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The Sea Urchin Profilin Gene Is Specifically Expressed in Mesenchyme Cells during Gastrulation

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Eggs and embryos of the purple sea urchin (*Strongylocentrotus purpuratus*) contain profilin that is partly supplied from maternal sources and partly produced by the gastrula. The maternal profilin protein content is about 13 μM and it persists in the embryo at least through gastrulation. Transcript quantitation from probe excess titrations show that very few profilin gene transcripts are present in the embryo during cleavage, but that they increase at the onset of gastrulation. By *in situ* hybridization, the newly synthesized profilin transcripts are localized in mesenchyme cells. Profilin gene expression increases when mesenchyme cells initiate migration and filopodial extension and retraction. We show that there are three isoforms of maternal profilin protein produced from the single copy gene during oogenesis. However, the blastula stage embryo only produces the major isoform, whereas the acidic isoform is produced in the early stages of gastrulation and the basic isoform appears by the end of gastrulation. Based on transcript prevalence and protein production rates, our calculations indicate that the amount of new protein produced in the mesenchyme cells in 12 hr is at maximum <2% of that supplied from maternal sources. Because of the large amount of maternally supplied profilin present in the egg and embryo, we suggest that it may be used in the cytokinetic processes of cleavage. Alternatively, because of the small amount of embryonically produced profilin, we suggest that it may function in the cytoskeletal shape changes required for filopodial extension and motility in the mesenchyme cells during gastrulation.

INTRODUCTION

The profilin gene from the purple sea urchin (*Strongylocentrotus purpuratus*) was initially cloned from a coelomocyte cDNA library (Smith *et al.*, 1992). Coelomocytes are amoeboid phagocytes that display an increase in the profilin transcript prevalence when they become activated in response to injury (Smith *et al.*, 1992), bacterial challenge, or lipopolysaccharide (unpublished data). Coelomocyte activation encompasses changes in shape and behavior that include increases in pseudopodial extensions involved in motility and chemotaxis to injuries (Karp and Hildemann, 1976; Coffaro and Hinegardner, 1977) and sites of infection (Johnson, 1969; Johnson and Chapman, 1970; Höbaus, 1979). In addition, foreign substances stimulate phagocytosis (Reinisch and Bang, 1971; Bertheussen, 1981; Coffaro, 1978; Yui and Bayne, 1983; Plytzcz and Seljelid, 1993), encapsulation (Johnson, 1969; Höbaus, 1979), degranulation (Service and Wardlaw, 1984), and clott formation (Edds, 1977, 1980), all of which require changes in the cytoskeleton.

Profilin is one of many actin binding proteins that interact with monomer (G-)actin and filaments (F-actin) to configure or modify the cell cytoskeleton (for review, see Aderem, 1992). The regulation of actin polymerization is largely mediated by a finely tuned interplay between profilin and thymosinβ_4, which compete for G-actin (Goldschmidt-Clermont *et al.*, 1992). Thymosinβ_4 sequesters actin monomers (Cassimeris *et al.*, 1992) and blocks the repolymerization of monomers into filaments, while profilin promotes their repolymerization (Pantaloni and Carlier, 1993) by catalyzing the ADP-ATP exchange on G-actin (Goldschmidt-Clermont *et al.*, 1992). Because free G-actin and the thymosinβ_4-G-actin complex are in equilibrium, the free G-actin pool is limited. Consequently, small changes in profilin concentration will produce large effects on the G-actin nucleotide exchange rate, and hence on the state of actin polymerization (Goldschmidt-Clermont *et al.*, 1992; Pantaloni and Carlier, 1993). Profilin thereby functions as a sensitive regulator of cytoskeletal organization.

Changes in the concentration of profilin itself are in turn affected by the inositol triphosphate (IP_3) second messenger system. Profilin binds to phosphoinositol-(4,5)-bisphosphate (PIP_2) and blocks the hydrolytic activity of nonphosphorylated phospholipase-Cγ1 (PLCγ1) on PIP_2 (Goldschmidt-Clermont *et al.*, 1991a). When the cell receives and transduces a signal by a transmembrane receptor with intracellular kinase activity, the PLCγ1 is phosphorylated and activated and can then displace profilin from PIP_2 (Goldschmidt-Clermont *et al.*, 1991a). This creates a localized increase in

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the profilin concentration (Lind *et al.*, 1987; Hartwig *et al.*, 1989) which could increase the nucleotide exchange rate on the ADP-G-actin, thereby promoting regional cytoskeletal modifications (Goldschmidt-Clermont *et al.*, 1992). Essentially, profilin can be viewed as a coupling device linking cellular signal transduction systems to the cytoskeletal mobilization system. Profilin is thus a key element in the mechanism by which cells respond with changes in shape or motility to extracellular signals (Goldschmidt-Clermont *et al.*, 1991b).

In this communication, we present patterns of profilin expression in the sea urchin embryo. Profilin protein is supplied to the egg from maternal sources and is present throughout gastrulation. Embryonic profilin gene transcripts are found in very low numbers until the onset of gastrulation, when expression increases sharply. New transcripts are localized in mesenchyme cells and their appearance correlates with changes in shape and behavior in these cells. This embryonic gene activity occurs despite the presence of about 13 μM maternally supplied profilin protein and can result, during the 12 hr required for gastrulation, in the synthesis of less than 2% of the total maternal profilin that is probably already present in these cells. We suggest that the large amounts of maternally supplied profilin may be involved in cytoskeletal modifications that occur during cleavage and that the small amount of newly produced embryonic profilin may be specifically required for the changes in cell shape that are a prominent aspect of mesenchyme cell behavior.

MATERIALS AND METHODS

Protein Quantitation

Profilin fusion protein. The coding region from SpCoel1, a *Bam*HI fragment of a cDNA clone (see Materials and Methods; Smith *et al.*, 1992), was subcloned into the *Bam*HI site of the pRSETA vector (Invitrogen), which incorporates a six-histidine metal binding site into the expressed fusion protein. The construct was transformed into BL21(DE3) bacteria containing the pLysS plasmid, and a protein of correct size and antigenicity was expressed after induction with 0.6 mM isopropyl β-D-thiogalactopyranoside (Bio-Rad Laboratories) (Studier *et al.*, 1990). The profilin-6(His) fusion protein was isolated from the bacterial lysate according to manufacturer's instructions for nondenaturing conditions on a nickel column (Invitrogen). The eluate was dialyzed against water, lyophilized, and the concentration was estimated (Bradford, 1976). Because the eluate contained a number of contaminating bands on a silver-stained protein gel (not shown), the percentage of the total protein mass that included profilin-6(His) was estimated by scanning and digitizing the gel on a computing densitometer (Molecular Dynamics, using ImageQuant, version 3.15 software). From this analysis, the mass amount of profilin-6(His) was estimated to construct a standard curve for quantitating protein blots analyzed with the anti-profilin antiserum. The provenance of this antiserum, which was raised against a bacterially expressed fusion protein, is described in our previous study (Smith *et al.*, 1992).

Protein gel blots and analysis by anti-profilin antiserum. Known amounts of profilin-6(His) and 200 eggs or embryos per lane were run on a sodium dodecyl sulfate (SDS) polyacrylamide gel (6% stacking, 15% resolving) and electroblotted onto a nitrocellulose-ECL filter (Amersham) in electroblotting buffer (20% methanol, 20 mM Tris, pH 8.8, 150 mM glycine, 0.05% SDS) for 2 to 4 hr, 300 mA, at 4°C). The filter was then incubated at room temperature for 1 to 2 hr in blotto [phosphate-buffered saline (PBS), i.e., 0.2 M phosphate, pH 7.4, 0.15 M NaCl, with 5% nonfat dried milk, 1% normal goat serum, 0.0001% tincture of merthiolate; filtered through No. 1 Whatman paper] followed by 1 hr in rabbit anti-profilin serum (1:1000 in blotto) and then 1 hr in goat anti-rabbit immunoglobulins labeled with horseradish peroxidase (EY Laboratory) (1:2000 in blotto). The antibody that was bound to the profilin bands in both the standard samples and the embryonic protein lanes was simultaneously identified with the luminol-based ECL reagents of Amersham, according to manufacturer's instructions. The film was then analyzed on a densitometer (E. C. Apparatus Corporation) with a chart recorder (Houston Instruments) to determine the mass of embryonic profilin by comparison to the profilin-6(His) standard.

Whole mount in situ hybridization. Whole mount *in situ* hybridizations were performed according to Harkey *et al.* (1992), Lepage *et al.* (1992), and Ransick *et al.* (1993), with the following minor modifications. The proteinase K treatment of the fixed embryos was changed from that in Harkey *et al.* (1992), in that the concentration of the enzyme preparation was increased to 10 μg/ml, with the incubation time for blastula and gastrula stages increased to 10 and 15 min, respectively. The viscous hybridization solution was infiltrated into and washed out of the embryos in multiple, small, incremental steps, in order to alleviate the problem of embryonic collapse, especially in older embryos. After hybridization, when the embryos were in the 1× SSC-1% Chaps wash (20× SSC is 3 M NaCl, 0.3 M Na citrate; Chaps (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate {Schwarz/Mann Biotech}), they were incubated for 25 min at 37°C in RNase solution [RNase A (Sigma) at 10 μg per ml and RNaseT1 (Boehringer-Mannheim) at 10 U per ml] in 1× SSC-Chaps (Lepage *et al.*, 1992). This step decreased the background that could not be removed with increased washes.

Digoxigenin-labeled antisense riboprobe. The template for producing the antisense riboprobe was a 609-bp region of the profilin cDNA that spanned the coding region and included most of the 5′ untranslated (UT) region and a small portion of the 3′ trailer sequence (see Smith *et al.*, 1992; Fig. 1; the probe extended from the *Xho*I site in the polylinker to the *Sac*I site in the 3′ UT region of clone Bsc9b.6). A large quantity of probe was produced (43 μg) using the Megascript kit (Ambion), following manufacturer's instructions, and as described in Ransick *et al.* (1993). The final concentration of probe used in the hybridization was 0.2 ng/μl.

Two-Dimensional (2D) Gels of Newly Synthesized Embryonic Profilin

Labeling sea urchin proteins with [^{35}S]methionine or [^{32}P]orthophosphate. To label embryonic proteins with [^{35}S]methionine (Amersham), 400 embryos were incubated in 450 μl millipore filtered sea water (MFSW) with 200 n*M* cold methionine (Sigma) and 50 μCi [^{35}S]-methionine (Amersham). Specific activity of the methionine in the seawater was 207 Ci/mmole. Unlabeled methionine was added to induce the sea urchin amino acid transporters to function maximally (Manahan *et al.*, 1989). To label embryonic proteins with [^{32}P]-orthophosphate (Amersham), 400 embryos were incubated in 400 μl mfSW with 100 μCi [^{32}P]orthophosphate. Development was allowed to continue for 2 hr at 16°C. The embryos were pelleted and lysed by freeze-thaw and sonication in 20 μl 2D gel buffer [1.5% SDS; 0.35% dithiothreitol; 7.65 *M* urea; 1.7% Nonidet-P40; 1.7% β-mercaptoethanol; 0.68% ampholine (Bio-Rad), p*I* range from 3 to 10]. Sonication was accomplished in a Branson 450 Sonifier, set at constant output setting No. 8, for 30 sec in a cup horn filled with ice water. Multiple aliquots of each preparation were stored at −70°C for less than 2 weeks before use and were not refrozen or reused after initial thawing.

Two-dimensional gels. The first dimension [isoelectric focusing (IEF)] was run as described (Harrington *et al.*, 1991a) in glass tubes (Wilmad Precision Glass) of 1.4 mm inner diameter and 210 mm long. Gels were polymerized to 160 mm. In normal IEF, 10 m*M* NaOH was loaded on top of the sample and 6 m*M* phosphoric acid was used in the bottom chamber. For nonequilibrium pH gel electrophoresis (NEPHGE) of basic proteins, the sample was loaded at the acidic end of the gel next to the phosphoric acid and was overlain with 20 μl of 4.5 *M* urea to prevent protein precipitation when in direct contact with the phosphoric acid. Focusing was performed at 200 V for 2 hr, 500 V for 4 hr, and then 800 V for 12 hr. NEPHGE was run for 3 hr at 500 V. The isoelectric points of proteins were determined by direct pH measurement of an IEF gel that was run concurrently with the sample containing gels, but instead of transferring this gel to an SDS-PAGE gel for the second dimension, it was cut into 3-mm pieces, soaked in water for 1 hr, and the pH of each sample measured. A plot of the pH gradient was then compared to the final position of the protein spots in the IEF dimension. The reproducibility of the pH gradient during the experimental conditions was ±0.05 pH units when different gels were compared. However, the accuracy of the pH measurements of the gels compared to absolute pH was at best ±0.5 pH units, due to the effects of urea on the mobility of the ampholines. Molecular weight of the proteins was determined by running low-molecular-weight standard protein markers (Bio-Rad) in the SDS-PAGE (second) dimension and comparing the profilin spots to a mobility plot of these standards. In the second dimension, gradient gels were used to separate the proteins by molecular weight. This was done to improve sample entry from the IEF tube gel into the separating slab gel without the use of a stacking gel. The SDS-PAGE gels were 160 by 200 mm and 1.5 mm thick. To facilitate the transfer of the tube gel onto the slab gel, 120 μl of 0.01% bromphenol blue solution were placed on the tube gel to lubricate it during the transfer and to visualize the conductivity front during the second-dimension run. SDS-PAGE was run at 40 mA, 12°C, until the dye front reached the bottom of the gel. Electroblotting the separated protein onto a PVDF membrane (Millipore) was accomplished using a TransBlot cell (Bio-Rad) using electroblotting buffer (25 m*M* Tris base, 192 m*M* glycine, 20% methanol; Towbin *et al.*, 1979) run at 200 mA for 3 hr or overnight. Because silver staining can decrease the ^{35}S activity by about 30% (Van Keuren *et al.*, 1981), the loaded membranes were first exposed to phosphorimager plates (Molecular Dynamics) for 1 to 6 hr and the images then digitized for analysis (Harrington *et al.*, 1991b).

Immunodetection of profilin on the membranes was then performed as described elsewhere (Towbin *et al.*, 1979). Briefly, after the radioactive image had been obtained, the blot was rewetted in TBST [10 m*M* Tris, pH 8.0, 150 m*M* NaCl, 0.05% Tween 20 (Sigma)] and incubated with the rabbit antiserum to sea urchin profilin (1:5000 in TBST). Detection of the primary antibody was achieved with an alkaline phosphatase-conjugated anti-rabbit (Promega) secondary antibody (1:7500 in TBST). The substrate reaction with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate produced a purple stain. These blots were then directly compared to the phosphorimager-derived image of the same radioactive proteins in order to identify which of the radioactive proteins were in the same charge and mass position as the immunostained profilin spots.

Digitized images from the phosphorimager plates

TABLE 1
PROFILIN PROTEIN IN SEA URCHIN EGGS AND EMBRYOS

Embryonic age	pg Profilin per embryo[a]	Molecules per embryo[b]	μM Profilin per embryo[c]
Egg	104	4.0×10^9	19.2
4 hr	59	2.3×10^9	10.9
8 hr	69	2.7×10^9	12.8
16 hr	62	2.4×10^9	11.5
22 hr	73	2.8×10^9	13.4
28 hr	83	3.2×10^9	15.3
31 hr	71	2.8×10^9	13.1
48 hr	77	3.0×10^9	14.2

[a] Calculated from the deduced molecular weight of sea urchin profilin; 15.3 kDa (Smith *et al.*, 1992).
[b] Mean = $2.9 \pm 0.53 \times 10^9$. Differences are due to sample variation.
[c] Mean = 13.8 ± 2.6 μM, based on an egg volume of 350 pl.

were analyzed using GALtool (Solomon and Harrington, 1993) on a Sun workstation. This software was used to determine the amount of radioactivity in each of the profilin spots on every blot examined. To do this, each spot was first identified by the edge-detector algorithm (Solomon and Harrington, 1993) using a kernel size of 13 and 15 for the filter and edge detector, respectively. The intensity of each spot was recorded and then normalized to all protein spots on the gel to correct for variations in the 2D gel procedure for each gel and between gels. This quantity was then used to obtain the relative differences of incorporated counts for the three profilin spots.

RESULTS

Estimation of Profilin Protein in Eggs and Embryos

The amount of profilin protein in sea urchin eggs and embryos was estimated by comparing the signal obtained from an enzyme-labled antibody reaction with the embryonic proteins to a standard curve prepared with known amounts of the profilin-6(His) fusion protein on a protein gel blot. Data from the densitometric analysis of this blot are shown in Table 1 and summarized in Fig. 1. The sea urchin embryo contains $2.9 \pm 0.53 \times 10^9$ molecules of profilin, the average concentration of which is 13.8 ± 2.6 μM (Table 1). This large amount of profilin is evidently generated during oogenesis since the protein synthesis apparatus is quiescent in the mature unfertilized egg. Consequently, the profilin mRNA must be fairly prevalent during oogenesis. For example, if all the maternal profilin were accumulated in the ~3-week terminal growth phase of oogenesis, about 10^5 profilin mRNAs would be required per oocyte (equivalent to ~10^2 mRNAs per cell at late embryonic stages). Even if the profilin were accumulated gradually, for example over the 6-month period from June to January when seasonal *S. purpuratus* return to fecundity, about 10^4 molecules of mRNA would be required per oocyte.

The distribution of the maternal profilin protein was studied immunocytologically in whole embryos. We observed that all cells in the embryo contain approximately equal amounts of maternal profilin from blastula through prism stages (data not shown).

Quantitation of Profilin Transcripts

Preliminary studies in which gel blots of embryonic RNA were probed for profilin transcripts indicated that transcript level is very low in the egg and in cleavage stage embryos, but that these transcripts increase substantially in gastrula and pluteus stages. In order to quantitate these changes, probe excess transcript titrations were performed, with the results summarized in Fig. 1 and Table 2. The hatched blastula has about 5800 transcripts per embryo, or about 14.5 transcripts per average cell. This concentration is assumed here to be the "base level" transcript prevalence in embryos analyzed by whole mount *in situ* hybridization; see below. The number of transcripts per embryo increases to about 11,500 by mid gastrula and to 21,000 by the completion of gastrulation.

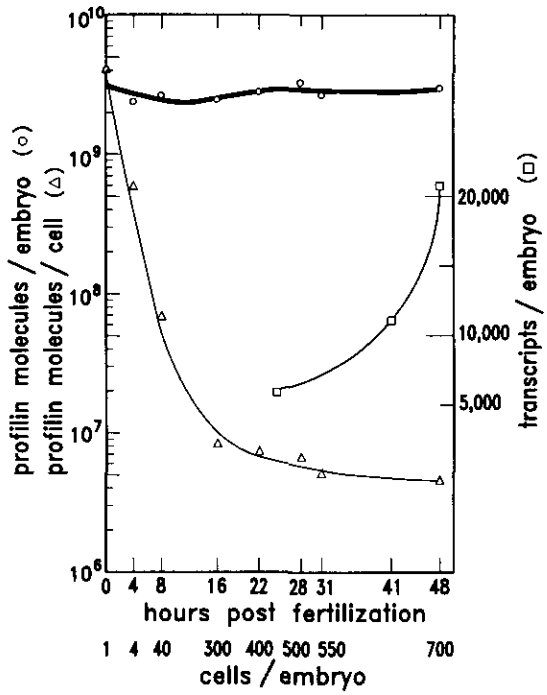


FIG. 1. Profilin in sea urchin eggs and embryos. Data for profilin were obtained from the densitometric analysis of immunoblots as described in text and listed in Table 1. Data for numbers of profilin transcripts are from Table 2.

TABLE 2
PROFILIN TRANSCRIPTS IN SEA URCHIN EMBRYOS

Embryonic stage	Cells per embryo	Transcripts per embryo[a]	Transcripts per average ectodermal cell	Transcripts in mesenchyme cells[b]	Mesenchyme cells per embryo	Minimum transcripts per average mesenchyme cell[c]
Hatched blastula	400	5800	14.5[e]	[d]	[d]	[d]
Mid gastrula	600	11500	[e]	5700	64 PMCs ~60 SMCs	46
Late gastrula	700	21000	[e]	15200	64 PMCs ~180 SMCs	62

[a] Correlation coefficients ≥0.9955.
[b] The number of transcripts per mesenchyme cell was calculated by subtracting the number of "base level" transcripts at the blastula stage, i.e., 5800/embryo, from the total number of transcripts for the later stages; that is, the mesenchyme cells are assumed to inherit no profilin mRNA from their progenitors. Since they probably do so, these are minimum estimates.
[c] For these calculations all mesenchyme cells are assumed to be the same and to express the profilin gene equally.
[d] Mesenchyme cells are not present at this stage of development.
[e] It is assumed that the average number of transcripts per ectodermal cell does not change during gastrulation.

Zygotic Profilin Transcripts Are Localized in Mesenchyme Cells

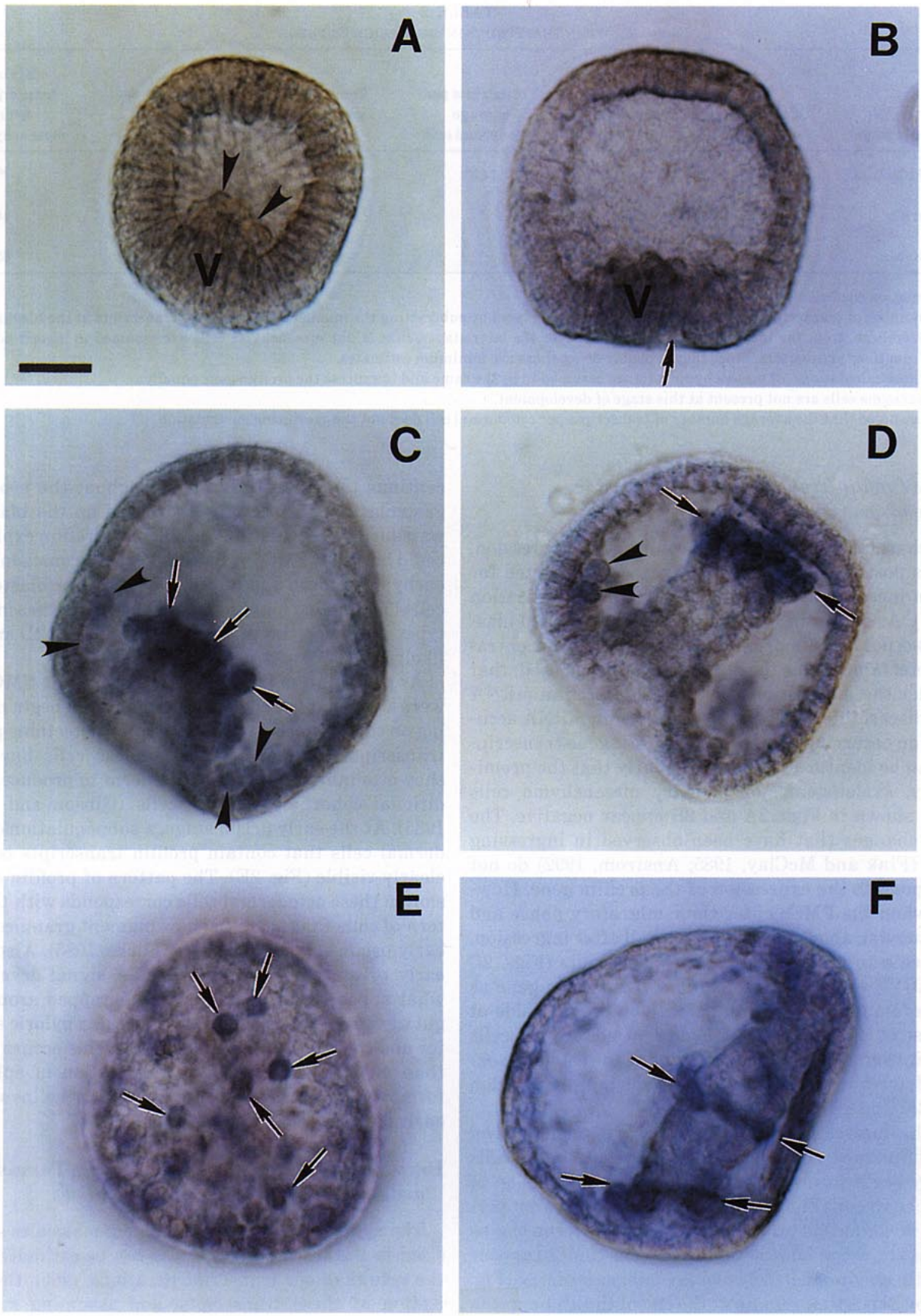
To characterize embryonic profilin gene expression, various posthatching embryo stages were analyzed for profilin message by whole mount *in situ* hybridization (Fig. 2). As shown in Figs. 2A and 2B the hatched blastula does not contain profilin transcripts at a concentration detectable under the staining conditions used; that is, under these conditions the maternal profilin mRNA is not discernible, and only further profilin mRNA accumulation occurring as a result of zygotic gene transcription can be identified. Note particularly that the premigratory, skeletogenic, or primary mesenchyme cells (PMC) shown in Figs. 2A and 2B appear negative. The shape changes that have been observed in ingressing PMCs (Fink and McClay, 1985; Anstrom, 1992) do not correspond to the expression of the profilin gene. However, when the PMCs enter their migratory phase and begin moving about the blastocoel wall after ingression, they begin to accumulate profilin transcripts (Figs. 2C and 2D). They continue to express the profilin gene as they accumulate in the two patches on the oral side at the base of the archenteron. Note that neither the cells of the invaginating archenteron nor of the ectoderm express detectable profilin transcripts during gastrulation (Figs. 2C and 2D).

At the onset of gastrulation, a barely detectable level of profilin transcripts appears in the vegetal plate cells as they begin to invaginate in the initial formation of the archenteron (Fig. 2B), but this staining does not persist as invagination proceeds. As the archenteron begins to elongate, secondary mesenchyme cells (SMCs) appear at the tip and immediately display intense staining (Fig. 2C). The SMCs that remain at the end of the archenteron continue to express profilin throughout the process of gastrulation (Fig. 2D). Cells located on the blastocoel wall during gastrulation that display profilin expression could be either PMCs or SMCs such as chromogenic mesenchyme (Gibson and Burke, 1985, 1987), or blastocoelar cells (Tamboline and Burke, 1992), or both mesenchyme types. However, those shown in Figs. 2C and 2D are most likely PMCs.

As gastrulation is completed, some of the SMCs that were located at the end of the archenteron begin migrating on the blastocoel wall and display detectable profilin transcripts (data not shown). These cells have been shown to infiltrate into the ectoderm to produce an additional cohort of pigment cells (Gibson and Burke, 1985). At the early prism stage, a subpopulation of ectodermal cells that contain profilin transcripts becomes clearly visible (Fig. 2E). The pattern of profilin expression in these ectodermal cells corresponds with the pattern of cells that begin to show pigment granules in the early prism stage (Gibson and Burke, 1985). Also in the early prism, a strong profilin RNA signal develops in what appear to be rings of SMCs wrapped around the gut at the positions where the muscular pyloric sphincter and the anus will form (Fig. 2F). This occurs earlier than any previously reported indication of sphincter formation and suggests that SMCs may be involved in early gut differentiation.

Estimation of Newly Synthesized Profilin Transcripts and Profilin Protein in Mesenchyme Cells

The number of new profilin protein molecules synthesized in the sea urchin gastrula can be estimated from the results of our transcript titrations, given the localization of these transcripts, and assuming standard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rates of translation for sea urchin embryos. Data for these calculations are shown in Table 2. We have seen that the profilin transcripts in the hatched blastula are not discernible by whole mount *in situ* hybridization, although the probe excess transcript titration shows that an embryo at this stage has about 14.5 transcripts per average cell, or 5800 transcripts in the whole embryo. We consider this the undetectable background level, which we also assume to remain present in the ectoderm cells that appear negative by *in situ* hybridization in later embryos. Thus, if none of the 5800 blastula stage transcripts are partitioned into the mesenchyme cells, the difference between this and the total number of transcripts present in mid and late gastrula stage embryos provides a minimum estimate of the number of transcripts that are localized in the mesenchyme cells, as shown in Table 2. There are about 64 PMCs in gastrulae (Cameron *et al.*, 1987; Ettensohn and Ingersoll, 1992), about 60 SMCs at mid gastrula, and about 180 SMCs in the late gastrula (Ettensohn and Ruffins, 1993). Accordingly, we estimate that there are at least 46 transcripts in an average mesenchyme cell at mid gastrula stage and 62 at late gastrula stage. When this calculation, which is based on the transcript titration data, is compared to the whole mount assay for a 609-bp probe under the standard conditions used in this investigation, the detection threshold for the *in situ* hybridization assay apparently falls between 14.5 and 46 transcripts per cell (greater sensitivity can be obtained by longer staining; unpublished data).

From the minimum number of profilin transcripts in the mesenchyme cells, the minimum numbers of newly synthesized profilin molecules (even if there is an equal partition of the prior profilin mRNAs to mesenchyme cells, i.e., an additional 14.5 transcripts/cell, the result is scarcely affected) can be estimated. We assume 54 transcripts per average mesenchyme cell during gastrulation and a profilin protein turnover rate of zero. The rate of protein synthesis measured for these embryos is about two molecules of protein min^{-1} $mRNA^{-1}$ (see legend to Table 3). Thus, during the 12-hr period required for gastrulation at 16°C in this species, the average mesenchyme cell will have synthesized over this time a maximum of 7.8×10^4 new profilin molecules (accuracy is based on the correlation coefficient of transcript titration linear regressions, see legend to Table 2). Comparing the number of profilin molecules that could be synthesized in a mesenchyme cell (7.8×10^4) to the number of maternally supplied profilin proteins present per average cell in the complete gastrula (4.1×10^6, calculated from molecules per embryo divided by 700 cells per embryo, see Table 1), a remarkable paradox emerges.

TABLE 3
CALCULATED SYNTHESIS OF PROFILIN ISOFORMS

A. Profilin protein molecules ($\times 10^6$) synthesized per embryo in 2 hr, calculated from mRNA prevalence

Hatched blastula	Early to mid gastrula	Late gastrula
1.4	2.8	5.0

B. Relative amounts of profilin protein isoforms ($\times 10^6$) per embryo synthesized in 2 hr, calculated from phosphorimager data

p*I* of profilin isoforms	Hatched blastula	Early to mid gastrula	Late gastrula
5.7	0	1.0	1.2
6.1	1.4	1.8	2.8
6.8	0	0	1.0

Note. A. The number of profilin proteins produced per embryo was calculated from transcript titration data (see Fig. 1 and Table 2), assuming about 2 protein molecules produced min^{-1} $mRNA^{-1}$ (i.e., for a translation rate of 1.5–1.8 codons sec^{-1}, and ribosome spacing of about 150 nt in active polysomes; reviewed by Davidson, 1986, pp. 74–78). The calculation was for 2 hr of synthesis, with no profilin turnover, and on the basis that all profilin transcripts are functional mRNAs.

B. The relative number of incorporated counts of [^{35}S]methionine in the profilin spots were averaged from three samples of phosphorimager digitized 2D gel data. This information was then used to estimate the relative amounts of the three profilin isoforms produced per embryo, based on the calculated amount of profilin produced from the numbers of transcripts present in the mesenchyme cells shown in (A).

FIG. 2. Whole mount *in situ* hybridization of profilin message in embryos. Embryos were photographed with a BH-2 Olympus photomicroscope equipped with an Olympus exposure meter. The magnification is the same throughout. Scale bar, 20 μm. (A) Hatched blastula. The vegetal plate (v) is positioned towards the bottom. At this stage, profilin gene expression is below detectable levels. Arrowheads, ingressed PMCs. (B) Very early gastrula. The vegetal plate (v), which is positioned toward the bottom, has initiated invagination (arrow). The vegetal plate cells show a very light staining, indicating that these cells have begun to express the profilin gene; the messages have accumulated to a level that is just above detection under the conditions used. (C) "One-quarter" gastrula. The focal plane passes through the archenteron and shows mesenchyme cells (probably PMCs, arrowheads) on the blastocoel wall and the SMCs (arrows) at the end of the archenteron, which have activated their profilin genes. (D) Late gastrula. The focal plane passes through the center of the embryo and the archenteron. The SMCs (s) (arrows) located at the end of the archenteron and the mesenchyme cells on the blastocoel wall (arrowheads) show pronounced expression of the profilin gene. At this stage, the SMC filopodia have contacted and bound to the future stomodeum. (E) Late gastrula. The focal plane passes along the ectoderm of the embryo. A subpopulation of cells in ectoderm, possibly pigment cells (see text) express profilin (arrows). (F) Prism. The focal plane passes through the archenteron and the center of the embryo. Mesenchyme cells encircling the gut express profilin (arrows) in two bands. These cells are located where the pyloric sphincter will form between stomach and hind gut and where the anal sphincter will form at the former blastopore.

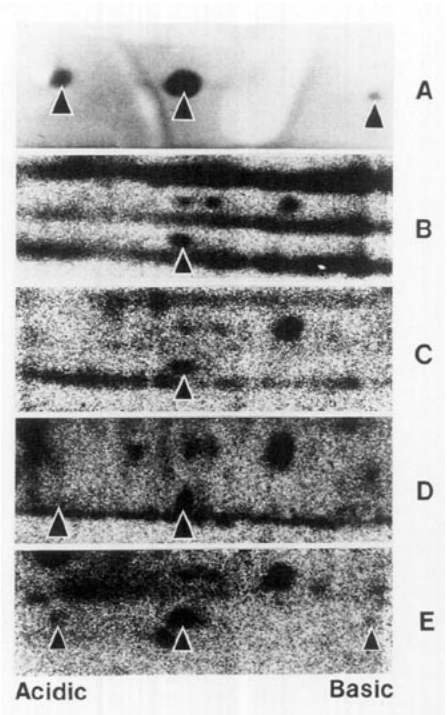

FIG. 3. Two-dimensional gel analysis of maternal and embryonic profilin proteins. The five panels in this figure show enlargements of small regions of 2D gels that were run on total embryonic proteins. (A) An immunostained membrane indicating the positions of the maternal profilins. (B-E) Incorporation, in a 2-hr incubation, of [^{35}S]methionine into newly synthesized embryonic profilins. Arrowheads indicate the positions of three species of sea urchin profilin. (A) Immunostaining of proteins of a blastula stage preparation displays three isoforms of maternal profilin, of p*I*'s 5.7, 6.1, and 6.8. (B) Prehatching embryo (16 hr); at this stage only the central, major isoform (p*I* = 6.1) of profilin is labeled with [^{35}S]methionine. (C) Hatched blastula (24 hr); still only the central, major profilin isoform (p*I* = 6.1) incorporates [^{35}S]methionine. (D) Early to mid gastrula (36 hr); in addition to the central 6.1 isoform, the acidic isoform (p*I* = 5.7) is labeled with [^{35}S]methionine. (E) The complete gastrula (48 hr); all three profilin isoforms, including the acidic and basic (p*I* = 6.8) isoforms are labeled.

The mesenchyme cells could produce, in 12 hr, less than 2% of the total amount of maternal profilin that is probably already present in them. Assuming, as we found immunocytologically, that the maternal profilin is more or less evenly distributed in the embryo, what then is the functional significance of the sharply confined accumulation of new profilin transcripts in mesenchyme cells?

Identification of Profilin Proteins by 2D Gels

When profilin from eggs or embryos of any stage (i.e., maternal profilin) is displayed by 2D gel electrophoresis, using the anti-profilin antiserum for identification, three spots are revealed which differ significantly in p*I*. An example, from a blastula extract, is shown in Fig. 3A; however, all embryonic stages produce identical patterns. The p*I* of the major, central isoform is 6.1, and the p*I*s of the others are 5.7 and 6.8. The molecular weights of the acidic and central spots are consistent with the previous sequence analysis, i.e., 15.3 kDa (Smith *et al.*, 1992). However, the basic isoform (p*I* 6.8) is consistently of slightly lower apparent molecular weight. We searched for a more basic isoform (p*I* > 8), as has been identified in other organisms (Pollard and Rimm, 1991) using NEPHGE, but no profilin-positive spots were observed (data not shown).

To determine which of the profilin isoforms are synthesized in the mesenchyme cells at gastrulation, embryos were labeled for 2 hr with [^{35}S]methionine, and the positions of the profilin isoforms in the array of radioactive proteins were identified immunologically. Figures 3B–3E display results for prehatched and hatched blastula, early to mid gastrula, and late gastrula stages. Unlike the immunostained blot of Fig. 3A, which reveals the maternal profilin isoforms that are present in all embryonic stages, the phosphorimages revealed a distinctly different distribution of embryonic profilin, which varied according to developmental stage. Only the major, central (p*I* 6.1) profilin protein is labeled with [^{35}S]methionine in the prehatched and hatched blastulae. The acidic isoform (p*I* 5.7) is barely detected in the early to mid gastrulae. By late gastrula stage all three forms are labeled. Counts of [^{35}S]methionine incorporated into the three profilin spots from phosphorimager data (three blots averaged) were transformed into relative numbers of each profilin isoform produced for each embryonic stage (Table 3). These data indicate significant differences between embryonic stages, and also imply an increase in the rate of profilin synthesis during development, in agreement with the increase in the number of transcripts present in older embryos as assayed by transcript titration.

Proteins with charge variants can arise from a variety of modifications to the amino acid side chains. Because profilin is known to catalyze the ADP–ATP exchange on actin monomers (Goldschmidt-Clermont *et al.*, 1992), we sought to detect profilin phosphorylation directly, by examining extracts from embryos that had been labeled *in vivo* with [^{32}P]orthophosphate. However, no evidence of phosphate incorporation in any of the profilin isoforms was obtained (Tseng *et al.*, 1984), although several hundred labeled phosphoproteins were easily observed in the 2D gels (data not shown).

DISCUSSION

Maternally Supplied Profilin and Cleavage

We show that maternally supplied profilin is present in sea urchin eggs and embryos in physiologically relevant concentrations (13.8 ± 2.9 μM) through gastrula-

tion. (For comparison, mammalian macrophages and platelets, which are very mobile cells, contain about 50 μM profilin (Hannapple and Van Kampen, 1987).) Maternal profilin in the embryo could be primarily involved in the repetitive cytoskeletal alterations that are involved in cleavage. In the ciliate *Tetrahymena*, profilin has been shown to localize in the division furrow and contractile ring during cytokinesis, in association with actin filaments (Edamatsu *et al.*, 1992). Sea urchin profilin could have a similar function with respect to the contractile cleavage furrows. Profilin may be associated with myosin, actin, and an actin cross-linking protein that has been shown to localize in the sea urchin embryo cleavage furrows (Mabuchi *et al.*, 1985; Tosuji *et al.*, 1992). Although maternally supplied profilin appeared evenly distributed throughout the late gastrula and early pluteus by whole mount immunofluorescence (data not shown), we predict that careful localization studies in the future on cleavage stage embryos would show profilin localization in the cleavage furrow in association with actin.

Profilin Gene Expression in the Mesenchyme Cells

Gastrulation involves substantial regional changes in the behavior of embryonic cells, requiring both cytoskeletal reorganizations and motility functions (Ettensohn, 1985; Hardin and Cheng, 1986; Hardin and McClay, 1990; McClay *et al.*, 1992). Mesenchyme cells in particular are highly mobile, utilizing exploratory filopodial extension and retraction (Karp and Solursh, 1985; Ettensohn, 1984; Hardin and McClay, 1990; McClay *et al.*, 1992; Ettensohn and Ruffins, 1993). The mesenchyme cells are the first embryonic cells that unequivocally display zygotic profilin transcripts, and by the end of gastrulation, profilin transcript prevalence has increased at least fourfold with respect to the average ectoderm cell. It is important to note that the level of profilin transcripts in mesenchyme cells is equivalent to that seen in activated adult sea urchin coelomocytes, which are amoeboid, mobile, phagocytic cells (Smith *et al.*, 1992). As do activated coelomocytes, the mesenchyme cells increase their profilin transcript prevalence as they initiate shape changes. Profilin gene expression is thus likely to participate in the mechanisms underlying cytoskeletal remodeling in mobile, responsive cells of both adult and embryonic sea urchins.

The PMCs do not begin to express detectable profilin transcripts until they begin their migratory phase. During the initial stages of PMC ingression there is a "purse string" localization of actin at the cell apex, which results in the establishment of the bottle shape that the cells assume as they loosen their prior contacts and invade the blastocoel (Fink and McClay, 1985; Anstrom, 1992). After they leave the vegetal plate, they then sit on the basal lamina before initiating migratory behavior. Their migration along the blastocoel wall requires the active extension and attachment of filopodia, which then retract, pulling the cell body forward (Karp and Solursh, 1985). The accumulation of detectable profilin transcripts is correlated with the activation of this filopodial extension and retraction process.

The most striking result reported in this communication is the accumulation of profilin gene transcripts in the SMCs that appear at the tip of the invaginating archenteron and are positive throughout gastrulation (Figs. 2C and 2D). These cells function to identify the stomodeal target and lead the archenteron toward fusion by a process of repeated filopodial extensions and retractions (Hardin, 1988; Hardin and McClay, 1990; McClay *et al.*, 1992). From the data presented here, it appears that profilin expression may occur in all but one of the SMC subsets even though they all utilize filopodial extensions. Several subsets appear during gastrulation (Cameron *et al.*, 1991; Ettensohn and Ruffins, 1993). Those that are positive for profilin expression include the early pigment cell precursors, or chromogenic mesenchyme, that migrate away from the forming archenteron during gastrulation (Gibson and Burke, 1985) and the blastocoelar cells present in the blastocoel (Tamboline and Burke, 1992). These two SMC subsets may make up part of the profilin positive cell population located on the blastocoel wall with the PMCs during gastrulation. When archenteron-stomodeal fusion occurs at the end of gastrulation, some of the SMCs located at the end of the archenteron migrate away from the gut, move along the blastocoel wall, pass through the basal lamina, and enter the ectoderm to become a later-appearing cohort of pigment cells (Gibson and Burke, 1985, 1987). During this migration, these cells constitute another set of profilin-positive cells on the blastocoel wall besides the PMCs. Finally, there is an SMC subset that remains on the foregut and participates in the formation of the coelomic pouches (Cameron *et al.*, 1991). It is this category of SMCs, the premyoblasts that come from the coelomic pouches (Burke and Alvarez, 1988; Ettensohn, 1990; Wessel *et al.*, 1990; Venuti *et al.*, 1991; Cameron *et al.*, 1991), that does not express profilin during the differentiation of the circumesophageal musculature. This suggests that the cytoskeletal transformations occurring as the myoblasts wrap filopodial extentions around the esophageal wall are different in nature from those taking place in amoeboid cells that engage in filopodia-driven mobility. This difference may be based in the process of myoblast differentiation into muscle. Once the filopodia are extended around the forgut, they are not retracted, which is unlike activities observed in other mesenchyme cell types.

In the ectoderm, no cells with elevated levels of profilin transcripts are detected by *in situ* hybridization until the completion of gastrulation, and we believe the positive cells seen in the early prism are probably pigment cells of SMC origin that migrate to their ectodermal locations at that time in development. Although we have no direct evidence that these profilin-positive cells are indeed pigment cells, unpublished data of the authors and D. Livant indicate that pigment cell-enriched ectodermal RNA from the early pluteus is positive for profilin transcripts on RNA gel blots. In summary, profilin expression occurs in most of the mesenchyme subsets of the sea urchin embryo when they extend and retract filopodia, activities which are intrinsic to mesenchyme differentiation.

The Role of Newly Produced Embryonic Profilin Protein

Given their modest content of profilin transcripts, the amount of new profilin that could be synthesized by a typical mesenchyme cell is less than 2% of the amount of maternal profilin that is retained in the average gastrula cell. Perhaps even such a small quantitative change could have a profound effect on cytoskeletal rearrangements, if all the newly synthesized profilin were localized to specific sites in the cytoskeleton where filopodial formation occurs. Profilin has been demonstrated in filopodia of spreading and locomoting fibroblasts (Bubb *et al.*, 1992). Along these lines, Goldschmidt-Clermont *et al.* (1992) suggested that very slight increases in profilin concentration localized near the plasma membrane, such as result from signal transduction, could greatly affect cell morphology. The correlations we observe imply that profilin gene expression, and the provision of a small amount of newly synthesized profilin in mesenchyme cells, could be required for the shape changes that are observed in these cells during gastrulation.

The appearance of three profilin isoforms in an organism with a single copy profilin gene is a new result and suggests post-translational modifications to the protein. [Genome blots, which were originally probed with a 3′ UT region of the cDNA (Smith *et al.*, 1992), were repeated under lowered wash stringency. The coding region probe that was used for the *in situ* hybridizations (see Materials and Methods) and the original 3′ UT probe were hybridized to *Hin*dIII-digested DNA from three individuals. The coding region probe identified bands corresponding to two polymorphic alleles in each genome, confirming that sea urchin profilin is a single copy gene (data not shown).] Documented post-translational modifications to profilins in other species include a trimethylated lysine at position 103 in *Acanthamoeba* profilin Ia/b (Ampe *et al.*, 1985, 1988) and a blocked N terminus in both the bovine protein (Nyström *et al.*, 1979) and *Acanthamoeb*a profilin II (Ampe *et al.*, 1988). The multiple isoforms we see must also be generated by post-translational modifications. We show that newly synthesized acidic and basic isoforms appear at gastrulation. Earlier than this only the central major isoform is produced by the embryo. Since these profilin variants appear in the embryo only after the onset of gastrulation, it is possible that only newly synthesized (i.e., zygotic) profilin can be modified, perhaps because of its subcellular location. This might explain why the filopodially active mesenchyme cells produce profilin, despite the relatively huge quantity of maternal profilin remaining present throughout the embryo.

In summary, this study reveals (i) that the onset of zygotic profilin transcript accumulation in the embryo occurs at the beginning of gastrulation; (ii) that the profilin gene is expressed in migratory skeletogenic and secondary mesenchyme cells; (iii) that the ratio of the amount of maternally supplied to embryonically synthesized profilin in the gastrula is approximately 50 to 1; (iv) that after gastrulation, sea urchin profilin appears in three isoforms; and (v) that the profilin gene is activated only in cells undergoing certain cytoskeletal modifications and changes in cell shape, viz mobile mesenchyme cells that extensively utilize filopodial extension and retraction.

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