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Phosphorylation-dependent regulation of skeletogenesis in sea urchin micromere-derived cells and embryos

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Sea urchin embryo micromeres when isolated and cultured *in vitro* differentiate to produce spicules. Although several authors have used this model, almost nothing is known about the signaling pathways responsible for initiating skeletogenesis. In order to investigate the potential involvement of phosphorylation events in spiculogenesis, the effect of inhibitors of protein kinases and phosphatases on skeleton formation was studied. Results obtained using both cultured micromeres and embryos revealed that protein tyrosine kinase and phosphatase inhibitors blocked skeleton formation, but not serine/threonine phosphatase inhibitors. The inhibitors showed a dose-dependent effect and when removed from micromere or embryo culture, spicule formation resumed. Inhibition of tyrosine phosphatases resulted in an increase in the tyrosine phosphorylation level of two major proteins and a modest decrease in the expression of the mRNA coding for type I fibrillar collagen. These findings strongly suggest that tyrosine phosphorylation and dephosphorylation is required for micromere differentiation and for normal skeletogenesis during sea urchin embryo development.

Key words: collagen, protein tyrosine kinase, protein tyrosine phosphatase, sea urchin embryo, skeletogenesis.

Introduction

Skeletogenesis is a key morphogenetic event in the embryonic development of both vertebrate and invertebrate organisms. Among invertebrates the sea urchin embryo offers a good model for studying skeletogenesis because of its transparency (i.e., the skeleton is easily visible upon microscopic examination). In sea urchin embryos primary mesenchyme cells (PMC), the skeleton-producing cells, are the descendants of the four micromeres of the 16-cell stage embryo. One important feature is that isolated micromeres, cultured in the presence of horse serum in sea water, divide and differentiate to produce spicules with the same timing as intact embryos (Okazaki 1975). This indicates that serum provides factors, not yet identified, necessary for micromere differentiation. Presumably, similar factors are naturally present in the blastocoele of the developing embryo. Although spicules can also grow *in vitro*, the correct skeleton patterning *in vivo* is a result of the interplay between the primary mesenchyme cells and the ectoderm (Ettensohn 1992; Ettensohn & Malinda 1993; Armstrong & McClay 1994; Zito *et al.* 1998). Recently, Guss and Ettensohn confirmed this idea, showing that the rate of skeleton growth and primary mesen-chyme gene expression are controlled by short-range signals emanating from ectodermal territory (Guss & Ettensohn 1997). One assumption is that the ectoderm may supply those factors needed for spiculogenesis. Recently, it has been shown that signaling mediated by platelet-derived growth factor (PDGF)-like and epidermal growth factor (EGF)-like receptors is required for spiculogenesis in the *Lytechinus* embryos (Ramachandran *et al.* 1995, 1997). Moreover, spiculo-genesis is inhibited by antibodies to human PDGF-B and transforming growth factor (TGF)-α growth factors (Govindarajan *et al.* 1995).

Extracellular matrix (ECM) components, such as collagens, are also necessary for spicule formation both during isolated cell differentiation and during embryo development (Blankenship & Benson 1984; Wessel *et al.* 1991). Extracellular matrix disruption prevents gastrulation and spiculogenesis, and also affects gene expression (Wessel & McClay 1987; Wessel *et al.* 1989). In the sea urchin four cDNA coding for collagens have been isolated and sequenced: two for

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fibrillar collagens, called COLL1α or Hpcol1 (D'Alessio *et al.* 1989; Tomita *et al.* 1994), COLL2α (D'Alessio *et al.* 1990), and two for non-fibrillar collagens, called COLP3α and COLP4α (Exposito *et al.* 1993, 1994).

A cascade of events, causing a variety of cellular responses, is initiated by the binding of growth factors, or of extracellular matrix components, to their own receptors. There have been few investigations into the role of signal transduction pathways in the spiculogenesis process. In particular, two papers from Mitsunaga *et al.* (1990a,b) have shown that protein kinase C activity is required for spicule formation in sea urchin embryos. In this report we studied the role of protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTPases) in the spiculogenesis of cultured micromeres and embryos of *Paracentrotus lividus*.

Materials and Methods

Reagents

Sodium orthovanadate (Na_3VO_4) dissolved in H_2O was used indifferently before or after being activated as reported (Mahadevan & Bell 1990). Genistein and okadaic acid were dissolved in dimethylsulfoxide (DMSO). All reagents were from Sigma (St Louis, MO, USA).

Isolation and culture of micromeres

For isolation and culture of micromeres the procedure reported by Benson *et al.* (1990) has been used with minor modifications. Briefly, in order to dissociate embryos at the 16-cell stage, eggs were fertilized in the presence of 3 mM *P*-aminobenzoic acid and passed through a nylon filter (60 µm mesh) immediately after the appearance of the fertilization membrane. Fertilized eggs were washed in Millipore filtered sea water (MFSW) and cultured in MFSW containing penicillin (30 µg/mL) and streptomycin (50 µg/mL) (MFSW + P/S). At the 16-cell stage embryos were washed twice with calcium- and magnesium-free sea water (CMFSW), resuspended in CMFSW and triturated gently until fully dissociated. Cells were layered on top of a linear gradient (250 mL of 5–25% (w/v) of 0.75 M sucrose in CMFSW) and allowed to separate at 1 *g* for 45–60 min at 15°C. The micromere band was removed and washed once in MFSW. Cells were plated onto 24-well tissue culture plates (Nunc Inc., Naperville, IL, USA). The micromeres were left to adhere for 1 h, after which MFSW was replaced with 1 mL MFSW + P/S containing 4% horse serum (HS) (Sigma) which had been dialyzed against MFSW. Cells were cultured at 15°C for the indicated periods of time.

RNA extraction and northern blot hybridization

RNA was extracted as reported by Spoerel *et al.* (1986) from normal embryos or from embryos treated with the different inhibitors for approximately 16 h. Total RNA (20 µg) was used for northern blot hybridization. For COLL1α mRNA analysis the probe used was the Uni11 clone (D'Alessio *et al.* 1989). As a control of the total amount of RNA loaded per lane, the same filter was re-probed using the prR03 DNA fragment, derived from the external transcribed spacer of the *P. lividus* ribosomal RNA precursor (Cantone *et al.* 1993) (kindly provided by Dr V. Izzo, IBS, CNR, Palermo, Italy). The signals obtained from the COLL1α probe were then normalized to the signal obtained from the 32S rRNA precursor probe, enabling changes in the level of the specific mRNA to be monitored.

Preparation of embryo extracts

Control embryos and embryos treated with 1 mM vanadate for 30 min at room temperature were washed in CMFSW and collected by centrifugation. Pellets were lysed with 1% Noniolet P-40 (NP-40) in 50 mM Tris, pH 7.4, 5 mM ethylenediamine tetraacetic acid (EDTA), 150 mM sodium chloride (NaCl) in the presence of 10 mM sodium fluoride (NaF), 100 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL leupeptin, 10 µg/mL aprotinin (all from Sigma) for 20 min on ice. Embryo extracts were clarified by centrifugation, and protein in the supernatants was determined by the Lowry method.

Electrophoresis and western blot

Equal amounts (50 µg) of embryo extracts were separated on 7.5% sodium dodecylsulfate (SDS)-polyacrylamide minigels (Laemmli 1970), and transferred to nitrocellulose paper. Peroxidase-conjugated monoclonal antibody against phosphotyrosine (Amersham Italia SrL, Milan, Italy) was diluted 1 : 500. Hybridization was visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Italia SrL), according to the manufacturer's instructions. Molecular weight markers were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa) and bovine serum albumin (66 kDa) from Sigma.

Results

Since a regulatory role for PTPases and for PTK has been suggested in the signal transduction cascade initiated by different stimuli, we decided to study the role of both PTPase and PTK activities in the process of spiculogenesis using isolated sea urchin micromeres and embryos. Micromeres were allowed to grow in a

24-well tissue culture plate at 15°C, and about 24 h after plating the culture medium was replaced and inhibitors, diluted in MFSW containing 4% HS, were added. Cells were cultured for an additional 18 h before observation. As shown in Fig. 1, after approximately 42 h untreated micromere-derived cells showed extensive spicule rod formation (Fig. 1A), as did cultures treated with 20 nM okadaic acid (OA), an inhibitor of serine/threonine phosphatases (Fig. 1B). Increasing OA concentration up to 40 nM had no visible effect. A dose-dependent inhibition of spicule formation was seen in cultures treated both with the PTK inhibitor genistein (Fig. 1C,D) and with the PTPase inhibitor sodium orthovanadate (Fig. 1E,F). As illustrated by the representative fields shown in Fig. 1C–F, the inhibitory effect was on both length and number of spicules formed. However, when the inhibitors were removed, cells were able to resume differentiation and form spicules (not shown), demonstrating that the inhibitor effect of both genistein and vanadate is reversible. These results suggest that protein tyrosine phosphorylation and dephosphorylation are involved in the signal cascade initiated by growth factors present in the horse serum.

We next tested the effect of inhibitors on spiculogenesis in entire embryos. Inhibitors were added to cultures of *P. lividus* embryos between the hatching blastula and mesenchyme blastula stages. As shown in Fig. 2, embryos treated for approximately 30 h with 0.5 mM vanadate formed small spicules (Fig. 2C), whereas no spicules were formed on increasing the concentration to 1 mM vanadate (Fig. 2D). By this time the untreated cultures (Fig. 2A) or the okadaic acid-treated embryos (Fig. 2B) had developed complete skeletons, suggesting again that serine/threonine phosphatases are not involved in spiculogenesis. In the presence of 20 μM genistein, treated embryos exhibited a certain level of invagination and small triradial spicules (Fig. 2E); at higher concentrations embryo development was arrested, and embryos showed a mesenchyme blastula-like morphology with an inappropriate number of cells in the blastocoelic cavity (Fig. 2F). The observed vanadate and genistein effect was not due to some toxic effect since embryos

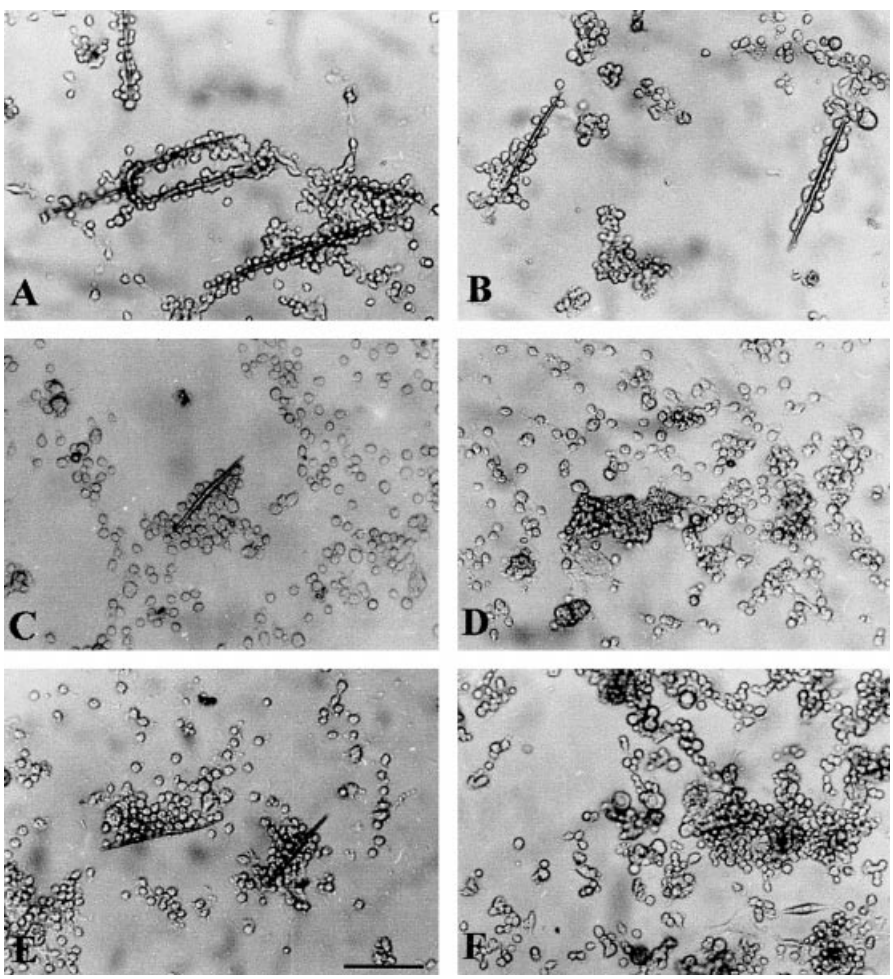

Fig. 1. Effect of protein phosphatase and protein kinase inhibitors on spicule formation in micromere-derived cell culture. Micromeres isolated from 16-cell stage embryos were cultured with 4% horse serum without inhibitor (A) and in the presence of 20 nM okadaic acid (B), 5 μM and 10 μM genistein (C,D) and 0.5 mM and 1 mM sodium orthovanadate (E,F). Pictures were taken after approximately 40 h of culture. Bar, 100 μm.

continued to swim and they appeared viable. Moreover, embryos seemed to recover upon vanadate removal, in that embryos with a prisma-like morphology were observed, although they had an incorrect spicule patterning (Fig. 3C). In contrast, the genistein effect was only partially reversible as embryos completed invagination and showed small spicules but did not grow further (Fig. 3B).

In order to determine the developmental stage at which embryos are sensitive to the inhibitors we conducted pulse studies where inhibitors were added and removed at different times during development. Figure 4 shows that the temporal window of sensitivity to vanadate was between the hatching blastula and early gastrula stages. No effect on development was observed if the inhibitors were removed before or added after the window, demonstrating again that the inhibitor has no toxic effect on embryo development. These data suggest that the signaling pathways inhibited by vanadate participate primarily in cell differentiation, and not in the early cell type specification. In the case of genistein, embryos treated from fertilization to morula (approximately 5 h) or to hatching blastula (approximately 10 h) did not survive, nor

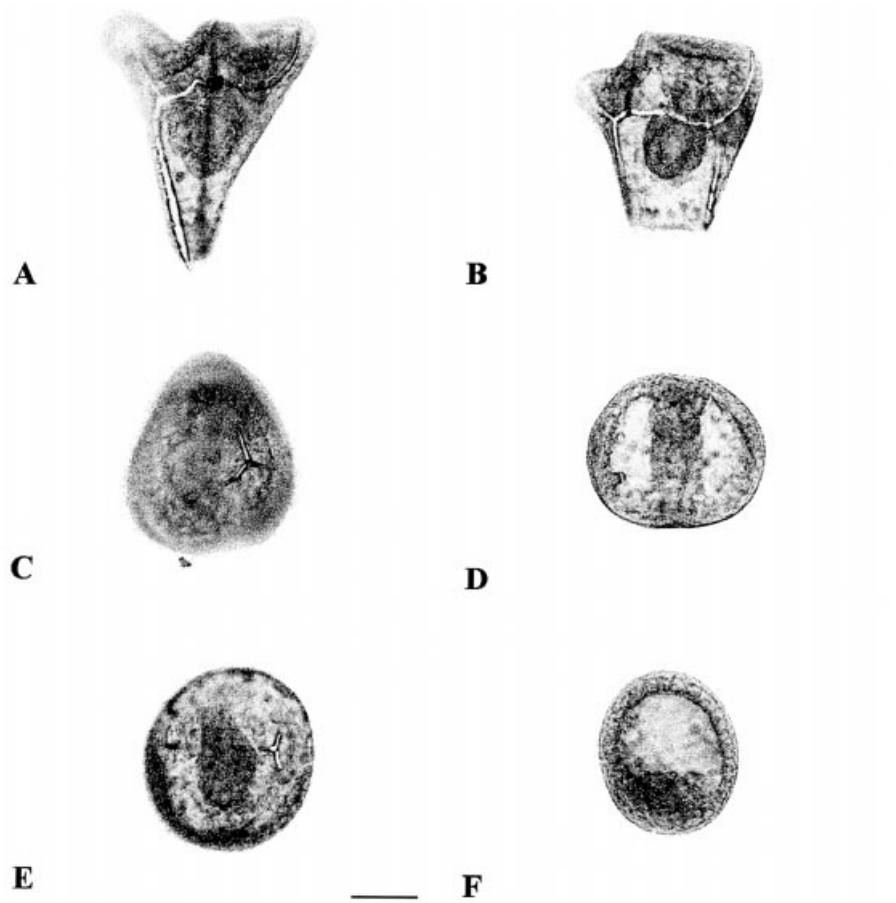

Fig. 2. Effect of protein phosphatase and protein kinase inhibitors on embryo development. Embryos were cultured in Millipore filtered sea water without inhibitor (A) and in the presence of 40 nM okadaic acid (B), 0.5 mM and 1 mM sodium orthovanadate (C,D), 20 μM and 40 μM genistein (E,F). Pictures were taken approximately 30 h after adding inhibitors. Bar, 50 μm.

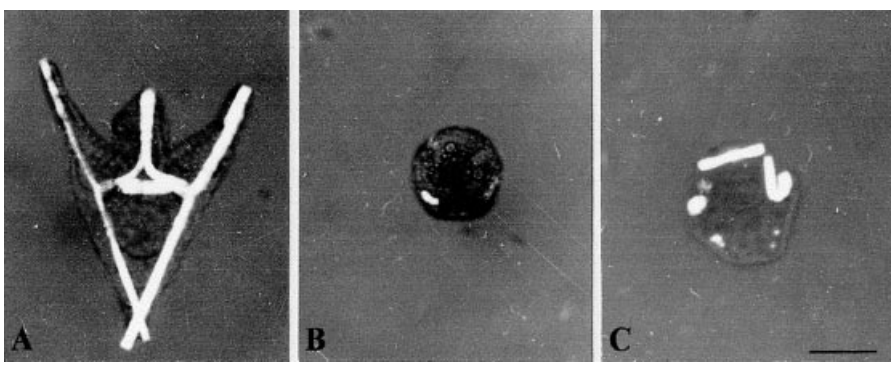

Fig. 3. Embryo recovery after drug removal. Embryos were cultured in Millipore filtered sea water (MFSW) without inhibitor (A) and in the presence of 40 μM genistein (B) or 1 mM sodium orthovanadate (C) for 30 h, after which drugs were removed by washing with MFSW. Embryos were allowed to grow for an additional 20 h. Bar, 100 μm.

did embryos treated from morula to hatching blastula. Treatment after the gastrula stage had no effect on embryo development. The inhibitor effect was observed only between the hatching blastula to early gastrula stages (not shown).

In order to study whether inhibitor treatment interfered with the expression of ECM components, we analyzed the expression of COLL1α mRNA by northern blot. As shown in Fig. 5, treatment of blastula embryos for 16 h with 0.5 mM or 1 mM vanadate inhibited mRNA expression by approximately 15 and 30%, respectively. No effect on collagen gene expression was observed using either 20 μM genistein or 20 nM OA, whereas at a dose of 40 μM genistein no collagen gene expression was observed.

In order to characterize potential substrates of PTPase activity equal amounts of embryo extracts from untreated and vanadate-treated embryos were analyzed by western blot using antiphosphotyrosine monoclonal antibody. As shown in Fig. 6, after treatment for 30 min with 1 mM vanadate there was a great increase in the phosphorylation level of two major proteins, with a molecular weight of approximately 180 and 140 kDa, respectively.

Discussion

Spiculogenesis in sea urchin embryos is a complex developmental event. During spiculogenesis the PMC produce the skeleton in an autonomously specified pattern, but PMC also respond to information provided by the ectoderm (Armstrong & McClay 1994). Skeletal rod growth is, indeed, controlled temporally and spatially by short-range signals, presumably growth factors, coming from the ectodermal epithelium (Guss & Ettensohn 1997). Furthermore, there is evidence to suggest that extracellular matrix components of the sea urchin embryo, such as collagens, are involved in the process of spiculogenesis, either by regulating cell adhesion (Blankenship & Benson 1984) or by binding signal molecules, such as growth factors (Govindarajan *et al.* 1995). The involvement of growth factor signaling, such as that initiated by PDGF-B and TGF-α in spiculogenesis has also been reported (Govindarajan *et al.* 1995; Ramachandran *et al.* 1995, 1997).

In consideration of all of the above findings spiculogenesis appears to be a very complex phenomenon in which several players are involved. In order to break down this complexity we took advantage of the use of a single cell population, the micromere. In order to

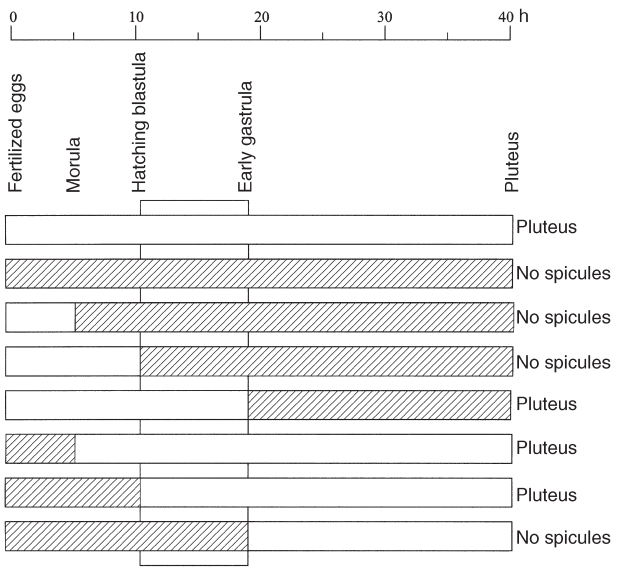

Fig. 4. Effect on *Paracentrotus lividus* embryo development of 'pulse' treatment with sodium orthovanadate. Embryos were cultured in the presence of 1 mM vanadate (▨) or in the absence of this inhibitor (□). The effect produced by the inhibitor is reported to the right of each treatment.

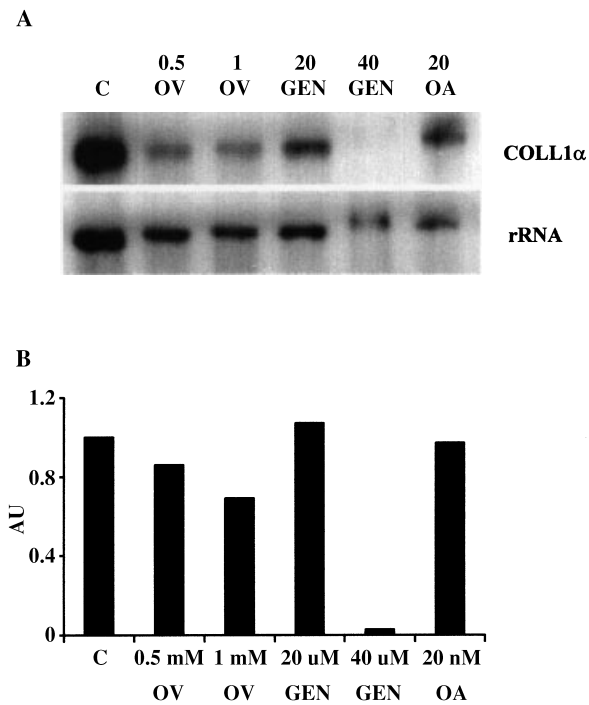

Fig. 5. Northern blot analysis and quantitation of COLL1α mRNA in untreated and treated *Paracentrotus lividus* embryos. (A) Total RNA purified from control embryos (C), 0.5 and 1 mM sodium orthovanadate (OV), 20 and 40 μM genistein (GEN), 20 nM okadaic acid (OA) was hybridized with Uni11 cDNA clone. The positions of COLL1α mRNA and rRNA are indicated. (B) Levels of COLL1α mRNA were determined as described in Materials and Methods. The relative abundance is expressed as arbitrary units (AU) giving a value of one to the level of COLL1α mRNA in the control.

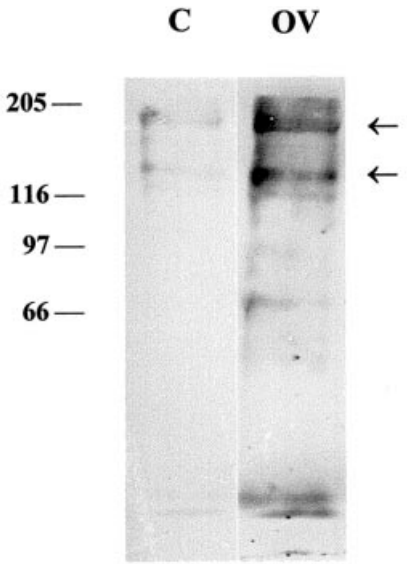

Fig. 6. Immunoblotting analysis of phosphotyrosine level in untreated and vanadate-treated *Paracentrotus lividus* embryos. Embryos at the mesenchyme blastula stage were either untreated (C) or treated with 1 mM sodium orthovanadate (OV) for 30 min at room temperature. Equal amounts of embryo extracts were loaded on a 7.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subjected to immunoblotting using antiphosphotyrosine monoclonal antibody. Molecular weight markers are indicated on the left. Arrows indicate the positions of the major phosphorylated proteins.

determine the different interactions in signaling pathways we used pharmacological agents able to inhibit the activity of specific components of a pathway.

The results obtained suggest that the phosphorylation of protein tyrosine residues plays a key role in the process of spicule formation. We showed that inhibitors of both PTK and PTPase were able to block the signaling of undefined factors present in the horse serum of the micromere culture or in the blastocoelic cavity of the developing embryo. In our experiments, it seems that cell adhesion was not compromised since, in the presence of inhibitors, cultured cells adhered to each other and to the substrate, as demonstrated by cell aggregates shown in Fig. 1(D) and by the presence of numerous spread cells in Fig. 1(F). Moreover, according to morphological criteria vanadate-treated embryos invaginated normally, suggesting that cell movements occurring during gastrulation are not impaired. The only morphological effect observed was the absence of skeleton. Impairment of collagen synthesis does not seem to be the primary cause of inhibition of skeleton formation as indicated by the expression level of the COLL1α mRNA. We showed that vanadate only partially affects collagen gene expression, in that it was expressed at approximately 70–85% of the value of control embryos.

The pulse study experiments in normal embryos showed that the window of sensitivity to vanadate is between the hatching blastula to early gastrula stage, suggesting an interference with signaling pathways involved in cell differentiation rather than on early cell type specification. In genistein-treated embryos it was difficult to ascertain whether the observed block of spiculogenesis was a consequence of growth arrest, rather than of a direct effect on primary mesenchyme differentiation. Although we have observed an inhibition of COLL1α mRNA expression, further studies examining the expression of a variety of genes are necessary in order to distinguish between the two hypotheses.

The results obtained with western blot analysis suggest that in treatment with vanadate, PTPase activity is blocked and the removal of phosphotyrosine residues from specific substrates is inhibited. This could result in a specific block in the signal transduction cascade necessary to activate the spiculogenesis process.

Taken together, these findings suggest that PTPase and PTK activities sustain micromere differentiation. Later in development, it seems that PTPases might be the key enzymes in the signal transduction cascade for mesenchyme differentiation, whereas the role of PTK remains less defined because of the block of embryo development observed in our experiments. Although we have not observed any effect of inhibitors of serine-threonine phosphatase during micromere differentiation and embryo development, other authors have shown that protein kinase C activity, which phosphorylates serine and threonine residues, is necessary for spicule formation (Mitsunaga *et al.* 1990a,b; Livingston & Wilt 1992). It is possible that either protein kinase C or protein tyrosine kinases/phosphatases are involved in skeletogenesis in sea urchins; however, further studies are needed in order to clarify this issue.

The observation that both inhibiting and increasing protein tyrosine phosphorylation during micromere differentiation results in the block of spiculogenesis suggests that a complex pathway is present during spicule formation, and that along the pathway PTPase and PTK activities are necessary. It is well documented that several pathways such as the one initiated by the binding of cytokine, growth factors, and antigens to their own receptors occur via the phosphorylation and dephosphorylation of appropriate substrates (Schlessinger & Ullrich 1992; Streuli 1996; Neel & Tonks 1997). Any imbalance in the phosphorylation/dephosphorylation level may affect normal cellular function (Sun & Tonks 1994). Depending on cellular type and cellular context, PTPases and PTK may either cooperate with or antagonize each other during the signal

transduction cascade. Therefore, it is not surprising that in a complex process like that of spicule formation both protein tyrosine phosphorylation and dephosphorylation events should be necessary. In the present study, we have shed some light on the signal transduction mechanisms that control skeletogenesis in sea urchin embryos. The next level of understanding will require knowledge of the genes that are affected by inhibitor addition and of the protein targets of PTPase and PTK activity.

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