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Different Heat-Shock Proteins Are Constitutively Overexpressed in Cadmium and Pentachlorophenol Adapted *Euglena gracilis* Cells

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To determine whether cellular resistance to a given stressor is related to induction of specific stress-proteins, responses of two adapted *Euglena gracilis* cell lines, one adapted to cadmium, the other adapted to pentachlorophenol, were analyzed. Our experiments showed that two sets of heat-shock proteins (hsps) were constitutively overexpressed in each cell line: while hsp90, hsp70, hsp55, and hsp40 were induced in cadmium-resistant cells, only hsp40 was induced in pentachlorophenol-adapted cells.

Stress response was commonly seen in all organisms from bacteria to higher vertebrates and was involved in protection from damages due to exposure to various environmental stresses. This response was characterized by the rapid preferential synthesis and accumulation of the so-called heat-shock (hsps) or stress proteins (1). These proteins have been shown to be induced by different unrelated agents including heat-shock treatment, drugs, amino-acid analogs or heavy metals (2,3,4). They have been classified mainly into five families: hsp100, hsp90, hsp70, hsp60, and the small hsps family. They have been described as having structural similarities among various organisms and conserved through evolution.

In our laboratory, we focused our interest on the response of achlorophyllous *Euglena gracilis* cells to fresh water pollutants. More precisely, to study molecular mechanisms settled following chronic exposure to these pollutants, we attempted to characterize specific proteins involved in tolerance to each of them.

For this purpose, we have established stable cell lines that are tolerant to normally lethal or sublethal concentrations of xenobiotics such as cadmium (Cd) or pentachlorophenol (PCP); their growing rate and their terminal density were similar to those of wild cells cultured without pollutant (5,6). We previously demonstrated that two polypeptides of 55 and 40 kDa are constitutively overexpressed in Cd-resistant cells. These polypeptides are also overexpressed in heat-shocked wild cells (7).

In the present report, we extended this study to other families of hsps, namely to hsp90 and hsp70, and we examined whether these hsps could also be overexpressed in PCP-tolerant *Euglena gracilis* cells. We found that two different sets of hsps are overexpressed in Cd or PCP-adapted cell lines. This study raises the possibility that resistance or adaptation of *Euglena gracilis* cells requires changes in levels of expression of hsps, and involves specific hsps, depending on the xenobiotic.

MATERIALS AND METHODS

Cell lines. Axenic cultures of *Euglena gracilis* W 100 ZUL (a wild achlorophyllous mutant derived from the wild-type Z strain of *E. gracilis* Klebs) were grown in a mineral medium supplemented with vitamins B1 and B12 and containing lactic acid (33 mM) as the carbon source (8). From this strain, a Cd resistant cell line (R-Cd) was established; this R-Cd cell line was subcultured in 2 mM Cd for 5 years. A PCP-tolerant cell line was also established and subcultured in 4 μM PCP for 2 years (6).

Cell culture and labeling. Euglena gracilis cells were grown at 23°C in lactate medium with or without 500 μM Cd for

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the wild strain and with or without 2 mM Cd for the Cd-resistant one. PCP-tolerant cells were grown in lactate medium containing 4 μM PCP. Cells were labeled for two hours at the midlogarithmic phase of growth (i.e. the third day of culture) with 0.37 MBq/mL of ^{35}S methionine and ^{35}S cysteine. Except for heat shock treatment, for which cells have been incubated at 35°C during labeling, all other labelings were carried out at 23°C. For Cd-shock, Cd concentration in the medium was adjusted to 500 μM at the time of labeling.

Cell extracts and acrylamide gel electrophoresis. After labeling, cells were harvested by centrifugation and washed twice with 10 mM Tris-HCl, pH 7.4. Cell pellets were incubated for 2 hours in lysis buffer (40,000 cells/μL) containing 9.5 M urea, 2% NP-40, 5% β-mercaptoethanol and 2% ampholines (0.4% pH 3–10, 1.6% pH 5–7). Then, after 10s. of sonication, protein samples were centrifuged for 3 min. at 6,000 g to release paramylum. Mono-dimensional electrophoreses in 12.5% gels were performed as previously reported (9). Samples were also analyzed by two-dimensional gel electrophoresis according to the method previously described (10). Briefly, a non equilibrium pH gradient (NEPHGE) was used for the first dimension. The second dimension was in 12.5% polyacrylamide gel containing 0.1% SDS. Following electrophoresis, gels were dried and exposed to Kodak X AR-5 films at −20°C.

RESULTS

We previously reported that hsp55 and hsp40 were constitutively overexpressed in achlorophyllous Cd-resistant *Euglena gracilis* cells, using NEPHGE/SDS PAGE 2D gel electrophoresis (7). In this study, we examined whether other hsps could be overexpressed in these cells. Results from monodimensional gel electrophoresis analyses are shown in Fig. 1. Compared to wild cells (Fig. 1, lane 1), heat-shocked wild cells (Fig. 1, lane 3) showed an increase in the rate of synthesis of two major polypeptides with apparent molecular weights of 90 and 70 kDa, respectively. These hsps were also induced in Cd-resistant cells labeled at the control growth temperature of 23°C, cultured with (Fig. 1, lane 5) or without 2mM Cd (Fig. 1, lane 6). It was noticeable that overexpression of these hsps became detectable in wild cells after a 500 μM Cd-shock, just prior cell labeling at 23°C (Fig. 1, lane 2) but not in a 3 day old cell culture with 500 μM Cd (Fig. 1, lane 4).

Taken together, these results indicated that at least four hsps, with molecular weights of 90, 70, 55 and 40 kDa, were constitutively overexpressed in Cd-resistant cells.

Next, we examined whether these hsps were overexpressed in an *Euglena gracilis* cell line adapted to PCP. For this study, protein synthesis levels in these cells were compared to those of wild and Cd-resistant cells, using 2D gel electrophoresis. Figure 2 shows the pattern of labeled polypeptides present in heat-shocked cells. Area being examined in more details was enclosed and those corresponding to other cell lines or culture and labeling conditions were joined together in Fig. 3. Unlike to heat-shocked (Fig. 3B) and to Cd-resistant cells (Fig. 3C), no detectable synthesis of the hsp55 was observed in PCP-adapted cells (Fig. 3D). However, when compared to control cells (Fig. 3A), PCP-adapted cells overexpressed the hsp40 (Fig. 3D).

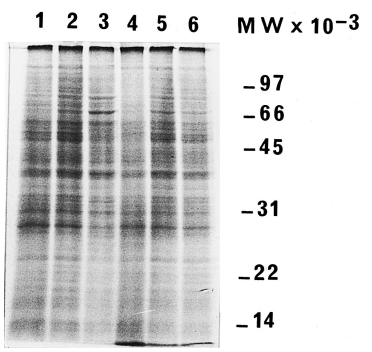

FIG. 1. Autoradiography of SDS-polyacrylamide gel of *Euglena gracilis* cellular proteins in: (1) wild cells grown and labeled at 23°C in lactate medium; (2) wild cells grown in lactate medium and labeled in 500 μm Cd lactate medium at 23°C (Cd-shock); (3) wild cells grown at 23°C and labeled at 35°C in lactate medium; (4) wild cells grown for 3 days in 500 μM Cd and labeled at 23°C; (5) Cd-resistant cells grown and labeled at 23°C in 2mM Cd lactate medium; (6) Cd-resistant cells grown and labeled at 23°C in lactate medium.

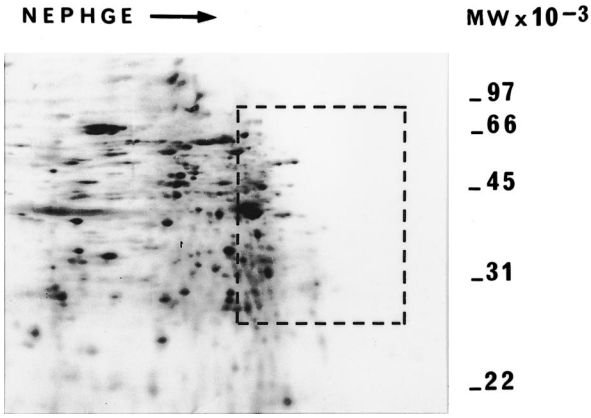

FIG. 2. Two-dimensional gel electrophoresis (NEPHGE) of total cellular proteins synthesized in heat-shocked *Euglena gracilis* cells. Area containing interesting spots was enclosed.

Finally, using one dimensional gel electrophoresis, we examined whether hsp90 and hsp70 were overexpressed in PCP adapted cells (Fig. 4). When compared to control wild cells (Fig. 4, lane 1) or to heat-shocked wild cells (Fig. 4, lane 2) or to Cd-resistant cells (Fig. 4, lane 3), PCP-adapted cells overexpressed neither hsp90 nor hsp70 (Fig. 4, lane 4).

DISCUSSION

In this study, we have compared cell-adaptation to chemicals that belong to distinct groups, Cd, a heavy metal, and PCP, a chlorinated chemical. These contaminants of ground water supplies (11) have been listed as priority pollutants by the US Environmental Protection Agency (12).

Two adapted *Euglena gracilis* cell lines have been used: a Cd-resistant cell line, subcultured with 2 mM Cd, and a PCP-adapted one, subcultured with 4 μM PCP. These two cell lines grow similarily to control in term of growing rate and terminal cell density (6). However, compared to the control cells, Cd resistant cells constitutively overexpress at least four hsps with molecular weights ranged from 90, 70, 55 to 40 kDa. In contrast, PCP-adapted cells only overexpress hsp40.

Induction of hsps in *Euglena gracilis* cells has been already reported in literature. Ortiz (13) has shown increases in the synthesis of polypeptides of 45, 58, 62, 70, 84 and 98 kDa in *Euglena*

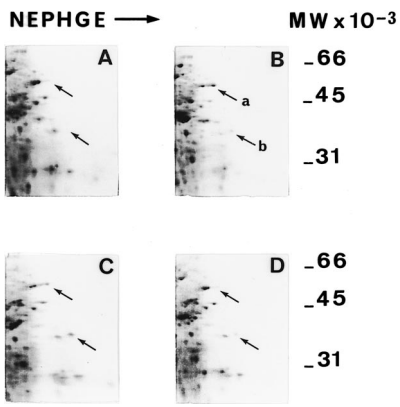

FIG. 3. Area containing the *Euglena gracilis* relevant polypeptides: (A) wild cells grown and labeled at 23°C in lactate medium; (B) wild cells grown at 23°C and labeled at 35°C in lactate medium (a, hsp55; b, hsp40); (C) Cd-resistant cells grown and labeled at 23°C in 2mM Cd lactate medium; (D) PCP-adapted cells grown and labeled at 23°C in 4μm PCP lactate medium.

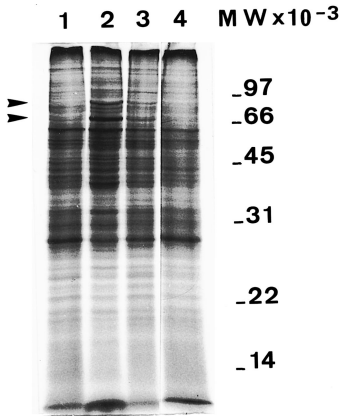

FIG. 4. Autoradiography of SDS-polyacrylamide gel of *Euglena gracilis* cellular proteins in: (1) wild cells grown and labeled at 23°C in lactate medium. (2) wild cells grown at 23°C and labeled at 35°C in lactate medium; (3) Cd-resistant cells grown and labeled at 23°C in 2mM Cd lactate medium; (4) PCP-adapted cells grown and labeled at 23°C in 4μm PCP lactate medium. Arrowheads, hsp90 and hsp70.

gracilis Klebs, Z strain, after 1 to 6 hours of incubation at 36°C. Amir-Shapira *et al.* (14) pointed out that the induction of 3 major polypeptides of 40, 70 and 80 kDa in *Euglena gracilis* Klebs var. *bacillaris* can be observed after 2 hours of incubation at 36°C. Thus, it is very likely that our 90, 70, 55 and 40 kDa hsps are part of these polypeptides.

Some biochemical studies have demonstrated that hsp90 associates with a variety of cellular proteins including tyrosine kinases, actin, tubulin as well as steroid hormone or dioxin receptors. It has been proposed that hsp70 family members associate with unfolded proteins for their translocation across the endoplasmic reticulum and mitochondrial membranes and also facilitate protein folding after partial denaturation induced by heat-shock. Hsp55 may be a member of the hsp60 family which acts sequentially in a common pathway with hsp70 in folding and assembly of proteins. To get insight for a possible identity of these proteins, use of specific antibodies should be necessary. Hsp40, a non classical hsp, was also described by Ohtsuka *et al.* (15) in mammalian and avian cells. This hsp has homology with the bacterial DnaJ (16) and is not only induced by heat-shock but also by other stresses including Cd. It is located in the basic region of the electrophoregram, as that is the case for *Euglena gracilis* hsp40.

Some data indicate that hsps can also be involved in the maintenance of a tolerant state against temperature or drugs (17). Sanchez *et al.* (18) have characterized in yeast a hsp104 of critical importance in tolerance to heat and ethanol, of moderate importance in tolerance to sodium arsenite, and of little or no importance in tolerance to copper and cadmium. For our part, we have recently reported that adaptation to Cd do not protect cells from PCP and *vice-versa* (6). Indeed, Cd- and PCP-adapted *Euglena gracilis* cells show an impaired pattern of growth when cross-cultured with the other pollutant. The hsp40, which is overexpressed in the two adapted *Euglena gracilis* cell lines, seems to be of great importance in PCP adaptation but of less importance in Cd adaptation. These observations suggest that detoxication pathways that are induced by one pollutant are specific for this pollutant. Our present data demonstrate that this specificity is detectable at the gene expression level, different hsps being overexpressed in each of the adapted cells. They also suggest that the cellular targets for Cd or PCP are fundamentally different. To ascertain this specificity, we are currently studying hsps responses of *Euglena gracilis* cell lines adapted to other metals and to other chlorinated chemicals.

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