p38 MAPK activation is required for *Paracentrotus lividus* skeletogenesis

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Abstract — We investigated the p38 MAPK role during sea urchin, *Paracentrotus lividus*, development. We found that at the morula stage, before the onset of skeletogenesis, p38 MAPK shows a peak of activity, and we tested whether p38 MAPK activity has any effect on skeletogenesis. By immunohistochemistry on whole-mount embryos we show the preferential localization of the active p38 form both in the presumptive PMCs and bilateral spiculogenesis centers in control embryos, and in the radialized supernumerary spiculogenesis centers induced by NiCl_2 treatment. By using SB203580, a p38 MAPK specific inhibitor, we show that p38 activity is required both for the initial triradiate spicule rudiments formation and for their correct elongation.

Key words: p38 MAPK, SB203580, sea urchin development, skeletogenesis.

INTRODUCTION

All the members of Mitogen Activated Protein Kinase (MAPK) family are involved in a wide variety of cellular processes such as inflammation, cell cycle, cell death, cell differentiation, development, senescence and tumorigenesis (ZARUBIN and HAN 2005). One of the main groups of MAPK is the p38 protein kinase group (p38), the activation of which by dual phosphorylation in the TGY motif, has been observed in response to various extracellular stimuli in different organisms and tissues. Four isoforms (α , β , γ , δ) of the p38 MAPK subfamily exist in mammals, some of which (α and β) seem to be expressed ubiquitously, while others (γ and δ) are differentially expressed in various tissues (FERRELL 1996; NEBREDÁ and PORRAS 2000).

The physiological activity of the p38 MAPK group is often transient, involving dephosphorylation in the downregulation of MAP kinase pathway (MILLWARD *et al.* 1999; SUNDARESAN and FARNDAL 2002; WU and BENNET 2005; ZARUBIN and HAN 2005).

In sea urchin, inhibition of MAPK signalling has been shown to affect gastrulation and spiculogenesis in *Strongylocentrotus purpuratus* (KUMANO

and FOLTZ 2003), and p38 activity was been found to be essential for secondary axis specification and patterning in *Lytechinus variegatus* sea urchin embryos (BRADHAM and McCCLAY 2006).

Skeletogenesis is one of the key events during sea urchin development (PENNINGTON and STRATHMAN 1990). Skeleton derives from primary mesenchyme cells (PMCs) that, after entering in the blastocoel, arrange into a ring parallel to the posterior wall of the larva and then fuse to form two centers of spiculogenesis (OKAZAKI 1975; HARDIN *et al.* 1992; HARDIN 1996; ETTENSOHN 1999; ANGERER and ANGERER 2003). GUSS and ETTENSOHN (1997) showed that the local interaction between PMCs and the neighbouring ectoderm regulates the correct skeletal morphogenesis, probably by modulating the expression of specific genes involved in spiculogenesis. Moreover, it was recently shown that, in *Lytechinus variegatus*, p38 activity is required not only for oral specification, through promotion of the Nodal expression in the oral territory, but also for skeletogenesis (BRADHAM and McCCLAY 2006).

We previously showed that hyperosmotic deciliation in *Paracentrotus lividus* causes (as well as other stresses) a thermotolerant response through the activation of a p38 MAPK-induced phosphorylation signalling cascade (CASANO *et al.* 2003); in addition, we found that during *P. lividus* development some members of the p38 subfamily are differentially activated (unpublished data). In this study, by using a p38 specific inhibitor, immunohistochemistry and NiCl_2 -treatment, we were

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able to show that p38 activation is specifically required for a regulated skeletogenesis.

MATERIALS AND METHODS

Embryo Culture and Treatment - *Paracentrotus lividus* embryos were cultured at 18°C in Millipore-filtered seawater with antibiotics, at the dilution of 5000/ml in glass beakers under continuous rotation, until the desired stage was reached, as previously described (CASANO *et al.* 1998). Fertilization was carried out in the presence of 6 mM PABA (Para-Amino-Benzoic Acid) to prevent hardening of the fertilization envelope in order to perform immunohistochemical observations at early cleavage stages.

A concentration of 60 μ M of SB203580 – a p38 MAPK specific inhibitor (Sigma) – was added to the embryo suspension at specific stages of development (CASANO *et al.* 2003). The SB treatments were blocked after 3 or 5 hours by washing embryos three times with seawater.

NiCl₂ was added to the embryo culture soon after fertilization at a concentration of 0.5 mM (HARDIN *et al.* 1992).

48 hours post-fertilization, aliquots of live embryos (200-300) were taken from the culture media to examine their morphology under a microscopy. Pigment cells and spicule rudiments were counted by compressing alive larvae.

RNA Extraction, RT/PCR, Sequencing Analysis - Total RNA was extracted from embryo cultures at the gastrula stage using phenol-guanidine thiocyanate method described by CHOMCZYNSKI and SACCHI (1987). Total RNA concentration was determined by measuring absorbance at 260 nm and the quality was detected by electrophoresis of the samples on formaldehyde agarose gel.

RT-PCR experiments were performed using the One Step RT-PCR system (Promega) and 1 μ g of total RNA. The cDNA primers, designed by GENE bank comparative analysis (Pl EST clone number PK0AAA40YF22RM1.SCF) were Plp38-Direct: 5'-TTCCAAACAGTCATTCATGCCAAG-3' (from 315 to 338) and Plp38-Reverse: 5'-TACGGGCCAATCCAAAGTCCAG-3' (from 654 to 633). Using these primers a 340 bp product was generated. RT conditions were those suggested by Promega supplier (45' at 44°C), PCR was performed on a (Eppendorf) thermal cycler using the following conditions: 94°C (2 min), [94°C (30 sec), 44°C (30 sec), 72°C (30 sec) -30 cycles], 72°C (5 min).

The RT-PCR product obtained was purified with a commercial kit (Wizard PCR Preps – DNA Purification System; Promega Italy) and then sequenced directly in both directions using BigDye Terminator Cycle sequencing kit 1.1 in ABI 3100 DNA sequencer and using the direct and reverse primers used for amplification.

Western Blotting - SDS-PAGE and Western blotting were performed as previously described (CASANO *et al.* 2003), with equal amounts of total proteins extracted from *Paracentrotus lividus* embryos at different stages of development. Protein concentration was measured by BRADFORD assay (1976). After electrophoresis, proteins were electro-blotted onto nitro-cellulose membranes (Hybond ECL, Amersham); then the membranes were stained with Ponceau red to verify that comparable amounts of proteins were loaded. Filters were pre-incubated for 3 hr with a blocking solution and then incubated with the specific antibody.

We used the following antibodies: i) Monoclonal anti non-activated p38 MAPK, clone p38-YNP, which reacts with the p38 MAPK non-activated form (Sigma 1:1000); ii) Monoclonal anti-activated p38 MAPK, clone p38-TY, (Sigma 1:500) which reacts only with the p38 MAPK activated (diphosphorylated) form, and whose reactivity has been observed with human, rat and mouse. After washing three times in TBS (150 mM NaCl, 10 mM Tris pH 7.4) to remove non-specific complexes, the membranes were incubated with anti-mouse IgG conjugated with alkaline phosphatase (AP) (Promega 1:7500) and finally stained with NBT-BCIP (Roche).

Immunostaining - Both treated and control embryos were fixed with Bouin's fixative at selected stages, and immunohistochemical observations were carried out as previously described (ROCCHERI *et al.* 2001).

Anti-diphosphorylated-p38 (active) MAP kinase monoclonal antibody was used at a dilution of 1:250; anti-p38 MAP kinase (inactive) mouse monoclonal antibody (Sigma) was used at a dilution of 1:250; anti-*Sp-(Strongylocentrotus purpuratus)*-msp130 antibody (kindly provided by Prof. G. SPINELLI) was used at a dilution of 1:250. Anti-mouse IgG, AP-conjugated secondary antibody was used at a dilution 1:1000, and staining was performed with NBT-BCIP mix (Roche). After staining and mounting them on glass slides, embryos were photographed with a Zeiss Axioscope 20 photomicroscope equipped with an automatic

MC 80 exposure system, using a Kodak gold colour film 100 ASA. Photo images were imported with a scanner using Adobe PhotoShop software.

RESULTS AND DISCUSSION

Paracentrotus lividus p38 MAPK is highly conserved – By a search of EST *Paracentrotus lividus* (Pl) (<http://goblet.molgen.mpg.de/cgi-bin/webapps/paracentrotus.cgi>) with *Lytechinus variegatus* (Lv) sequence (GenBank Accession Number AY445029) we found one sequence corresponding to p38 MAPK (clone PK0AAA40YF22RM1, SCF).

On the basis of comparative analysis of *Lytechinus variegatus* and *Paracentrotus lividus* EST p38 nucleotide sequences, we designed two primers to reverse transcribe RNA extracted from gastrula embryos. The obtained 340 bp product was sequenced (GenBank Accession Number AM295340). It exhibited a sequence highly homologue to *P. lividus* EST coding for p38 MAPK (from the amino acidic residue 60 to 172, the sequence in bold in Figure 1).

The amino acid alignment of both Pl EST and Lvp38 sequences is shown in Figure 1. General similarity between the two sequences is about 82%; higher similarity, about 89%, (both with *Lytechinus variegatus* and human p38) was found at the level of the nine residues that specifically interact with p38 SB203580 inhibitor (SB); moreover, the degree of homology at the level of

the non-specific SB-interacting elements is 74%; finally the phosphorylation motif TGY is totally conserved, and the amino acids surrounding the TGY motif are well conserved. All these features indicate that *Paracentrotus lividus* contains a p38 MAPK that may cluster, like Lvp38, with α (or β) subtype, and suggest that human p38, Lv-p38 and Pl-p38 MAPK specifically might interact with SB in a similar way.

p38 MAPK activity during development – A number of studies suggested that echinoderm oocytes, eggs and embryos have multiple MAPKs (CHIRI *et al.* 1998; PHILIPOVA and WHITAKER 1998; KATOW and HAIZU 2002). We previously demonstrated that p38 activation has an effector role in the deciliation stress signalling, at gastrula stage of *P. lividus* (CASANO *et al.* 2003). Therefore, we studied the p38 activity during development of the sea urchin *P. lividus*. At this aim we tested – in a time-course experiments with synchronous embryo cultures – the activity of p38 in whole-embryo extracts. In particular, extracts collected at different times after fertilization were separated by electrophoresis, blotted and probed with two different antibodies, one specific for the active p38 MAPK (diphosphorylated) and the other directed against the inactive forms of p38 MAPK (non-phosphorylated or mono-phosphorylated). The anti-activated p38 MAPK-antibody revealed two peaks of activity during development: the first at the morula stage, the other at the early gastrula stage (Fig. 2). The high degree of homology of p38 phosphorylation

Lv	1	MSSKSETNPL	PSGFHVLELN	GTKWEIPIRY	VRLEPVGSGA	YGQVCGADDT	50
Pl	1	MSS-----L	PADFHHELN	KTIWEVPNRY	VQLEPVGSGA	YGQVCSAVDT	44
Lv	51	KQGG-RVAIK	KLSRPFQTVI	HAKRTYRELRL	LHKMKHENV	ICLLDCFTPN	100
Pl	45	HQKGLKVAIK	KLARPFQTVI	HAKRTYRELRL	LLHKMKHENV	ISLLDCFTPD	94
Lv	101	P-SCL-DVYM	VTILMGADLN	NIIKCQKLTN	EHVQFLIYQV	LRGLKYIHSA	148
Pl	95	PVNFS-NVYM	VTHLMGADLN	NIIKCQKLSD	DHVQFLIYQV	LRGLKYIHSA	143
Lv	149	GVIHRDLKPS	NIAVNEDCEL	KILDFGLARS	TDEMTGYVA	TRWYRAPEIM	198
Pl	144	GVIHRDLKPS	NIAVNEDCEL	RILDFGLARS	TDVEMTGYLA	PKWYRAPEIM	193

Fig. 1 — Alignment of deduced p38MAPK amino acid sequences in *Paracentrotus lividus* and *Lytechinus variegatus*. Partial amino acid sequences of Plp38 and Lvp38 are shown. The phosphorylation motif TGY is boxed; specific SB-interacting residues are in dark grey, and non specific SB-interacting elements are in light grey. In bold are the sequences that we have reverse transcribed and amplified.

Fig. 2 — Changes in amount of active p38 during *P. lividus* development. (A) Western blot analysis of total embryo protein extracts (100 µg/lane), performed with either anti-active-p38 or anti-inactive-p38 antibodies. (1) Two-Blastomeres; (2) Morula; (3) Hatching Blastula; (4) Mesenchyme Blastula; (5) Early Gastrula; (6) Late Gastrula. (B) Histogram obtained by densitometry of the stained filters with Image J software. Band intensity is reported in arbitrary units.

motif TGY and its surrounding amino acids with the corresponding sequences of human and Lv p38 MAPK, allowed us to use the commercial heterologous antibodies (CASANO *et al.* 2003).

p38 MAPK is involved in PMCs and SMCs differentiation - Since the analysis of p38 activity in embryo extracts does not allow to localize cell-specific activity, whole-mount embryos were tested with the anti-p38 antibodies by immunohistochemistry. As shown in Fig. 3, the results confirmed stage dependence of p38 activation

and in addition demonstrated that at the morula stage, (where we demonstrated by immunoblot analyses the peak of activity), the p38 active form was mainly present in the micromeres (Fig. 3 A) while the inactive form was uniformly distributed (Fig. 3 E). At the early gastrula stage, the active p38 was localized both in micromere-derived cells -the PMCs- and in the tip of the archenteron (Fig. 3 B), (from which the secondary mesenchyme cells [SMCs] derive), while the inactive form was especially abundant in the apical region of the embryo (Fig. 3 F). Finally, at the late gastru-

Fig. 3 — Immunohistochemical localization of p38 in whole mount sea urchin embryos during development and after NiCl_2 treatment. Localization of the active (A-D) and inactive forms (E-H) of p38 assessed by using monoclonal anti-active or anti-inactive p38 MAPK specific antibodies. (A, E) Morula; (B, F) Early Gastrula; (C, G) Late Gastrula; (D, H) NiCl_2 -treated embryos. Bar = 20 μm . Note that for anti-inactive p38 MAPK antibody the staining time was shorter in order to show any difference in amount.

la stage, both active and inactive p38 forms were preferentially and differentially localized in the apical regions (Fig. 3 C, G), in particular the active form in the oral and the inactive in the aboral region. The presence of the active form of p38 in micromeres and PMCs suggested its involvement in skeletogenesis.

As it is well known, the treatment of the embryos with NiCl_2 affects number and skeletal pattern, with over-representation of oral ectodermal cells. The skeleton shows a radial instead of bilateral symmetry: in particular, the ring of ingressed PMCs gives rise to many as a dozen spicule rudiments, arranged in a radial pattern, instead of the two ventrolateral clusters (HARDIN *et al.*, 1992). In order to demonstrate the p38 involvement in spiculogenesis, we probed p38 activation in NiCl_2 -treated embryos. Whole-mount immunolocalization of p38 MAPK in treated embryos showed that, at the early gastrula, an array of radialized PMCs reacted with the antibody specific for the active form of the enzyme (Fig. 3 D) but not with the anti-inactive p38 antibody (Fig. 3 H).

The skeletogenic mesenchyme cells, radialized in NiCl_2 -treated embryos or clustered in control embryos, in which p38 MAPK was active, also reacted with an antibody specific for the skeletogen-

ic marker msp130 (ANSTROM *et al.* 1987; GUSS and ETTENSOHN 1997; data not shown), thus better correlating p38 activation with spiculogenesis.

It is worth noting that the staining of inactive p38 (Fig. 3 G) disappears from the apical aboral ectoderm when the embryos are "oralized" with NiCl_2 (Fig. 3 H), demonstrating the aboral ectoderm nature of these cells.

The data discussed above suggested a role of the p38 kinase in skeletogenesis. In order to further demonstrate the involvement of p38 in *P. lividus* skeletogenesis we incubated the embryos with SB203580, a specific p38 MAPK inhibitor. We previously showed that the activation of p38 in twice deciliated embryos was inhibited by SB (CASANO *et al.* 2003), and moreover, as discussed above, we recognized in *P. lividus* p38 putative SB-interaction sites similar to the ones present in human and *L. variegatus* p38 (BRADHAM and MCCRAY 2006).

It is well-known that skeletogenesis is a process regulated both in the initial phase, i.e. the formation of the rudimental triradiate spicules existing in the two centers of spiculogenesis, and during the extension of such rudiments in order to form embryo's definitive skeleton (GUSS and ETTENSOHN 1997), therefore we decided to inves-

tigate how p38 is involved in the various phases of skeletogenesis process in *P. lividus*. To this aim, we treated embryos at different developmental stages with SB 203580 only for short time lapses (wash out experiments). The optimal conditions for using the SB203580 inhibitor in *P. lividus* sea urchin system were determined in a previous paper (CASANO *et al.* 2003).

We found that treatment with SB since 1 hour after fertilization and throughout development, affected a regular gastrulation and impaired spiculogenesis (data not shown). In order to define the time at which the spiculogenesis was most sensitive to the SB inhibitor, the zygotes were cultured in the presence of SB203580 up to different developmental stages and then the inhibitor was washed out and the embryos were checked for their ability to develop up to the pluteus stage.

In *P. lividus* the PMCs ingression depends on ERK activation (ROTTINGER *et al.* 2004); on the other hand, we did not observe any abnormal PMC ingression in SB-treated embryos. Therefore, we monitored spiculogenesis, gastrulation and the number of pigmented cell in these embryos; the results are summarized in Figure 4. The treatments with the inhibitor between 1 and 7 hours after fertilization variably affected gastrula-

tion, spiculogenesis and pigmented cell differentiation, in agreement with the idea that the skeletal pattern is in part autonomously programmed but also controlled by the overlying ectoderm.

In particular we found that, inhibiting the p38 activity during the early hours of development (up to the morula stage) anomalies were observed mainly in gastrulation (exogastrula). Later treatments (at the morula/blastula transition) impaired skeleton formation and morphogenesis. We also observed that the number of pigment cells (one of the SMCs derivatives) was reduced upon SB treatment, suggesting an involvement of p38 activity also in SMCs differentiation.

The time lapse in which, despite a regular gastrulation occurs, the SB effect on spicule formation was more significant, ranges from 5 to 8 hours after fertilization. It is worth noting that treatments performed later (between 6 and 10 hours of development) are less and less effective on both spicule morphogenesis and pigmented cell differentiation.

In conclusion, our results show that for production of the skeletal spicules in *P. lividus* p38 activation is required, and also that p38 activity is necessary, during the initial cleavage stages, for regular embryo gastrulation. However, we point-

Fig. 4 — *Effect of SB203580 on P. lividus development.* Sea urchin embryos were cultured in presence of SB203580 (60 μ M) for the time indicated (solid lines on the left of figure), and then observed at 48 hours of development, when control embryos were at the pluteus stage. The data regarding the percentage of exogastrulae (EXO), spicules (SPIC.) and pigmented cells (PIGM.) observed for each treatment, are summarized in the table on the right of figure (N.B. the observations were also made by squashing the embryos to highlight even spicule rudiments). The stars indicate the presence of only spicule rudiments. See text for details.

ed out that at later stages, when its activity is no longer necessary for gastrulation, p38 is still required for spiculogenesis *per se*, that is both for the initial triradiate spicule rudiments formation and for their correct elongation. Inhibition of p38 activity between 5 and 8 hours of development (at the morula/blastula transition), did not invalidate gastrulation but completely impaired spicule formation, whereas later treatments with the inhibitor allowed some spicule rudiment formation, while they still impaired the correct skeleton morphogenesis (stars in Fig. 4). Finally, if treatment started at 8 hours of development, spiculogenesis was almost regular.

These results suggest a crucial role of p38 activity between 4 and 6 hours of development especially for gastrulation and spiculogenesis determination (light grey band in Fig. 4), p38 activity is also necessary, between 6 to 8 hours from fertilization, for regular spicule elongation (dark grey band in Fig. 4).

Taken together, our data suggest a p38 effector role in a signaling pathway on which correct spiculogenesis depends, that is possibly responsive to growth factor(s) (KATOW and AIZU 2002; KUMANO and FOLTZ 2003; ZITO *et al.* 2003; KIYOMOTO *et al.* 2004).

Acknowledgements — Many thanks are due to Prof. I. Di Liegro for critical evaluation of the manuscript. Grant Sponsor: MIUR (E.G. 60% MIUR) and PRIN; Grant number: 2004057282.

REFERENCES

- ANGERER L. M. and R. C. ANGERER, 2003 — *Patterning the sea urchin embryo: gene regulatory networks, signaling pathways, and cellular interactions*. *Curr. Top. Dev. Biol.*, 53: 159-198.
- ANSTROM, J. A., J. E. CHIN, D. S. LEAF, A. L. PARKS and R. A. RAFF, 1987 — *Localization and expression of msp130, a primary mesenchyme lineage-specific cell surface protein in the sea urchin embryo*. *Development*, 101: 255-265.
- BRADHAM C. A. and D. R. McCLAY, 2006 — *p38 MAPK is essential for secondary axis specification and patterning in sea urchin embryos*. *Development*, 133: 21-32.
- BRADFORD M. M., 1976 — *A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding*. *Anal. Biochem.*, 72: 248-254.
- CASANO C., M. C. ROCCHERI, K. ONORATO, D. CASCINO and F. GIANGUZZA, 1998 — *Deciliation: a stressful event for Paracentrotus lividus embryos*. *Biochem. Biophys. Res. Commun.*, 248: 628-634.
- CASANO C., M. C. ROCCHERI, L. MAENZA, S. MIGLIORE and F. GIANGUZZA, 2003 — *Sea urchin deciliation induces thermoresistance and activate p38 MAP kinase pathway*. *Cell Stress & Chaperones*, 8: 70-75.
- CHIRI S., C. DE NADAI and B. CLAPA, 1998 — *Evidence for MAP kinase activation during mitotic division*. *J. Cell Sci.*, 111: 2519-2527.
- CHOMCZYNSKI P. and N. SACCHI, 1987 — *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction*. *Anal. Biochem.*, 162: 156-159.
- ETTENSohn C.A., 1999 — *Cell movements in the sea urchin embryo*. *Curr. Opin. Genet. Dev.*, 9: 461-465.
- FERRELL J.E., 1996 — *MAP kinases in mitogenesis and development*. *Curr. Top. Dev. Biol.*, 33: 1-60.
- GUSS K. A. and C. A. ETTENSohn, 1997 — *Skeletal morphogenesis in the sea urchin embryo: regulation of primary mesenchyme gene expression and skeletal rod growth by ectoderm-derived cues*. *Development*, 124: 1899-1908.
- HARDIN J., J. A. COFFMAN, S. D. BLACK and D. R. McCLAY, 1992 — *Commitment along the dorsoventral axis of the sea urchin embryo is altered in response to NiCl₂*. *Development*, 116: 671-685.
- HARDIN J., 1996 — *The cellular basis of sea urchin gastrulation*. *Curr. Top. Dev. Biol.*, 33: 159-299.
- KATOW H. and G. AIZU, 2002 — *Essential role of growth factor receptor-mediated signal transduction through the mitogen-activated protein kinase pathway in early embryogenesis of the echinoderm*. *Dev. Growth Differ.*, 44: 437-455.
- KIYOMOTO M., F. ZITO, S. SCIARRINO and V. MATRANGA, 2004 — *Commitment and response to inductive signals of primary mesenchyme cells of the sea urchin embryo*. *Dev. Growth Differ.*, 46: 107-114.
- KUMANO M. and K. FOLTZ, 2003 — *Inhibition of mitogen activated protein kinase signaling affects gastrulation and spiculogenesis in the sea urchin embryo*. *Dev. Growth Differ.*, 45: 527-542.
- MILLWARD T.A., S. ZOLNIEROWICZ, B.A. HEMMINGS, 1999 — *Regulation of protein kinase cascades by protein phosphatase 2A*. *Trends Biochem. Sci.*, 24: 186-191.
- NEBRED A. R. and A. PORRAS, 2000 — *p38 MAP kinases: beyond the stress response*. *Trends Biochem. Sci.*, 25: 257-260.
- OKAZAKI K., 1975 — *Spicule formation by isolated micromeres of the sea urchin embryo*. *Am. Zool.*, 15: 567-581.
- PENNINGTON J.T. and R.R. STRATHMANN, 1990 — *Consequences of the calcite skeletons of planktonic echinoderm larvae for orientation, swimming, and shape*. *Biol. Bull.*, 179: 121-133.
- PHILIPPOVA R. and M. WHITAKER, 1998 — *MAP kinase activity increases during mitosis in early sea urchin embryos*. *J. Cell Sci.*, 111: 2497-2505.
- ROCCHERI M.C., M. PATTI, M. AGNELLO, F. GIANGUZZA, E. CARRA and A.M. RINALDI, 2001 — *Localization of mitochondrial HSP56 chaperonin during sea urchin development*. *Biochem. Biophys. Res. Commun.*, 287: 1093-1098.

- ROTTINGER E., L. BESNARDEAU and T. LEPAGE, 2004 — *A Raf/MEK/ERK signaling pathway is required for development of the sea urchin embryo micromere lineage through phosphorylation of the transcription factor Ets*. Development, 131: 1075-1087.
- SUNDARESAN P. and R. W. FARNDALE, 2002 — *p38 mitogen-activated protein kinase dephosphorylation is regulated by protein phosphatase 2A in human platelets activated by collagen*. FEBS letters, 528: 139-144.
- WU J. J. and A. M. BENNET, 2005 — *Essential role for mitogen-activated protein (MAP) kinase phosphatase-1 in stress-responsive MAP kinase and cell survival signaling*. J. Biol. Chem., 280: 16461-16466.
- ZARUBIN T. and J. HAN, 2005 — *Activation and signaling of the p38 MAP kinase pathway*. Cell Res., 15: 11-18.
- ZITO F., C. COSTA, S. SCIARRINO, V. POMA, R. RUSSO, L. M. ANGERER and V. MATRANGA, 2003 — *Expression of univin, a TGF-beta growth factor, requires ectoderm-ECM interaction and promotes skeletal growth in the sea urchin embryo*. Dev. Biol., 264: 217-227.

Received April 13th 2007; accepted January 2nd 2008