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CADMIUM RESISTANCE OF ACHLOROPHYLLOUS *EUGLENA GRACILIS* CELLS: CONSTITUTIVE OVEREXPRESSION OF TWO HEAT-SHOCK PROTEINS

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SUMMARY: The heat-shock response of *Euglena gracilis* was studied by cell labeling at both the normal growth temperature (23 °C) and an elevated temperature (35 °C). Analysis of the labeled proteins by two-dimensional polyacrylamide gel electrophoresis indicated that the rate of synthesis of two polypeptides p55 (55 kDa) and p40 (40 kDa) increased in cells labeled at the highest temperature studied. These polypeptides are also overexpressed in Cadmium-resistant *Euglena gracilis* cells labeled at the normal growth temperature (23°C). On the basis of these results, p55 and p40 appear to be heat-shock proteins involved in some steps of the acquired Cd-resistance process in *Euglena gracilis* cells.

When cells or organisms are exposed to elevated temperature, they trigger the synthesis of a small group of proteins called the heat-shock proteins (hsps) (1,2). This response is highly conserved and presumed to be universal, serving a general protective function in the stressed cells (3). Principal hsps have molecular masses ranging from 28 to 110 kDa. Among these hsps, those having a molecular weight between 70 and 90 kDa are seen in response to most situations of stress (4). The other proteins appear to be induced more selectively: for example, hsp 47 is found to be a collagen-binding protein (5), hsp 32 and hsp 8 are identified as heme oxygenase and ubiquitin, respectively (6,7). In most cases, an increased expression of hsps is also accompanied by an increased tolerance to more extreme temperatures. This has been observed in a wide variety of plants, animals, fungi, bacteria (8,9) and algae (10).

Hsps are also induced by other forms of stresses including drugs, amino-acid analogs and heavy metals (11,12,13). Cellular exposure to cadmium (Cd) is known to elicit a number of responses in addition to metallothionein induction. A mechanistic understanding of these processes may allow the development of biological indicators of Cd exposure. Among the potential indicators of exposure and effects for Cd, stress proteins or hsps are good candidates.

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Although the phenomenon of increased synthesis of hsps upon subjection of a stress induced by Cd is well known, little or none is settled about the possible involvement of hsps in the maintenance of a resistant state to this metal.

In order to relate specific stress proteins to adaptation / protection in Cd-tolerant cells, we report here the characterization by two-dimensional gel electrophoresis of two hsps constitutively overexpressed in an achlorophyllous Cd-resistant *Euglena gracilis* cell line. This strain was selected in our laboratory and continuously subcultured for more than 5 years.

MATERIALS AND METHODS

Cell lines

Euglena gracilis W100 ZUL, a wild achlorophyllous mutant strain, was derived from the wild-type Z strain of *Euglena gracilis* Klebs. From this strain, Cd-resistant cells were selected by progressively elevating the Cd concentration from 500 µM to 2 mM in the culture medium. These selected cells were subcultured in 2mM Cd for 5 years. They were able to grow in the same way as done sensitive cells on a Cd free medium.

Cell culture and labeling

Euglena gracilis cells were grown at 23° C in lactate medium (14) with or without 500 µM Cd for the sensitive strain and with or without 2 mM Cd for the Cd-resistant one. Cells were labeled for 2 hours at the midlogarithmic phase of growth 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 culture 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% 3-10, 1.6% 5-7). Then, after 10 s. of sonication, protein samples were centrifuged for 3 min. at 6,000 g to release paramylum. Supernatants were analyzed by two-dimensional gel electrophoresis according to the method previously described (15). 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

In our culture conditions, growth curves of *Euglena gracilis* cells showed a short lag-phase during a few hours, followed by an exponential growth from day 1 to day 5, and then a plateau phase occured (not shown). According to these data, metabolic cell labelings were achieved at the midlogarithmic phase, i.e., the third day of culture. The response at the protein synthesis level of *Euglena gracilis* cells was analyzed by two-dimensional gel electrophoresis (NEPHGE). Equal amounts of protein were submitted to electrophoresis. The pattern of labeled polypeptides of *Euglena gracilis* cells grown in control conditions (23 °C, without Cd) is shown in Fig. 1. The area containing interesting spots was enclosed. To examine in more details the polypeptide spots, this area (Fig. 2 A) and those corresponding to other cell lines or culture and labeling conditions (Fig. 2 B-F) were enlarged.

Compared to the control (Fig. 2 A), the polypeptide pattern of heat-shocked (35 °C) *Euglena gracilis* cells (Fig. 2 B) showed two novel spots (arrows) designated a and b, in the basic region of the electrophoregram. These two spots were not detected in *Euglena gracilis* cells

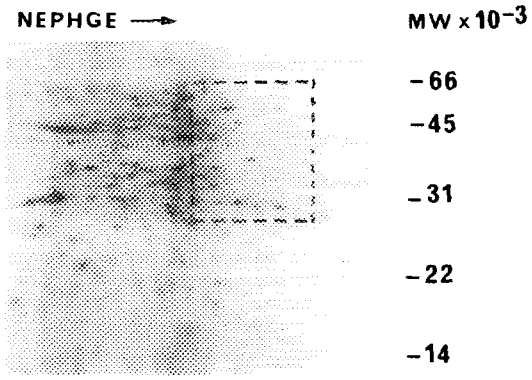

Figure 1. Two-dimensional gel electrophoresis (NEPHGE) of total cellular proteins synthesized in wild *Euglena gracilis* cells grown and labeled at 23 °C in lactate medium. Area containing relevant spots was enclosed.

cultured in 500 μM Cd for 3 days (Fig. 2 C). However, when Cd was added at this same concentration to a 3 days old culture at the outset of the labeling, these two spots became visible (Fig. 2 D). Finally, the spots a and b were detected in Cd-resistant *Euglena gracilis* cells growing and being labeled at 23 °C , with or without 2 mM Cd in the culture medium (Fig. 2 E, F). The apparent molecular weights of the polypeptides a and b were 55 kDa and 40 kDa, respectively.

DISCUSSION

Two polypeptides with apparent molecular weight of 55 kDa (p55) and 40 kDa (p40) are overexpressed in Cd-resistant *Euglena gracilis* strain (Fig. 2 F). These polypeptides have the same

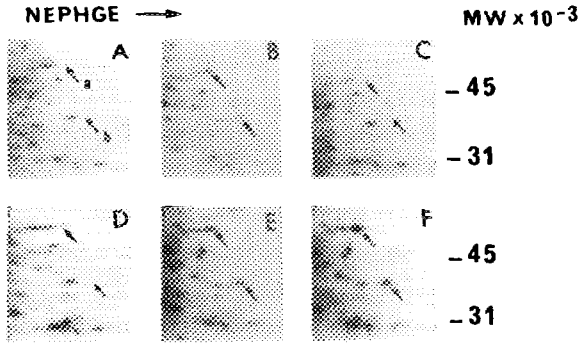

Figure 2. Enlarged area containing the altered spots (arrows) in :
A - wild *Euglena gracilis* cells grown and labeled at 23 °C in lactate medium (a: p55, b: p40).
B - wild *Euglena gracilis* cells grown at 23 °C and labeled at 35 °C in lactate medium.
C - wild *Euglena gracilis* cells grown in 500 μM Cd lactate medium for 3 days and labeled at 23 °C.
D - wild *Euglena gracilis* cells grown in lactate medium and labeled in 500 μM Cd lactate medium at 23 °C.
E - Cd-resistant *Euglena gracilis* cells grown and labeled at 23 °C in 2 mM Cd lactate medium.
F - Cd-resistant *Euglena gracilis* cells grown and labeled at 23 °C in lactate medium.

two-dimensional electrophoretic behaviour than two polypeptides whose synthesis levels increase in wild *Euglena gracilis* strain in response to elevated temperature (Fig. 2 B). On the basis of this observation, these polypeptides may represent the same or closely related molecules, i.e., hsps.

A lot of data indicate that various forms of stress, including heavy metal exposure, can induce synthesis of a discrete set of proteins analogous to hsps. In this respect, polypeptides p55 and p40 became detectable when 500 µM Cd is added just prior cell labeling at 23 °C (Fig. 2 D). In contrast, these polypeptides are not observed when 500 µM Cd is present for 3 days in the culture medium (Fig. 2 C), thus indicating that the Cd induction of p55 and p40 do not persist in wild *Euglena gracilis* cells. These data also suggest that 500 µM Cd induces a moderate stress which result in a transient response in *Euglena gracilis* wild strain. However the p55 and p40 polypeptides are detectable in Cd-resistant *Euglena gracilis* cells growing for 3 days in lactate medium with or without 2 mM Cd (Fig. 2 E, F). Consequently, this expression is not due to the presence of 2 mM Cd in the lactate medium. It is not also due to remaining trace amounts of Cd in culture medium: Cd-resistant cells being analyzed in Cd free culture medium conditions (Fig. 2 F) were previously subcultured every 3 days for 2 weeks in normal lactate medium. Moreover, eventual Cd accumulation into Cd-resistant cells is unlikely because no Cd was detected into these cells even when cultured with Cd (unpublished data). Finally, keeping in mind that Cd-resistant cells were subcultured for more than 5 years in 2 mM Cd lactate medium, it can be argued that chronic exposure to this more elevated Cd concentration may result in a sustained response to hsps. However, up to now, no data have reported a so long (2 weeks) kinetics of recovery for the stress response. Althought reports are contradictory in the literature, most researchers now estimate that hsps have a half-life of a few days in a number of organisms (1, 16).

Taken together, these data provide strong evidence that the polypeptides p55 and p40 are constitutively overexpressed in Cd-resistant *Euglena gracilis* cells. These polypeptides are also overexpressed in heat-shocked wild *Euglena gracilis* cells. This study raise the possibility that resistance to Cd of *Euglena gracilis* cells require changes in expression of hsps.

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