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Cadmium induces the expression of specific stress proteins in sea urchin embryos

Maria Carmela Roccheri [a,*], Maria Agnello [a], Rosa Bonaventura [b], Valeria Matranga [b,*]

[a] *Dipartimento di Biologia Cellulare e dello Sviluppo "A. Monroy," Università di Palermo, Viale delle Scienze, Parco d'Orleans, 90128 Palermo, Italy*
[b] *Istituto di Biomedicina e Immunologia Molecolare "A. Monroy," Sezione Biologia dello Sviluppo, C.N.R., Via Ugo La Malfa 153, 90146 Palermo, Italy*

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Abstract

Marine organisms are highly sensitive to many environmental stresses, and consequently, the analysis of their bio-molecular responses to different stress agents is very important for the understanding of putative repair mechanisms. Sea urchin embryos represent a simple though significant model system to test how specific stress can simultaneously affect development and protein expression. Here, we used *Paracentrotus lividus* sea urchin embryos to study the effects of time-dependent continuous exposure to subacute/sublethal cadmium concentrations. We found that, between 15 and 24 h of exposure, the synthesis of a specific set of stress proteins (90, 72–70, 56, 28, and 25 kDa) was induced, with an increase in the rate of synthesis of 72–70 kDa (hsps), 56 kDa (hsp), and 25 kDa, which was dependent on the lengths of treatment. Recovery experiments in which cadmium was removed showed that while stress proteins continued to be synthesized, embryo development was resumed only after short lengths of exposure.

Keywords: Stress proteins; Embryo development; Gel electrophoresis; Western blot

Recent evidence suggests that exposure to different environmental toxicants, including heavy metals, may cause a cascade of events, including the production of reactive oxygen species. Among the heavy metals, cadmium is considered an important toxicant, whose mutagenic, embryotoxic, and teratogenic potentials have been extensively investigated [1]. Since it is a permanent metal ion, which cannot be degraded by bacteria, it is accumulated by many organisms and causes a series of toxic effects such as: increased production of reactive oxygen species [2], depletion of glutathione [3], inhibition of enzymes involved in DNA synthesis and repair [4], and DNA single-strand breaks [5].

Higher invertebrates and vertebrates, when exposed to heavy metals, usually activate protection systems by increasing the expression of metal binding proteins such as metallothioneins and heat shock proteins (hsps) [6]. The increased expression of hsps (hsp70, hsp60, and hsp27) in response to cadmium was demonstrated in tissues and cells of many organisms, e.g., sponges [5], *Drosophila* [7], nematodes [8,9], and human cells [10].

For many years the effects of various toxicants, such as cadmium, on marine invertebrate embryos, and in particular on echinoderm larvae have been studied [11,12]. Most of these studies examined the developmental defects due to the presence, in rearing media, of this specific toxicant [13,14]. However, few studies consider the ability of sea urchin larvae to accumulate contaminants during development [15]. Therefore, the sea urchin developmental model offers an excellent opportunity to investigate the possible adaptive response of cells exposed to cadmium during differentiation. Furthermore

* Corresponding authors. Fax: +39-091-6577430 (M.C. Roccheri), +39-091-6809557 (V. Matranga).
E-mail addresses: mariroc@unipa.it (M.C. Roccheri), matranga@ibim.cnr.it (V. Matranga).

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sea urchin embryos, as the majority of organisms, are able to respond to many stresses by synthesizing a set of highly conserved proteins, the hsps [16,17] and/or metallothioneins [18,19]. We have recently shown that continuous exposures of *Paracentrotus lividus* sea urchin embryos to subacute/sublethal cadmium concentrations cause a number of abnormalities, such as a general developmental delay, reduced gut elongation, and skeleton defects [19].

In this paper we investigated if cadmium chloride induces the synthesis of a specific set of stress proteins (hsps) in exposed embryos. In addition, we examined the relationship between the abnormalities triggered by cadmium chloride on embryo development and the expression of hsps. Lastly, we explored the possibility that hsps over-expression may be able to rescue embryo development after cadmium removal.

Materials and methods

Embryo culture, treatment, and labeling. Gametes were collected from gonads of the sea urchin *P. lividus* harvested from the West coast of Sicily. Briefly, eggs were fertilized and embryos, at a concentration of 5000 ml^{-1}, were grown at 18 °C in Millipore filtered seawater containing antibiotics (50 mg/L streptomycin sulfate and 30 mg/L penicillin), under gentle rotation. For treatment with $CdCl_2$, embryos were continuously cultured, after fertilization, in the presence of 10^{-5}, 10^{-4}, and 10^{-3} M $CdCl_2$, and their development was monitored by optical microscopy. Embryos were treated for different times as indicated in Results. During the last 45 min of each treatment, aliquots of embryo cultures were labeled with 40 μCi/ml L-[^{35}S]methionine (Amersham, specific activity 1000 Ci/mM). To obtain information on the reversibility of the treatment we performed some experiments in which cadmium was removed by washing the embryos in normal seawater after 15, 18, and 21 h of exposure. Embryos were then allowed to develop in seawater for 24 h and metabolically labeled during the last 45 min, as above described.

One- and two-dimensional electrophoretic analysis of samples. Control and treated embryos were Dounce homogenized in lysis buffer [20] including the protease inhibitor cocktail (complete, Mini, EDTA-free protease inhibitor cocktail tablets—Roche). The amount of radioactive [^{35}S]methionine incorporated into proteins was determined by TCA precipitation and measured by a scintillation counter (Beckman). Equal amounts of samples (40 μg) were analyzed by one-dimensional SDS–PAGE, performed on 10% slab gels. Two-dimensional gel electrophoresis was carried out as described [17]; the first dimension was performed on cylindrical gels containing 1.6%, pH 5–7, and 0.4%, pH 3–10, ampholytes (LKB), loaded with equal amounts (150 μg) of proteins, and run overnight at 300 V. The second dimension was performed on 10% SDS–polyacrylamide slab gels and run for 5 h at 100 V. Molecular weights for mono- and two-dimensional gels were evaluated by comparison with a set of ^{14}C-labeled standard proteins (Rainbow ^{14}C-methylated protein molecular weight markers, Amersham). Gels were then soaked with 2,5-diphenyloxazole in dimethyl sulfoxide solution, dried, and finally exposed for autoradiography to films (Hyperfilm MP—Amersham).

Western blotting. After electrophoresis in 8% SDS–PAGE, proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham) using a semidry apparatus (Novablot, Pharmacia), in 24 mM Tris–1 M glycine–20% methanol, at 0.8 mA/cm^2 for 2 h. Filters were first pre-incubated for 2 h in a blocking solution: 5% non-fat dried milk in Tris-buffered saline–0.05% Tween 20 (TBS-T), washed once in TBS-T, and then incubated for 1 h with the specific antibody:anti-hsp60 (human) and anti-hsp70 (bovine) mouse monoclonal antibodies (Sigma) diluted 1:500 and 1:5000, respectively, in TBS-T. After removal of non-specific complexes by three washes in TBS-T and two washes in TBS, filters were incubated for 1 h with anti-mouse IgG-horseradish peroxidase linked whole antibody (Amersham) diluted 1:10,000 in TBS. Bands were detected by chemiluminescence using a SuperSignal West Pico Chemiluminescent Substrate (Pierce) and Hyperfilm ECL films (Amersham). Chemiluminescent markers used were Magic Mark (Invitrogen).

Results

Paracentrotus lividus embryos were divided into three different batches just after fertilization, continuously cultured in the presence of 10^{-5}, or 10^{-4}, or 10^{-3} M $CdCl_2$, and harvested after 9 or 24 h of development. At the end of the treatments, some embryos were metabolically labeled with [^{35}S]methionine, during the last 45 min, and others were utilized for morphological observations. Fig. 1A shows that the treatment of embryos with 10^{-3} M $CdCl_2$ for 9 h induces the synthesis of 72–70 kDa proteins. After 24 h of treatment, a general reduction in the protein synthesis pattern of embryos treated with 10^{-4} and 10^{-3} M $CdCl_2$ was detected. Moreover, the activation of the specific synthesis of 90, 72–70, 56, 28, and 25 kDa proteins in embryos treated with 10^{-3} M $CdCl_2$ was found.

In order to correlate the observed stress protein induction to defects in embryo development, we performed a statistical analysis of the morphological observations carried out on 100 embryos for each experimental point: the frequencies of developmental defects are reported in Figs. 1B and C. We found that embryos exposed to 10^{-5} M cadmium concentrations developed until the swimming blastula stage (about 9 h of development), with no significant differences with respect to controls, while some embryos exposed to 10^{-4} or 10^{-3} M cadmium showed a delay in embryo development (Fig. 1B). When control embryos reached the late gastrula stage (about 24 h of development), cadmium-exposed embryos exhibited defects in development (not shown). The frequency and the severity of abnormalities increased with the increase of $CdCl_2$ concentration in the seawater. At the highest cadmium concentration used (10^{-3} M $CdCl_2$) about 70% of the embryos were found delayed at the early gastrula stage and about 30% at blastula stage (Fig. 1C).

On the basis of these results it appears that the highest cadmium chloride concentration and 24 h of treatment have the most significant effects both on biochemical and morphological parameters. However, it should be considered that such a high cadmium concentration is needed in order to produce an effect in a fast-developing embryo, as the sea urchin.

Thus, we decided to treat embryos with 10^{-3} M $CdCl_2$ with exposure times for 15–30 h in order to better define

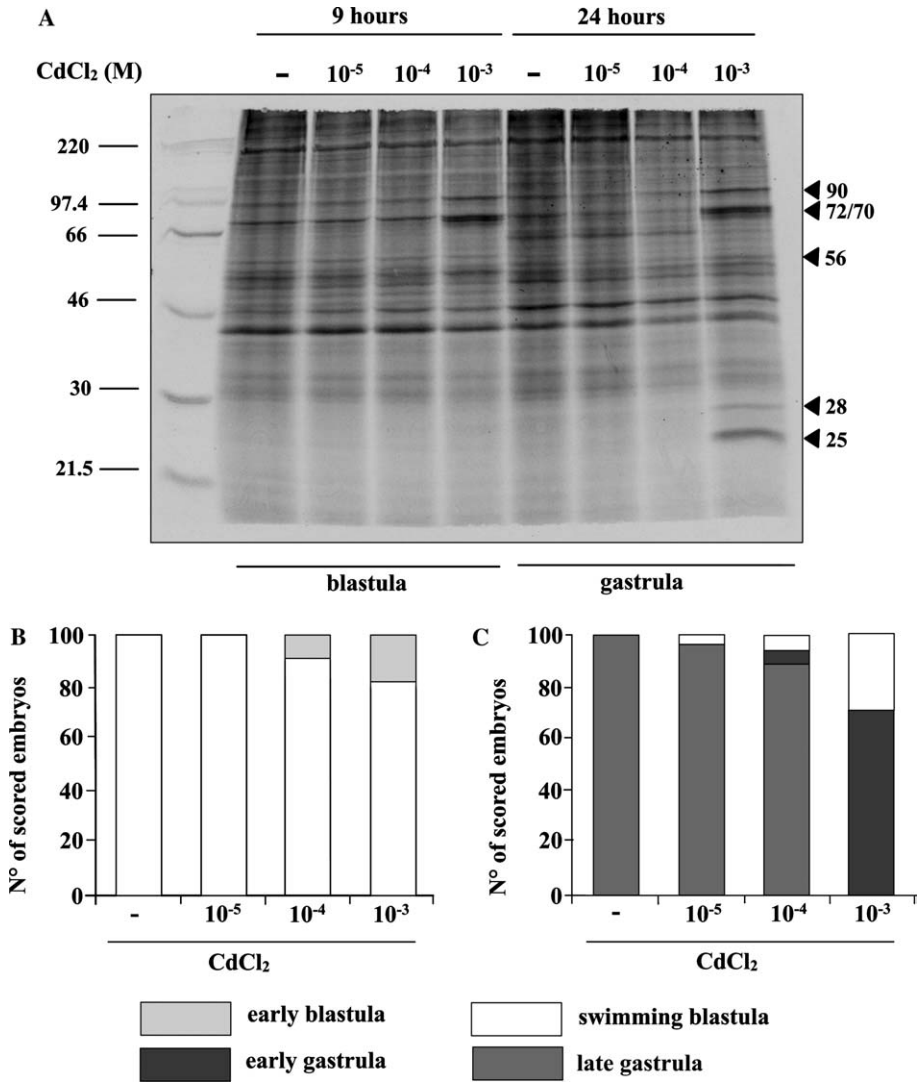

Fig. 1. Continuous exposure to different cadmium chloride concentrations induces the synthesis of specific stress proteins and affects embryo development. Autoradiography of mono-dimensional gel electrophoresis of lysates from ^{35}S-metabolically labeled embryos continuously exposed to 10^{-5}, 10^{-4}, and 10^{-3} M cadmium chloride and harvested after 9 and 24 h post-fertilization, when controls reached the blastula and gastrula stages, respectively. (A) Morphological analysis of sea urchin embryos exposed continuously to different $CdCl_2$ concentrations for 9 h (B) or 24 h (C).

the putative correlation between stress effects and cadmium accumulation. Results of experiments in which embryos were treated with 10^{-3} M $CdCl_2$ for 15, 18, and 21 h are shown in Fig. 2. SDS–PAGE analysis of same amounts of proteins per lane showed a general reduction in the protein synthesis pattern and the over-expression of specific 72–70, 56, and 25 kDa proteins (Fig. 2A). The proteins at 90 and 28 kDa were not found because they can be detected only after a lengthy exposure to cadmium. Quantification of major stress-induced proteins obtained by densitometric scanning of the auto-radiographic film is reported in Fig. 2B. In the histogram, the values, expressed in arbitrary units, show that the levels of 72–70, 56, and 25 kDa proteins increased during time of exposure. Specifically, 72–70 and 56 kDa levels show a major increase after 15 h and continue to increase at a lower rate at 18 and 21 h of exposure. On the contrary, an increase in the levels of 25 kDa protein is found in all cases, in a direct relationship to times of exposure to cadmium. Since prolonged times of treatment (30 h) produced a lethal effect on embryos, it was not possible to evaluate their protein synthesis pattern.

As we found that, an 18 h treatment with cadmium was sufficient enough to produce a strong induction in stress protein expression, but, at the same time, did not have harmful effects on embryo development, we choose this length of treatment for further analyses. Then, proteins were analyzed by a 2D gel as shown in Fig. 3. The major findings were: first, the 56 kDa band observed in the mono-dimensional electrophoresis is resolved in a series of isoforms ranging from neutral to acidic isoelectric points; second, the 25 kDa band

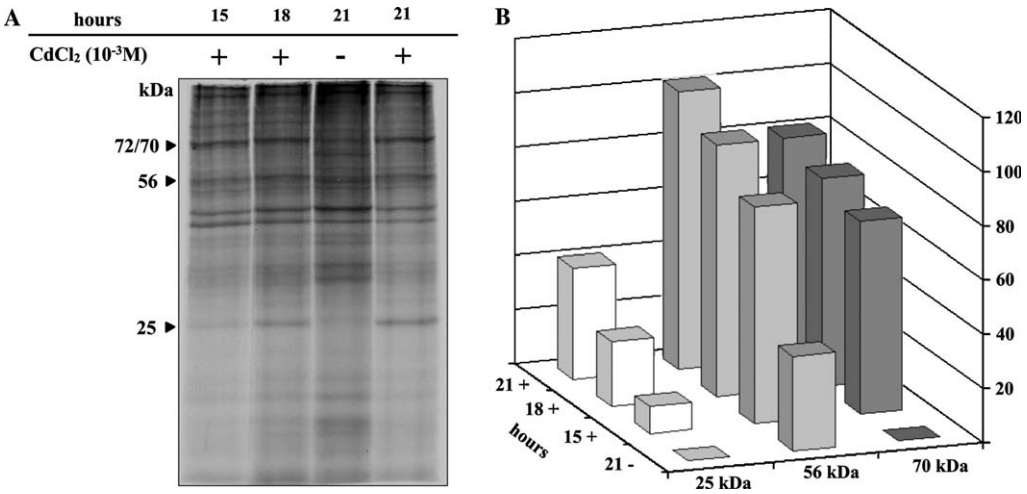

Fig. 2. Definition of the temporal window of sensitivity to cadmium chloride exposure. Mono-dimensional gel electrophoresis of lysates from ^{35}S-metabolically labeled embryos exposed to 10^{-3} M $CdCl_2$ for the indicated time lengths (A). The histogram (B) shows the quantification of the major stress-induced proteins, obtained by a densitometric scanning of the filter shown in (A).

observed previously was resolved in at least two discrete isoforms having basic-neutral isoelectric points. The 72–70 and 90 kDa proteins were resolved in a limited number of isoforms having more acidic isoelectric points than other proteins activated by cadmium treatment. The above-mentioned findings seem to indicate that the proteins induced by cadmium treatment could belong to the heat shock protein class and known to be differently induced in response to various stress events, as previously shown [17,21–23]. To confirm this hypothesis, Western blot analysis using heterologous anti-hsp70 and anti-hsp60 antibodies, previously shown to cross-react with sea urchin embryo proteins, was performed on lysates from embryos treated with 10^{-3} M $CdCl_2$. It should be pointed out that the anti-hsp70 antibody used is able to recognize both hsp70 constitutive and inducible forms [24] and the anti-hsp60 antibody used recognizes as homologous a sea urchin hsp56 [25]. We found that antibodies recognize specifically the 72–70 and 56 kDa proteins, respectively, demonstrating in addition a discrete increase in the protein levels in embryos treated for 15–21 h (Fig. 4).

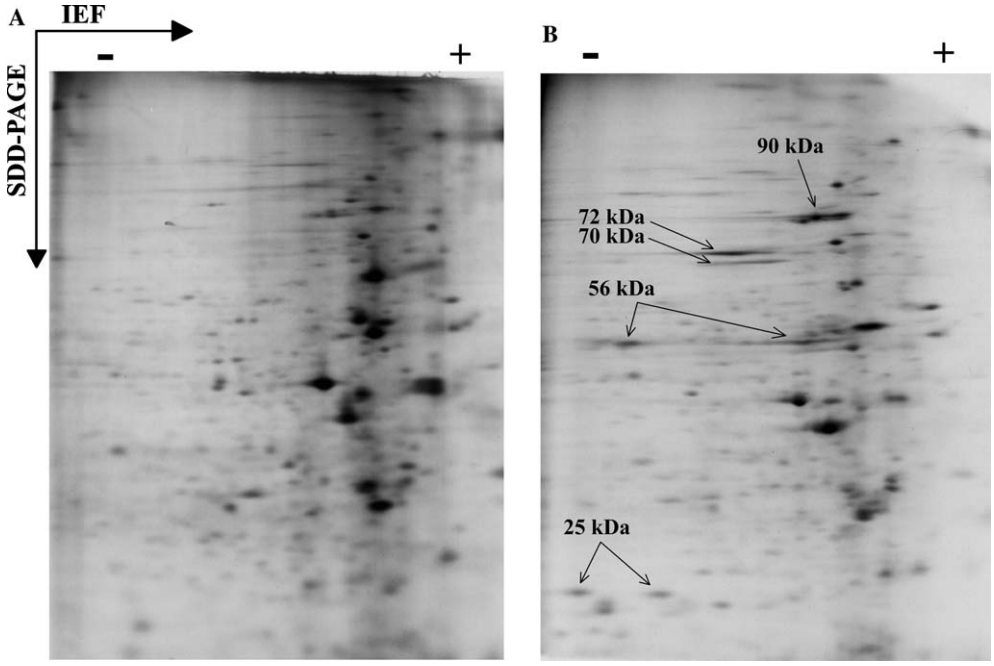

Fig. 3. Cadmium chloride induced the synthesis of several stress proteins isoforms. Autoradiography of two-dimensional gel electrophoresis of lysates from ^{35}S-metabolically labeled 18-h-old embryos, control (A) and exposed to 10^{-3} M $CdCl_2$ (B).

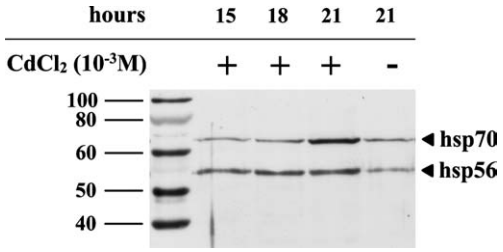

Fig. 4. Sea urchin embryos respond to cadmium chloride exposure by the expression of heat shock proteins. Western blot analysis of total lysates from embryos continuously treated with 10^{-3} M $CdCl_2$ for 15, 18, and 21 h and 21-h-old controls reacted with anti-hsp60 and anti-hsp70 specific antibodies.

In order to investigate if the hsps proteins switch-on is conserved after cadmium chloride removal, embryos were first treated with 10^{-3} M $CdCl_2$ for 15, 18, and 21 h, then washed with fresh seawater, allowed to develop for other 24 h, and analyzed. We found that, while there was a general recovery in the rate of total protein synthesis, the 25 kDa protein continued to be expressed at high levels; the 90 kDa, the 72–70 hsp, and the 56 hsp were still synthesized, even if at low levels (Fig. 5). This finding was observed in all the samples tested, regardless of the time of exposure to cadmium.

Interestingly, the recovery of total protein synthesis was not always coupled to a recovery of embryo morphological development. Embryos treated for 15 h with cadmium, observed 24 h later, showed a general delay in development; while 48 h later they were similar to controls (not shown). Embryos treated for 21 h and cultured for other 24 or 48 h under physiological conditions (no cadmium) showed a general delay in development as they were found at the gastrula stage. In addition, aberrant morphologies for at least 30% of the scored embryos were found: in fact, while control embryos were well-formed late pluteus (Figs. 6A and B), embryos from which cadmium was removed for 24 h (Figs. 6C and D) or 48 h (Figs. 6E and F) had a severe impairment of skeleton development.

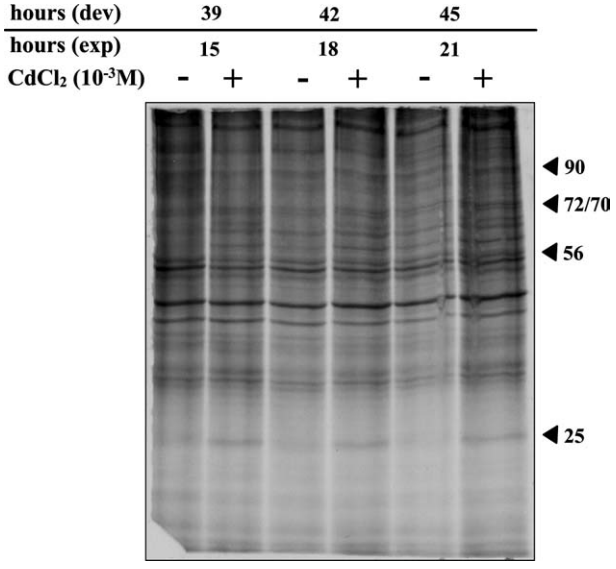

Fig. 5. Recovery of protein synthesis pattern and partial induction of stress proteins after cadmium removal. Autoradiography of monodimensional gel electrophoresis of lysates from ^{35}S-metabolically labeled embryos continuously exposed to 10^{-3} M $CdCl_2$ for the indicated time lengths and allowed to develope in physiological conditions for another 24 h.

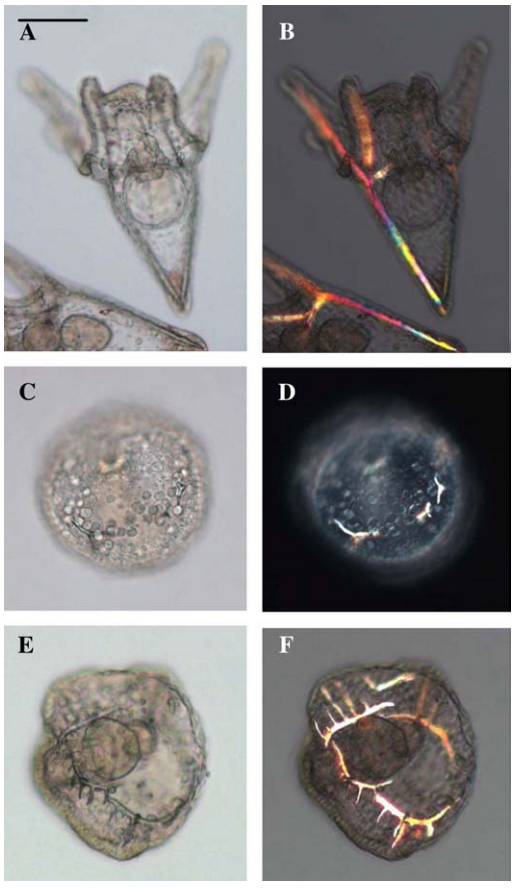

Fig. 6. Perturbations of skeletogenesis in partially recovering embryos after cadmium removal. Control pluteus embryos cultured in normal seawater for 45 h (A,B); after exposure to 10^{-3} M $CdCl_2$ for 21 h, embryos were cultured in normal seawater for another 24 h (C,D) and 48 h (E,F). Bar=25 μm (A,B) and 50 μm (C–F).

Discussion

In this study we demonstrate that cadmium chloride induces the synthesis of a specific set of stress proteins in embryos continuously exposed to sublethal concentrations of this metal. The correlation between the induced hsps and cadmium concentrations coupled to periods of exposure has been demonstrated by mono- and two-dimensional electrophoretic analysis on metabolically labeled embryos. We found that 9 h of exposure are sufficient to induce the synthesis of 70 and 72 hsps,

while at least 15 h are necessary to observe also the induction of hsp56 and 25 kDa synthesis. In all cases an increase in their synthesis levels is observed for longer times of exposure (from 18 to 24 h).

Moreover, the levels of protein synthesis remain high even when the stimulus is withdrawn from the culture for 24 h. This finding is in agreement with reports describing the capacity to induce stress protein production in the recovery period, even after the removal of cadmium, in another marine invertebrate [26]. When the accumulation of hsps during time was monitored by Western blot analysis, we found that their amounts were higher than those of controls for long periods of treatment.

Several authors demonstrated that cadmium is likely to involve mitochondria as a target, since micromolar concentrations have a direct effect on increasing permeability of the inner membrane in rat liver mitochondria [27,28]. However, little is known about the cause/effect relationship between the cadmium-induced response and the mitochondrial chaperonine (hsp60) expression. Some authors have demonstrated the hsp60 induction in response to cadmium in hepatoma cells [29]; in addition, others have proposed hsp60 as a potential biomarker to toxicant stress in nematode [9]. We have previously shown that *P. lividus* embryos subjected to heat shock increased the synthesis levels of hsp56, a mitochondrial chaperonine belonging to the hsp60 family [25,30].

In the present study we demonstrated for the first time that the induction of hsp56 is related to increased concentrations of cadmium to which sea urchin embryos are exposed and described also the induction of a new stress protein, having an apparent molecular size of 25 kDa. It should be noted that the increase in the rate of synthesis observed for the last is continuous and constant over time of treatment; moreover, the synthesis levels remain high even after the removal of cadmium. Therefore, at least in our model system, it appears that the 25 kDa is specifically enhanced by cadmium. Interestingly, in renal epithelial cells a hsp27 has been described to be induced, in association with hsp70 and metallothionein, by micromolar cadmium concentrations [31]. If the sea urchin 25 kDa protein, shown to be induced by cadmium in this study, belongs to the small hsp family remains to be verified. Confirmation of this hypothesis would necessitate performing immune-detection studies, thus requiring antibodies specific for sea urchin or cross-reacting, which are not currently available.

It has been demonstrated that the ability of sea urchin embryos to synthesize hsps represents a general protective strategy against many stress-inducing agents including heat shock [16,17,32], heavy metals [17,21], and others [22,23]. Moreover, different stress-inducing agents enhance the expression of different hsps [33–35]. For example, it is known that in the chick embryo, the induced protein expression patterns are depending on the kind of heavy metal used for the assay [36]. In the sea urchin embryo subacute/sublethal cadmium concentrations are also able to enhance the expression of the metallothionein gene, as demonstrated in our previous paper [19]. Similarly, the expression of a gene encoding for metallothionein was also shown to be strongly increased after exposure to cadmium in the marine sponge *Suberites domuncula* [37]. It is known that together with hsp70 and metallothionein genes, a member of the BAG-3 gene family is over-expressed in cells exposed to heavy metals and appears to play a dual role in preventing cell death and contributing to the cellular defence response to stress [38]. It should be recalled that hsp proteins increase resistance to apoptosis as shown by several authors which demonstrated that an increase in the cellular levels of hsp27 and/or hsp70 allows cells to survive to otherwise lethal conditions [39,40].

Last, analysis of morphological defects upon cadmium exposure demonstrated no impairment of embryo development at low $CdCl_2$ concentration tested. On the other hand, very high $CdCl_2$ concentrations for long times of exposure lead to abnormal development with serious defects in skeleton elongation and patterning. These results are in agreement with those reported previously and matching with measurements of heavy metal contents found in *P. lividus* embryos [14,15,19]. Furthermore, we defined the temporal window within which the effects of cadmium were reversible: embryos exposed for shorter than 21 h to cadmium could be rescued by the removal of the ion; thus, reinforcing the concept of a time-dependent effect of cadmium exposure. In conclusion, the hsps differential time-dependent expression suggests the existence of a specific sequential cross talk among stress proteins in response to exogenous stress conditions. Since these chaperones modulate cell death and survival pathways, the overall balance of the pathways as well as their interplay determine whether embryos exposed to cadmium will die or become stress tolerant and survive. In addition, our findings confirm that in the sea urchin embryo hsps expression represents a sensitive biomarker to detect environmental pollution caused by heavy metals.

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