purpuratus coelomocyte poly(A)⁺ RNA (see MATERIALS AND METHODS). The initial isolate, pSPT6, was selected on the basis of preliminary data that indicated that the transcript from which it is derived is present at significantly higher levels in activated than in control coelomocytes obtained from undisturbed animals. Genome blots demonstrated that pSPT6 contains a single copy sequence. However, as indicated in Figure 1, pSPT6 includes only a section of the 3` untranslated region (UT) of a coelomocyte message. Further clones were isolated using pSPT6 as a probe, and a composite map of the complete 4 kb transcript, which we have termed SpCoelI, is shown in Figure 1. The sequence of the SpCoelI mRNA is given in Figure 2. An open reading frame (ORF) begins at nt 599 (as numbered from the 5` end of the transcript), and the first stop codon is located at nt 1025. This defines an ORF of 426 nt, which encodes a rather small 15.3-kDa protein of 142 amino acids. A second stop codon does not occur until nt 1301. Were the stop signal at nt 1025 read through, the protein would then include 92 additional amino acids and its size would be 25.9 kDa. Numerous stop codons occur immediately after nt 1301. The stop codon at position 1025 is real and functional, however, at least in an in vitro translation assay. RNA was transcribed in vitro from clone Bsc9b (see Figure 1), which contains the whole of the relevant region, including some of the 5` UT region, the start codon, the first and second stop codons, and about half of the 2.7 kb 3` UT sequence. When added to a commercial wheat germ lysate, this RNA directed the translation of a 15.3-kDa protein, just as expected if translation indeed terminates at nt 1025.

**SpCoelI Amino Acid Sequence Comparisons**

The SpCoelI amino acid (a, a) sequence shown in Figure 2 was used to search the protein sequence database. The best match to the protein encoded by the SpCoelI ORF is with the widely distributed protein profilin. As shown in Figure 3A, 72 of the 142 amino acids encoded
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GACACATGTC CCCTCTCCGG AAGCACCTCA TCCGCTCAAATG TTAGGCTAGT ATGAGGGTGAA 60
TGCGTGCCAGT CTGGTCTGCTATG CACGCTTCTAA ATAGAGGAGGA GGGCTTGAA 120
CATGAACTCT CAGCTTCTGCTC TCTGACTTTT CATGAAACCG AATCCCCCAATCGCNGAGNNCGC 180
TCTGACTCTCAGCTCCTAGAGCANNCTGAGCAGCCTGATGAGCTTGGTACTGCTGCTGGATCC 240
TCCTGCTGGGAA CACTGTGAGA ATCTCTTCTGAC ATCTCTTCTGAC ACTATATCTCA TCTCTCTC 300
CAAGCTCAATG GTAAACCTGCA GCCTAGAGAATGTGAGGTTGGT ATGAATGCTG 360
ATTACCTCTC TCTGACCTCA CTCTCTCTCTGAAATCCGATG CTGCTCTTCTGCTGCTGGATCC 420
TGACTCTGGCT GCATGCTCTC ATCTCTGAAACTT GATGATGTAATGCTGCTGCTGGATCC 480
GTGAAAGCTC ACCCTCCCAATACGAGGTC ACCGGTGATTGAC ATCTATATACTGAT 540
TGACTGCGG CTGTATTACAAG TTACGCGGCTGCTGCTGGATCC 598

ATG TCT TGT GAT TCA TAC GTC GAC AAT CTT ATA GCT CAG TCC AAA GAT 646
Met Ser Trp Asp Ser Tyr Asp Leu Ile Ala Gin Ser Lys Asp 16

GCG TCA GCT ACC ACC CAT TGC GAC AAG GCC GTG ATA ATC GGT AAA GAT 694
Ala Ser Gly Thr Thr His Cys Asp Lys Ala Cys Ile Ile Gly Lys Asp 32

GGA TCT TGG ACC AGC ATG CGG ACA TCT GAT ACC AGC AAC AAT TTA 743
Ser Gly Ala Trp Thr Thr Met Pro Thr Ser Thr Asn Asn Leu 48

AAG GAT GCC GAA GAG ATG GCA AAT ATA GCA AAA TGT TTT AAG TCG 790
Lys Leu Thr Pro Glu Glu Met Ala Asn Ile Ala Lys Cys Phe Lys Ser 64

AAG GAT TCT GCA GCT TCT TGT TCC TCT GAT GTA TAT GAA GCA ACA 838
Thr Ala Ala Phe Ala Ala Phe Met Ser Ser Gly Ile Tyr Val Asn Gly Thr 80

AAA TAC CAA TTC TTA AGG GAA GAC TCA AAG TTG GTG TTG GGA AAA 886
Lys Tyr Gin Phe Leu Arg Glu Gly Asp Ser Lys Leu Val Leu Gly Lys 96

AAG AAA GAT GAA GGA TCA CTC ACA TGG CAA AGC AGC AAG ACA GGG ATT 934
Lys Lys Gly Gly Ser Leu Thr Leu Gin Ser Ser Thr Ala Ile 112

GTA ATC GAT CAT TGC CCA GAA GGA GCC CAG CAA GGG AAT TTT TAT AAA 982
Val Ile Gly His Cys Pro Glu Gly Gly Gly Asp Leu Asn Lys 128

GCA GTT GCC GTA ATA GCA GAA TAT TGG GAG ATG TGT TCA TGT TAA TGG 1030
Ala Gly Val Gly Val Ile Gly Leu Ser Leu Ser Met * [Cys 143

ATT CCC AAC CAT AGG ATA TTT GAT ATG CCA AGA AGA TTT GGC TGG AGG 1078
Met Pro Asn His Arg Ile Leu Tyr Met Ala Arg Arg Phe Gly Leu Arg 159

TTG GAT GAC AGG AAA GGA GCT CAA GAA AAA CCG AAG ATT CAA GGA ATA 1126
Leu Asp Arg Gly Ala Gin Gly Lys Arg Lys Gin Ile Gly Gin Ile 175

ATT TTA AGA ACA CCA TGT CTT CTC GAC GAC TAT TGG TAT GAG GCA 1174
Ile Leu Arg Thr Phe Leu Leu Leu Asp Phe Met Cys Leu Asn Gly 191

TGG TTA ACT GAC CAT GTC TGG TAT ATG AAG TAA TCA CAC 1222
Thr Leu Thr His Ile Ser Phe Leu Gly Met Leu His Ser Leu 208

AAA ACT ATA CTT ACA TAT TAT CAA GCT GCA GGA TCA GAA TAA TAT 1270
Lys Thr Ile Leu Thr Phe Tyr Thr Ala Ile Gly Thr Ser Arg Phe Ser 224

ATA AAG TTA TGT GAC AAA ACC CTT CTA GCA TAA AACTTAA AAGGGCACA 1320
Ala Lys Leu Cys Asp Lys Thr Leu Leu Gly] * 234

CACAAGTGGT ATTGTTTATG TTCAAGAAGCAC AAAATAGTGA TGCTAGATAC AATGAGGAAT 1380

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Figure 2. SpCoel1 mRNA sequence and deduced amino acid sequence. The first ATG of the ORF occurs at nt 599. The ORF terminates with a stop codon located at nt 1025. A second stop codon occurs at nt 1301; both are denoted by an asterisk. The ORF is thus 426 nt in length (142 aa). If utilization of the initial stop codon at nt 1025 was in some way suppressed, the cryptic additional peptide indicated in brackets would be added to generate an ORF of 699 nt (234 aa).

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by SpCoel1 (i.e., 51%) can be found in at least one of the four bona fide profilins at homologous positions in the protein. The comparison is anchored by agreement with all four profilins in 14 positions located throughout the protein. To achieve this alignment, three short regions of the sea urchin sequence were considered as "insertions" with respect to all four other profilins: a 7 amino acids sequence (positions 16–22 of SpCoel1), a 3 amino acids sequence (positions 45–47), and a 2 amino acids insertion (positions 66 and 67). On the other hand, the mouse and cow profilins possess a 16 amino acid insertion (at position 120 of the SpCoel1 sequence) with respect to the sea urchin, amoeba, and yeast profilins. All registry changes other than the three small sea urchin insertions utilized in the optimized alignment shown in Figure 3A are supported by occurrence of the same changes in at least two of the four profilins used for the comparison. Of the 72 shared amino acids resulting from this alignment, 23 are shared both with at least one mammalian profilin and at least one of the lower eu-karyote profilins; 12 are shared only with one or more of the mammalian profilins; and 36 are shared only with one or more of the lower eu-karyote profilins. Figure 3A indicates convincingly that SpCoel1 is a sea urchin profilin or a closely related protein. No other comparisons were discovered that approach the >50% level of similarity with a "consensus" protein that is revealed by the multiple profilin alignment illustrated.

Even though the SpCoel1 sequence contains a genuine termination signal after nt 1025, it was interesting to explore sequence similarities in the ORF that follows nt 1025, because it is possible that this termination codon could be spliced out or suppressed in some cell types or stages of the life cycle not yet examined. This comparison, shown in Figure 3B, reveals a surprising 28% similarity, in almost perfect registry, with a portion of the human and rabbit low-density lipoprotein (LDL) receptor that includes the transmembrane sequence. Identities between SpCoel1 and the human and rabbit sequences are found throughout this portion of the sequence. The human and rabbit proteins include four amino acids, NPYV, that are located just inside the plasma membrane. The tyrosine (#805 in the rabbit) at the end of this diagnostic sequence element is necessary for binding the LDL receptor to clathrin-coated pits during internalization (Chen et al., 1990). The SpCoel1 sequence lacks the NPYV element, except for the tyrosine, which occurs in the SpCoel1 sequence in exactly the same relative position as in the LDL receptor (Figure 3B, amino acid 215; this tyrosine is highlighted on a black background).

**SpCoel1 Expression in Adult Tissues**

An RNA blot hybridization study of SpCoel1 expression in adult tissues is shown in Figure 4 (see MATERIALS AND METHODS). Testis, ovary, and gut showed almost

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**Figure 3.** SpCoel1 encodes a profilin. Protein similarities were identified by searching the Protein Identification Resource database base Release 29.0, June 3, 1991 (National Biomedical Research Foundation). Amino acid sequence alignments were generated with the FAST A program (Pearson and Lipman, 1988), improved with the Macaw alignment program (Schuler et al., 1991) for Windows 3.0 (Microsoft), and minor adjustments were then made by hand. Amino acid numbers refer to the SpCoel1 sequence. (A) Multiple alignment comparison with selected profilin sequences. Shaded letters denote matches to SpCoel1. Within the "consensus" sequence shown on the bottom row, an amino acid letter appears if there is at least one match to SpCoel1; the letter is shaded if the sea urchin sequence matches two or more of the four profilins and is underlined if the amino acid is found in all four profilins. The sequences on this table are from the following sources: Amoeba, *Acanthamoeba castellani* profilin-1 sequence from Ampe et al. (1988b) and Pollard and Rimm (1991); Yeast, *Saccharomyces cerevisiae* sequence from Oechsner et al. (1987); Cow, profilin sequence from Ampe et al. (1988a). The mouse profilin sequence is from Wieda et al. (1989). In this comparison, R is considered equivalent to K, but no other equivalences between chemically similar amino acids have been accepted, and the comparison is thus a conservative one. Some pairwise similarity results for SpCoel1 versus other profilins are as follows: sea urchin (*Anthocidaris crassispina*) 75%; sand dollar (* Clypeaster japonicus*) 73%; amoeba (*A. castellani*) 37%; yeast (*S. cerevisiae*) 35%; slime mold (*Physarum polycephalum* 35%; human (*Homo sapiens*) 23%; cow (*Bos bovis*) 21%; mouse (*Mus musculus*) 17%. These were obtained using the McCaw program (Schuler et al., 1991) for Windows 3.0 (Microsoft). References for the additional sequences are as follows: for the sea urchin (*A. crassispina*) and sand dollar (*C. japonicus*), Takagi et al. (1990); for slime mold (*P. polycephalum*), Binette et al. (1990), Takagi et al. (1990); for human (*H. sapiens*), Kwiatkowski and Bruns (1988). (B) Comparison of the cryptic SpCoel1 ORF after nt 1028 with a portion of mammalian LDL receptor sequences. Shaded letters denote a match between SpCoel1 and either of the sequences listed. The boxed region denotes the amino acids located within the membrane in the mammalian sequences. The consensus sequence is shown on the bottom row. The black shaded Y, located in the consensus sequence, is found in all three sequences and in the LDL receptor is involved in receptor internalization (Chen et al., 1990). The human sequence is from Yamamoto et al. (1984); the rabbit sequence is from Yamamoto et al. (1986).
no expression compared with the coelomocyte samples. Control RNA gel blots and other evidence showed that these RNAs were not degraded, and the experiment indicates at least a major difference in profilin transcript concentration comparing coelomocytes with other cell types (this, of course, does not indicate the relative concentrations of stable profilin protein). On very long exposures, faint bands could be seen in the other adult tissues, which could have been due either to very low expression in those tissues or, possibly, to expression in migratory coelomocytes that were present in or on these tissues when they were isolated from the animal. Note that the band in the activated coelomocyte lane of Figure 4 is more intense than that for the normal coelomocytes (see below).

To determine which type of coelomocyte expressed the SpCoel1 protein, rabbit antiserum was raised against an SpCoel1 fusion protein. Coelomocyte types were differentially enriched in Percoll step gradient fractions according to their intrinsic density differences, as described in MATERIALS AND METHODS. Proteins extracted from equal numbers of the major cell types in each fraction were separated by SDS-polyacrylamide gel electrophoresis, electroblotted, and incubated with the rabbit antiserum. Figure 5 shows that all four cell fractions produce the SpCoel1 protein. However, the phagocyte-enriched fractions appear to contain more do than the other types of coelomocyte (compare lanes 1 and 2 with lanes 3 and 4 on Figure 5). The small phagocytes (lane 1) appear to have more SpCoel1 protein than do the larger phagocytes (lane 2). The coelomocytes display only the 15.3-kDa protein, indicating that the first stop codon is indeed utilized by these cells and that a transmembrane region is not present in the protein.

To obtain a more detailed analysis of SpCoel1 representation in coelomocytes of different types, the rabbit antiserum was used for a cytoplasmic immunofluorescence study. As shown in Figure 6B, most coelomocytes produced the SpCoel1 protein. All of the red and the colorless spherule cells and all of the vibratile cells are uniformly positive. However, only some of the phago-

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**Figure 4.** SpCoel1 blot hybridization to adult sea urchin RNAs. One microgram of poly(A)+ RNA was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde in 1X MOPS buffer (20 mM 3-[N-morpholino]propanesulfonic acid, 5 mM NaOAc, 1 mM EDTA, pH 7), blotted onto Genescreen Plus (Millipore, Bedford, MA), and probed with 32P-labeled antisense RNA under standard hybridization conditions (see MATERIALS AND METHODS). The probe spanned nt 1107 to 489 of the Bsc9b clone (Sac1 site to the 5' end of the cdNA clone, 609 nt long; see Figure 1). Lane 1, ovary; lane 2, testis; lane 3, gut; lane 4, coelomocytes; lane 5, activated coelomocytes. Marker sizes are shown on the left.

**Figure 5.** Immunological detection of SpCoel1 protein in coelomocyte fractions. Equal numbers (3 x 105) of the major coelomocyte type from each Percoll fraction were lysed by boiling in SDS sample buffer (4% SDS, 0.2% glycerol, 20% beta mercaptoethanol, trace of bromophenol blue). Samples were loaded onto an SDSPolyacrylamide gel (4% stacking gel, 15% running gel), electrophoresed at 25 mA for 2.5 h, and electroblotted onto an immulon-P filter (Millipore) in blotting buffer (20% methanol, 20 mM Tris, pH 8.8, 150 mM glycerine, 0.05% SDS) at 0.3 A for 2 h at 4°C. The filter was subsequently incubated in blocking buffer (phosphate buffered saline with 1% normal goat serum, 1% bovine serum albumin [BSA], 0.1% nonidet P-40), followed by rabbit anti-SpCoel1 antiserum, diluted 1:40 in blocking buffer. The secondary antibody, goat anti-rabbit Ig labeled with alkaline phosphatase, was diluted 1:400 in Tris-buffered saline containing 3% BSA. The enzyme substrate reaction was run according to instructions from the Boehringer-Mannheim Genius kit. Lane 1, 20% Percoll fraction, mostly small phagocytes with some large phagocytes. Lane 2, 30% Percoll fraction, mostly large phagocytes with some small phagocytes and 2.4% contamination with other cell types. Lane 3, 50% Percoll fraction vibratile cells with 13.6% colorless spherule cells. Lane 4, 70% Percoll fraction, red spherule cells with 4.5% contamination of various cell types. Lane 5, gel-purified fusion protein used to inject the rabbits. Lane 6, bacterial lysate containing the fusion protein. Marker standards are shown on the left.
cytes displayed SpCoel1 protein, whereas some clearly do not. No fluorescence was observed over a subpopulation of the phagocytes, consisting of most of the large phagocytes and some of the small ones (compare Figure 6, A and B). Although it is not easily seen in Figure 6B, the small phagocytes stain more intensely than do the other cell types, as expected from the antibody blot shown in Figure 5. Because positive and negative cells are adjacent to each other on the same slide, it seems unlikely that any difference in fixation or treatment could account for failure of some of the large phagocytes to stain, although local effects cannot be formally excluded. The SpCoel1 protein is clearly not mounted on the cell surfaces, because unfixed live cells appear uniformly negative.

SpCoel1 transcripts were also detected in coelomocytes by in situ hybridization (Figure 6C). An antisense RNA probe made from clone Bsc9b was hybridized to Percoll separated coelomocytes. Transcripts are present in all cell types, including all of the phagocytes. Because some cells appear to fail to react at all with the antibody, while all contain the SpCoel1 message, this suggests that some of the phagocytes either do not translate the SpCoel1 transcript, have in some way eliminated the SpCoel1 protein, or that the determinants recognized by the antisera are masked when the SpCoel1 protein is associated with other molecules.

**SpCoel1 Transcript Titrations**

Preliminary analyses on coelomocyte RNA collected from sea urchins that had been injected with *S. franciscanus* coelomic fluid indicated that this treatment induces an increase in SpCoel1 transcript level, as compared with "unactivated" or "normal" coelomocytes (e.g., in Figure 4, compare lanes 4 and 5). In further experiments, we found that four injections of *S. franciscanus* coelomic fluid over 5 d resulted in a higher transcript level per cell than one injection alone. Preliminary cell count data indicated that there are no gross variations in coelomocyte populations in response to this physiological challenge. Nor have proliferative responses of mixed coelomocytes in vitro been found (Bertheussen, 1979). The increases in SpCoel1 transcripts after injection are thus due to changes in the number of transcripts per average responding cell, rather than to changes in the relative sizes of the coelomocyte populations.

Groups of six sea urchins were used to analyze in more detail the time course of SpCoel1 transcript changes in response to various treatments (see MATERIALS AND METHODS). Probe excess titrations (Lee et al., 1986) carried out on total RNA extracted from these pooled coelomocytes yielded the average values of SpCoel1 transcripts per cell shown in Figure 7. All experimental groups displayed elevated levels of SpCoel1 transcript, compared with controls, as early as 3 h after the final treatment. Animals receiving either puncture injuries or reinjected coelomic fluid maintained their elevated transcript levels at 24 h. SpCoel1 transcript levels had returned to normal by 3 d. The group receiving 50 µl of coelomic fluid from *S. franciscanus* displayed a peak transcript level at 24 h, and the level has returned to normal by 6 d.

The sea urchins used in this study were from wild populations collected subtidally between Corona del Mar and San Diego, California. Though they were held under carefully controlled conditions in our large-scale culture system (Leahy et al., 1978) for several weeks before use, they could have retained significant physiological differences that might have affected our results. To examine individual responses, SpCoel1 titration measurements were carried out on coelomocytes prepared from single experimental and control animals treated as above. All the coelomocytes from an individual were needed for a single transcript titration measurement, and thus only one time point per animal could be examined. The coelomocytes were collected 1 d after the final treatment, i.e., the time at which the maximal response had been observed in the previous experiments (Figure 7). The results are displayed in Figure 8. Pairwise comparisons using Student's *t* test and Duncan's multiple range test were used to determine the significance of the differences observed between groups. The results of both tests showed that the experimental groups had significantly elevated mean transcript levels per cell, compared with controls, with a hierarchy of statistically significant differences among these groups. Animals receiving a single needle-hole injury per day, and those that had 50 µl of their own coelomic fluid withdrawn and reinjected, displayed levels of SpCoel1 per cell that were slightly above normal. (By Student's *t* test, the one-time injury and sham-injected distributions are both significantly different from the pooled controls at the *p* = 0.02 level of significance but are indistinguishable from one another.) When foreign cells were injected or if the animals were injured 10 times per day, a much greater significant difference was noted in comparison with the normals. (By Student's *t* test, the 10 times injury and the 150-µl *S. franciscanus* coelomic fluid injection distributions differ from the controls at *p* ≤ 0.005 and *p* ≤ 0.0005 levels of significance, respectively.) These data suggest that the sea urchin coelomocyte defense system is extremely sensitive to physiological challenges, that it is easily activated, and that it responds to varying amounts of injury but does not differentiate between different types of injury. The most likely interpretation is that the animals are only responding to injuries from the needles and irritations from the foreign cells.

In general, as Figure 8 shows, the results for the individual animals within an experimental group were coherent. However, within the group receiving 50 µl of *S. franciscanus* coelomic fluid, one animal had 138 transcripts per cell, by far the highest value seen in any of
the groups. This animal may simply have mounted a sharper response to *S. franciscanus* coelomic fluid than do most animals. Or, it may have been in a pathological or disturbed condition before the experiment was begun. Furthermore, because the surfaces of these animals cannot be "sterilized" with alcohol before being injected because they do not have a dead protective epidermal layer as do mammals, surface microbes may have been introduced during injections of foreign coelomic fluid. This could occasionally result in a combined response to the injury of injection, the foreign coelomic fluid, and unknown microbes. Whatever the cause, the occurrence of this result is interesting because it suggests that there are forms of stimuli that elicit far greater coelomocyte responses than those we have utilized. Identification of these stimuli would provide a more direct indication of the types of physiological challenge against which the coelomocyte defense reaction is actually armed.

**DISCUSSION**

We have identified a sea urchin gene, SpCoel1, that is expressed specifically in coelomocytes and at an enhanced level in response to injury. This is the first molecular parameter of lower deuterostome cellular defense systems so far reported. It is fascinating that SpCoel1 appears to encode an intracellular protein that in other organisms is believed to link cytoskeletal and signal transduction systems.

**SpCoel1 and Other Profilins**

Profilins have been cloned and sequenced from a variety of species. Although their amino acid sequences are similar, the transcript sizes for the other profilins are substantially smaller than that for SpCoel1, ranging in size from 0.5 kb in *Physarum* to 0.8 kb in humans, versus 4 kb for SpCoel1. None of the profilin mRNAs previously described include a 5' untranslated region as long as 0.6 kb nor a 3' trailer 2.7 kb in length. Pairwise amino acid comparisons of other profilins to SpCoel1 are summarized in Figure 3. The most closely related proteins are those from other echinoderms, which display a 73 to 75% similarity to SpCoel1. Profilins from *Acanthamoeba* and the fungi are ~35% similar, and individual mammalian profilins are ~20% similar.

It has been shown that profilins bind to actin and to polyphosphoinositides (Lassing and Lindberg, 1985, *Sea Uchinn Profilin*).

**Figure 6.** SpCoel1 expression in coelomocytes by immunofluorescence and in situ hybridization. (A) Four types of coelomocytes by Nomarski optics. Coelomocytes were removed from the animal, diluted into an equal volume of CMPSW-EDTA, and separated on a Percoll step gradient. (Cells used for negative controls, shown in the bottom photographs, were not fractionated.) The cells were then spun onto poly-L-lysine-coated slides using trunion carriers in an IEC refrigerated centrifuge at 576 × g for 5 min at 4°C, fixed immediately in cold 4% paraformaldehyde in CMPSW-EDTA for 2 min, and washed in CMPSW. These are paired photographs to the immunofluorescent photos in B. Arrow heads indicate the subpopulation of phagocytes that are positive for SpCoel1 protein expression. All cell types were photographed at 40× magnification; scale bar = 10 μM. (B) Immunofluorescent staining of coelomocytes. Separated fixed cells were repreincubated in a humidified chamber at room temperature in blocking buffer (CMPSW-EDTA containing 10% normal goat serum, 1% BSA, 0.01% nonidet P-40) for 40 min, followed by a 1:200 dilution of rabbit anti-SpCoel1 in blocking buffer for another 40 min. The secondary antibody was goat-anti-rabbit IgG labeled with Cy3, diluted 1:120 in blocking buffer. The unfraccionated cells in the bottom photograph were stained with normal rabbit serum diluted 1:200 in blocking buffer, followed by the goat anti-rabbit IgG secondary antibody as above. Cells were observed in an Olympus photomicroscope equipped with an Olympus exposure meter and an ultraviolet light source. Kodak ASA 400 print film was exposed for 2.5-5 s depending on staining intensity. All cell types were photographed at 40× magnification; scale bar = 10 μM. (C) In situ hybridization of SpCoel1 on coelomocytes. Hybridization of antisense riboprobes labeled with 35S-rUTP to mRNA in density centrifugation separated coelomocytes was carried out according to Rothenberg *et al.* (1990). The bottom photograph shows background using the sense strand probe. Slides were autoradiographed for 6 d and photographed on black and white (ASA 64) film under dark field conditions. All cell types were photographed at 20× magnification and were from a different cell preparation than those seen in A and B; scale bar = 20 μM.
the SpCoel1 protein might be expected to bind both actin and PIP₂ (Machesky et al., 1990).

### Possible Functions of SpCoel1

Profilins are thought to provide a regulatory linkage between the phosphatidyl inositol signal transduction pathway and cytoskeletal transformations, because binding of polyphosphoinositide metabolites is exclusive of binding to actin subunits (Lassing and Lindberg, 1985, 1988). Transient or prolonged interactions between profilin and actin can promote or block filament polymerization (Goldschmidt-Clermont et al., 1991b). When bound to polyphosphoinositides, profilin also prevents their hydrolysis by phospholipase C (Goldschmidt-Clermont et al., 1991; Machesky et al., 1990), except when the phospholipase C has been phosphorylated by tyrosine kinases such as are activated by growth factor receptors (Goldschmidt-Clermont et al., 1991a). Profilin may thus regulate interactions between different signal transduction pathways, the stimulation of which cause changes in cytoskeletal form (Stossel, 1989; Goldschmidt-Clermont et al., 1991b). In yeast, profilin mutants display defects in cell shape as well as a variety of other morphological abnormalities, and profilin interacts with signal transduction pathways that are activated by environmental nutrients (Haarer and Brown, 1990; Vojtek et al., 1991).

The profilin-like SpCoel1 protein could be involved in biochemically similar aspects of the response mechanisms mounted by sea urchin coelomocytes on activation. Coelomocytes are sensitive to a variety of physiological challenges, including infection or injury (Coffaro and Hinegardner, 1977; Höbaus, 1979) or the presence of bacteria, foreign cells, or inert objects (Johnson, 1969; Bertheussen, 1981). The activation of coelomocytes involves dramatic changes in behavior that are controlled by changes in the actin cytoskeleton, which in turn may be initiated by signals from the cell surface. In this study, we demonstrate that SpCoel1 transcript concentrations per cell increase significantly in response to very minor puncture injuries made at peripheral locations remote from most of the coelomocytes. A diffusible injury signal must therefore be involved in the response we have measured. Perhaps, by analogy with previously studied systems, the coelomocyte profilin functions at the intersection between the intracellular system that informs coelomocytes that a remote injury has occurred, and the cytoskeletal reorganization involved in its response.

### Evolutionary Implications

The echinoderm cellular defense system responds to challenges with a form of nonspecific cellular activation, which exhibits some similarities to the nonspecific primary line of cellular defense in mammals mediated by granulocytes, macrophages, and perhaps natural killer...
cells. Sea urchins apparently survive quite well without immune recognition specificity. The effectiveness of their defense systems is suggested by the long evolutionary history of this taxon and by the current large population size of species such as S. purpuratus. Furthermore, these animals are individually long lived. Some have survived many years in our laboratory, and they display remarkable abilities to recuperate from infections and injuries. The intrinsic usefulness of this nonadaptive cellular defense systems is clearly rooted in coelomocyte responsiveness, of which we here provide an initial molecular parameter. Sea urchins share a deuterostome ancestry with the chordates, and their coelomocytes may function in a way that is homologous with some elements of the nonspecific primary cellular defense system of chordates. Perhaps the adaptive components of the vertebrate immune system also evolved from simpler migratory cells endowed with various effector functions activated in response to diffusible systemic signals, i.e., from cells much like the coelomocytes of modern echinoderms.

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