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METALLOTHIONEINS AND RESISTANCE TO CADMIUM POISONING IN DROSOPHILA CELLS

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SUMMARY : Toxicity of cadmium on Drosophila cell lines has been studied. Maximal tolerance for cadmium chloride is 10 µM. Metallothioneins are induced in Drosophila cells following cadmium addition. A stable cadmium resistant cell line (Cd R200) has been selected starting from the haploid D clone. The Cd R200 cells are diploid and display metallothionein levels 22 times higher than cells of the original line fully induced with cadmium. The 200 µM $CdCl_2$ tolerance upper limit in Cd R200 line is overcome if L-cysteine is supplemented to the medium. It is thus possible, in the presence of 5 mM L-cysteine, to select cells able to resist 800 µM $CdCl_2$. These cells produce 4 times more metallothioneins than Cd R200 cells.

Homeostasis of heavy metal ions and detoxification of cadmium or mercury poisoned cells are the two main physiological functions attributed to the small proteins called metallothioneins (MT). This class of proteins is characterized by an exceptionally high content (around 30%) of cysteine residues. They are known from vertebrates as well as from invertebrates (1). MT have been found in some insect species (2,3), but reports also suggest that they are replaced in other insect species by small glycoproteins with lower cysteine content (4,5). We have looked for MT in Drosophila melanogaster. The interest of this species arises from the possibility of using genetics in the study of MT function.

We have found a protein which has the characteristic properties of MT in Drosophila cells grown in the presence of 10 µM $CdCl_2$. Furthermore, we have selected a cell line able to grow in the presence of cadmium concentrations lethal for normal cells. These cells produce 22 times more MT than cells of the original line fully induced with cadmium. An even better resistance to cadmium poisoning was obtained by supplementing the culture medium with L-cysteine.

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MATERIALS AND METHODS

Drosophila melanogaster cell lines

Clone 89K is a diploid clone derived from the Kc line, established from Drosophila embryos (6). Cells are cultured as monolayers in plastic flasks at 23°C in D_{22} medium (7) supplemented with 5% decomplemented fetal calf serum (FCS).

Clone D is a stable haploid clone derived from the 1182-6 line (8). Line 1182-6 was established from lethal haploid embryos produced by the female sterile mutant mh 1182 (9). Cells are cultured in a mixture (half/half) of D_{22} and M3 medium (10) supplemented with 10% FCS.

Labelling of proteins

Cells were incubated in culture medium with 10 µCi [^{35}S] cysteine/ml (1080 Ci/mmol, Amersham) for 16 h.

Cadmium-binding proteins were detected by their capacity to bind $^{109}Cd^{++}$ ions. $^{109}CdCl_2$ (0.1 µCi, 87 Ci/mmol, Amersham) was added to the protein extract prior to chromatographic analyses.

Characterization of MT

Cells were homogenized in 10 vol of 100 mM ammonium formate, pH 8.8, 0.1% β-mercaptoethanol (0-4°C). The homogenate was centrifuged at 10,000 g for 15 min , then at 100,000 g for 6 h (4°C). The supernatant was heated to 65°C for 10 min, cooled to 0°C and centrifuged at 10,000 g for 15 min. The supernatant was concentrated by lyophilization, redissolved in 10 mM ammonium formate buffer, pH 8.8, 0.1% β-mercaptoethanol and fractionated on a Sephadex G-75 column (2.6 x 90 cm). Fractions were eluted in this buffer at a rate of 25 ml / h. Those fractions corresponding to MT were analysed on a DEAE-cellulose column (Whatman DE52) equilibrated with 10 mM Tris-HCl, pH 8.8 and eluted with a linear gradient of 10-500 mM Tris-HCl, pH 8.8.

Analytical acrylamide gel electrophoresis

Proteins were analysed on 20% acrylamide, 6 M urea, 0.1% SDS gels (11). Pellets of about 10^7 cells were lysed in 0.1 ml of ice-cold 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, 1% Triton X-100. The lysate was heated to 65°C for 10 min, then centrifuged at 15,000 g for 15 min. Carboxymethylation of thermoresistant proteins was performed as described by Hamer and Walling (12). Proteins were stained with 0.2% Coomassie blue dissolved in 45% methanol, 10% acetic acid after a 1 h fixing step in 10% trichloroacetic acid, 30% methanol, 10% acetic acid. Destained gels were soaked in Amplify (Amersham), dried and fluorographed at -70°C.

RESULTS

Cadmium toxicity on Drosophila cells

We have compared two cell clones for their ability to resist cadmium poisoning. One of these (89K) is a diploid clone while the other (D) is a stable haploid clone (at least 80% of haploid cells).

A concentration of 5 µM $CdCl_2$ has no visible effect on cell morphology and growth. A slight decrease in the rate of division was observed at 10 µM $CdCl_2$. All cells died within few days above 30 µM $CdCl_2$. Differences between the two clones became apparent at a concentration of 20 µM. In several trials the entire culture of clone 89K died. For clone D, some colonies of living cells appeared after three weeks.

Induction of MT in Drosophila cells

We have looked for MT synthesis in Drosophila cells poisoned by cadmium. We detected MT-like molecules using two different properties which are

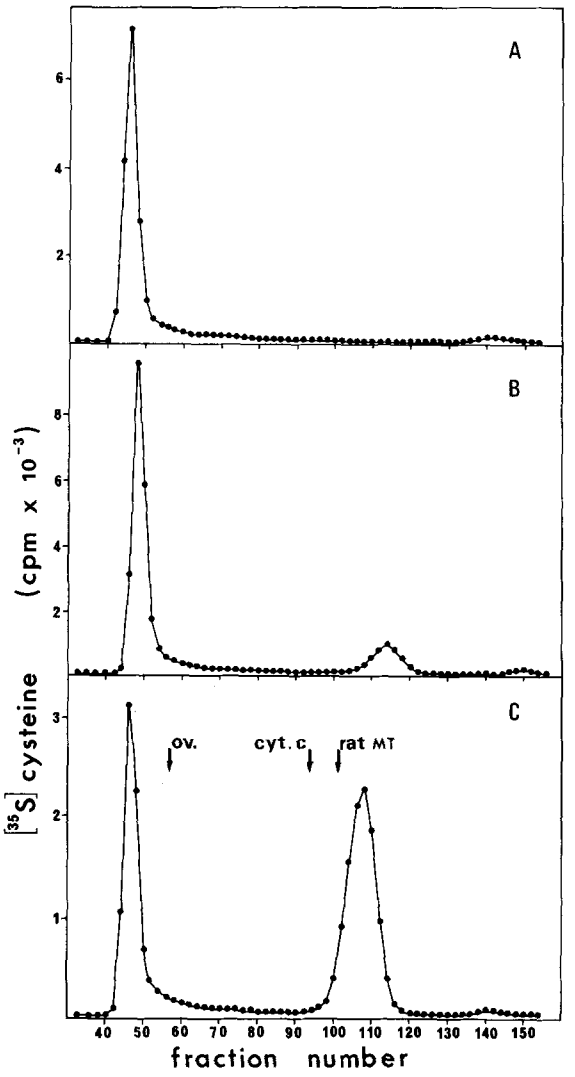

Figure 1. Sephadex G-75 elution profiles of [^{35}S]cysteine-labelled proteins from control D cells (A), D cells treated with 10 µM $CdCl_2$ for 32 h (B) and Cd R200 cells cultured in 200 µM $CdCl_2$ (C). Total proteins (no heat denaturation treatment) were first passed through a small Sephadex G-25 column in order to remove free[^{35}S]cysteine, then applied to the Sephadex G-75 column. Positions of ovalbumine, cytochrome c and rat MT were determined and are indicated in C. A residual amount of free[^{35}S]cysteine accounts for the small peak at positions 140-150. Fractions in B were slightly smaller, explaining the shift between the peaks in B and C.

characteristic of these proteins (1). Cells were labelled with[^{35}S]cysteine, which is known to be the major amino acid of MT. The capacity to bind cadmium ions is another criterium for identification.

Proteins extracted from control cells and from cadmium treated cells grown in[^{35}S]cysteine-supplemented medium were fractionated on Sephadex G-75 columns (Fig. 1). In cadmium poisoned cells (Clone D, 10 µM $CdCl_2$), 15% of

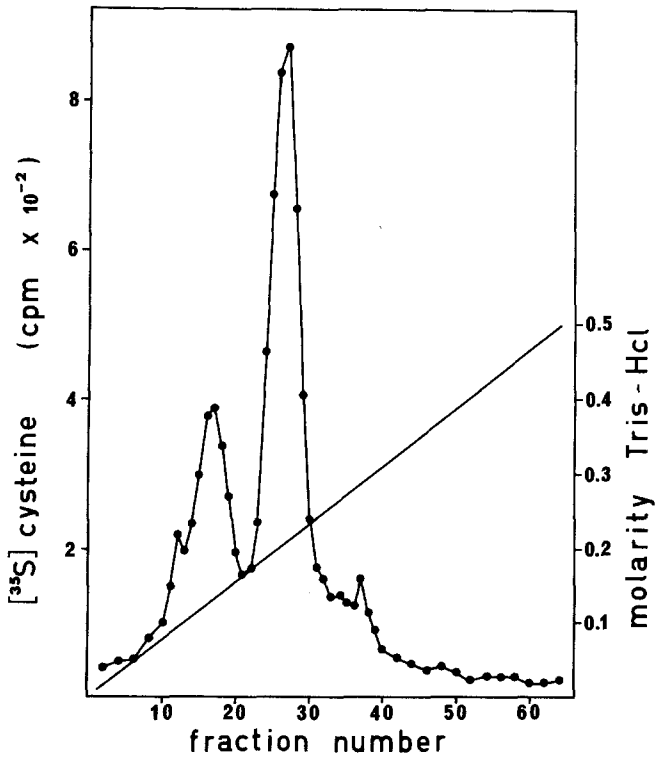

Figure 2. DEAE-cellulose chromatography of MT from Cd R200 cells. Proteins eluting at the expected position for MT (Fig. 1C, fractions 102-112) were pooled and lyophilized. Proteins were dissolved in 10 mM Tris-HCl, pH 8.8, then applied to a column (5 x 2 cm) of DE52 cellullose equilibrated with the same buffer. Elution was performed with a linear gradient of 10-500 mM Tris-HCl, pH 8.8.

$[^{35}S]$cysteine incorporated in total proteins is recovered in a protein fraction which elutes at the position expected for MT (Fig.1B). Rat MT was shown to elute slightly faster on a Sephadex column run in parallel. There is no radioactive peak at this position when proteins are extracted from control cells (Fig.1A). The characteristic peak of induced cells was further analysed by DEAE cellulose chromatography. As shown in Fig.2, two peaks were detected, eluting at a Tris concentration of 125 mM and 205 mM, respectively. This again is a chromatographic pattern typical of MT (1). We also obtained characteristic MT peaks when proteins were labelled in vitro by addition of $^{109}CdCl_2$ prior to the Sephadex or DEAE cellulose chromatographic analyses (not shown). MT levels in protein extracts can be determined with this technique by measuring the proportion of $^{109}Cd^{++}$ bound (see below). We found that a maximal level of MT in the cells induced continuously with 10 µM $CdCl_2$ is reached in about 32 h (not shown).

In mammals, glucocorticoids are known to induce MT (13,14). In insects, ecdysteroids play an essential role in regulating many biological processes. Several proteins are known to be induced by ecdysterone in Drosophila cell

lines (15). We have thus looked for an inductive effect of ecdysterone on the MT Drosophila system but were unable to detect any induction after three days of exposure to 10^{-7} M ecdysterone.

Selection for cadmium resistance

We tried to select cadmium-resistant lines by exposure of Drosophila cells to increasing levels of cadmium. Selection was initiated at 10 µM $CdCl_2$. As soon as the cells were capable of growth at a given cadmium concentration, cells were subcultured in a 1:5 dilution (20 x 10^6 cells / flask) with increments of 10 or of 20 µM $CdCl_2$ added to the medium. We obtained totally different results with the two lines tested.

a)Clone 89K

Cells were first grown in a medium containing 10 µM $CdCl_2$ for a few days and then, when confluent, shifted to a medium containing 20 µM $CdCl_2$. In all of our attempts, cells died and no resistant colonies were obtained.

b)Clone D

With this clone, resistant colonies appeared after a prolonged period of culture in the 20 µM $CdCl_2$ medium. We thus progressively increased the cadmium concentration and were able, after 140 days of selection, to isolate a cell population growing at a concentration of 200 µM $CdCl_2$ (Cd R200 cell line). It was not possible to select cells growing at a higher cadmium concentration.

MT synthesis in the Cd R200 line

Cysteine is the predominant amino acid of MT (1). We found that 54% of [^{35}S]cysteine incorporated into proteins of the Cd R200 line is recovered in the MT fraction (Fig.1C).[^{35}S]cysteine-labelled proteins of non induced D cells and Cd R200 cells have been compared on 20% acrylamide gels (Fig.3). MT is visible as a small molecular weight protein (around 7000 daltons) only in Cd R200 extracts. This protein appears as a faint band by Coomassie blue staining, but is the major radioactive component (Fig.3).

We have developped a system which enables us to quantify (in relative values) the amount of MT in Drosophila cells. When proteins are heated to 65°C for 10 min, most proteins precipitate and can be removed by a short centrifugation. We found that radioactive cadmium ($^{109}CdCl_2$) added to the remaining thermo-resistant proteins is taken up for more than 99% by MT (not shown). If these proteins are filtered on a small Sephadex G-25 column, all the radioactivity eluting in the excluded peak thus can be considered to be bound to MT. In Figure 4, 50 µg of thermally resistant proteins extracted from control cells (clone D, 0 µM $CdCl_2$), cadmium-induced cells (clone D, 10 µM $CdCl_2$, 32 h) and Cd R200 cells (200 µM $CdCl_2$) were compared for their ability to bind cadmium. Less than 1% of $^{109}Cd^{++}$ was found to bind to proteins in control cells (Fig.4A). In cadmium-induced and Cd R200 cells, 31%

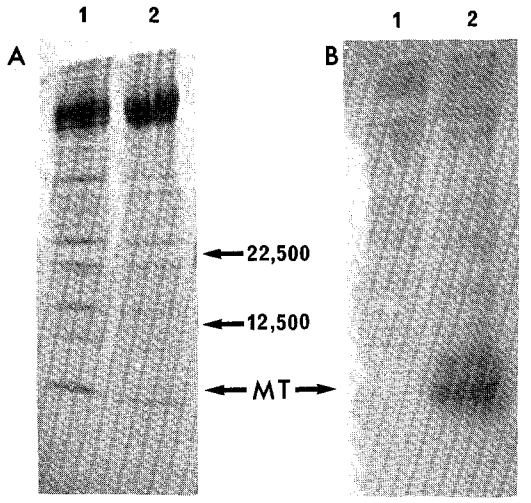

Figure 3. Analytical gel electrophoresis of Drosophila thermo-resistant proteins. [^{35}S] cysteine-labelled proteins were carboxymethylated and electrophoresed on 20% acrylamide, 6 M urea, 0.1 % SDS gels. Proteins of D cells (A1, B1) and Cd R200 cells (A2, B2) are compared. Left panel (A) : Coomassie blue staining. Right panel (B) : fluorography. Molecular weight markers are indicated by arrows (cytochrome c, 12,500 daltons and soybean trypsin inhibitor, 22,500 daltons).

and 74% respectively of radioactive cadmium was detected in the excluded peak (Fig.4B,C). A calibration of the system has been established to extrapolate

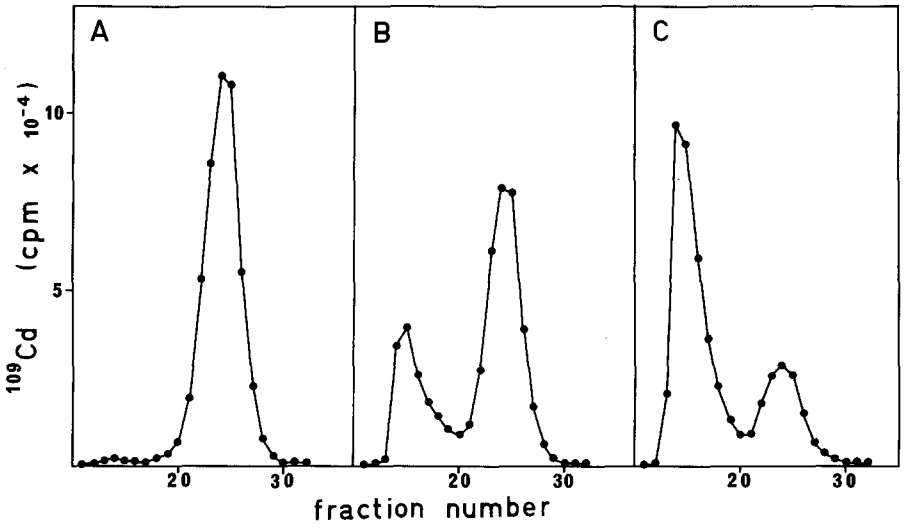

Figure 4. Quantification of MT in control D cells (A), 10 µM $CdCl_2$ induced D cells (B) and Cd R200 cells (C). Fifty µg of proteins extracted as described in Materials and Methods were dissolved in 200 µl of 100 mM ammonium formate, pH 8.8, 2.5 % β-mercaptoethanol , 0.1 µCi $^{109}CdCl_2$ (5×10^{-3} µM) and fractionated on a Sephadex G-25 column (55 x 0.7 cm) equilibrated in 100 mM ammonium formate, pH 8.8, 0.1% β-mercaptoethanol. Elution fractions : 0.75 ml. Excluded proteins elute in fractions 13-18 and free $^{109}CdCl_2$ in fractions 21-27.

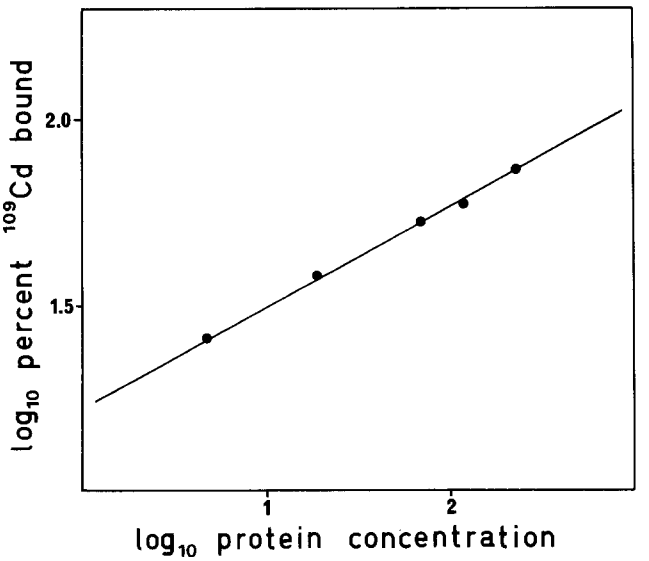

Figure 5. Calibration of the Sephadex G-25 columns shown in Fig.4. Increasing amounts (1, 5, 10, 25 and 50 µg) of proteins extracted from Cd R200 cells were dissolved in 200 µl of buffer as described in Fig.4 and chromatographed on Sephadex G-25 columns. The percent of ^{109}Cd excluded from the Sephadex (bound to MT, see the text) was plotted against protein concentrations ($\log_{10}$). Relative amounts of MT molecules in Drosophila cells have been calculated from these data (Table 1).

these results in quantitative terms (Fig.5). It can thus be calculated that Cd R200 cells contain 22 times more MT than fully cadmium-induced D cells.

Stability for cadmium resistance

The stability of cadmium resistance has been tested by culturing Cd R200 line in the absence of cadmium for two months. Cells were then shifted back to the 200 µM $CdCl_2$ medium. We found that these cells survive and synthesize as much MT as cells of the Cd R200 line. Resistance to cadmium poisoning thus appears to be a stable phenotype.

The karyotype of the Cd R200 cell line was checked. These cells, selected from the haploid D clone, are now perfectly diploid. We did not detect any minute chromosome or specific changes in the karyotype.

Selection for super-resistant cells

The inability to select cells resistant to concentrations superior to 200 µM $CdCl_2$ could be due to a limited availability of cysteine (endogenous level of cysteine in our medium is about 1 mM). To test this possibility, we have checked the effect of L-cysteine on the growth of Drosophila cells. For cells grown in normal medium without cadmium (clones 89K and D), the addition of 2 mM cysteine to the medium appears to be highly toxic. A cysteine level of 5 mM is lethal. In the case of the Cd R200 line, grown in the presence of 200 µM $CdCl_2$, there is no lethal effect of cysteine at 5 mM. Furthermore, the growth rate is greatly increased if the cells are grown in medium

supplemented with 3 mM cysteine. In these conditions, it is possible to increase the cadmium chloride concentration to 400 µM. Renewed selection experiments using these cells produced, after 5 steps, cells capable of growing in medium supplemented with 5 mM L-cysteine and 800 µM $CdCl_2$. As shown in Table 1, these cells synthesize 4 times more MT when compared to cells growing in the 200 µM $CdCl_2$ medium.

DISCUSSION

We show in this paper that cadmium chloride can induce a Drosophila melanogaster cell line to synthesize proteins possessing the properties of MT, i.e. of small molecular weight, with strong incorporation of cysteine, and the capacity to bind cadmium (Figs.1 and 2). Kinetics of MT accumulation in the cells shows that a maximal level is reached around 32 h after continuous induction. Similar results have been obtained with cadmium-induced mouse cells (16).

As in other systems studied so far (1), two MT peaks were recovered following DEAE-cellulose chromatography (Fig. 2). Relative proportions of these two peaks is highly variable in different experiments. It is so far difficult to state if these two peaks represent true molecular species or are due to artifactual modifications of a single MT form. We also detected MT in adults and larvae of D.melanogaster. Amino acid composition of their predominant form is characteristic of MT: i.e. the absence of aromatic amino acids and a high cysteine content (Wegnez, manuscript in preparation). As shown in Fig.1, rat MT (61 residues, ref. 17) elutes from Sephadex G-75 significantly faster than Drosophila MT. It is also known that the two MT isoforms of the crab Scylla serrata consist of 57 and 58 amino acids, respectively (18). A common feature of MT from arthropods thus could be a smaller size relative to MT from vertebrates.

Cell lines resistant to cadmium poisoning have been obtained in vertebrates (14,19,20). We also selected a line (Cd R200) able to resist high cadmium concentrations. The resistant Cd R200 line shows a duplication of the original chromosome set as was found for the resistant mouse cell line (19). The maximal tolerance for cadmium in Cd R200 line is 200 µM. One of the many reasons which can be proposed to explain this plateau value is that cysteine is a limiting factor. Cysteine is the major component of MT as it usually makes up 30% of the total amino acids of these proteins (1). We have shown that the mere addition of cysteine to the medium allows the Cd R200 line to grow at a 400 µM $CdCl_2$ concentration. This could be explained by the binding of cadmium ions to cysteine molecules in the medium, resulting in an apparent higher resistance. However the cells growing in 800 µM $CdCl_2$ with 5 mM cysteine actually possess 4 times more MT molecules as compared to the

Table 1. Quantification of MT in Cd R200 cells cultured in cysteine-supplemented medium. Thermo-resistant proteins (5 µg) were dissolved in 200 µl of formate buffer containing $^{109}CdCl_2$ and analysed as described in Fig.4. Levels of MT were calculated using the calibration shown in Fig.5.

Culture conditions		
$CdCl_2$ (µM)	L-cysteine (mM)	MT levels (relative values)
200	0	1.0
200	2	1.2
400	3	2.3
500	3	1.8
800	5	4.2

Cd R200 line grown in 200 µM $CdCl_2$ (Table 1). The availability of a high cysteine pool appears to be a critical requirement in establishing cadmium super-resistant cell lines.

Cadmium resistance in vertebrate cells has been correlated with a small amplification of MT genes (14,19). Cloning of the Drosophila MT cDNA is in progress and will allow us to see if a similar mechanism accounts for the results we obtained with the Cd R200 line.

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