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# INTRACELLULAR COPPER TRANSPORT IN CULTURED HEPATOMA CELLS

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The distribution of copper in lysates prepared anaerobically from copper-resistant hepatoma cells radiolabeled with $^{67}Cu$ was examined in pulse-chase experiments. Initially, the majority of the radioactivity (>85%) coeluted with copper-metallothionein. As the chase time increased there was a gradual loss of $^{67}Cu$ from metallothionein, with a concomitant increase in the level of $^{67}Cu$-labeled glutathione. There was also an increase in $^{67}Cu$ incorporation into superoxide dismutase. These results suggest that the chelation of copper by metallothionein from a copper-glutathione complex (Freedman, J. H., Ciriolo, M. R., and Peisach, J. (1989) J. Biol. Chem. 264, 5598-5605) is a reversible process. Further, they demonstrate that the copper bound to metallothionein is not permanently sequestered, but can be incorporated into other copper proteins. 

Metallothionein is a member of a class of low molecular weight, cysteine-rich proteins capable of binding a variety of transition metal ions (for a review see ref. 1). Although this protein is present in all mammals (1) and in some invertebrates as well (2, 3), its role *in vivo* remains under debate. It has been suggested that the primary function of MT is metal detoxification (4). This proposal is based on the high affinity of MT for transition metals (5), and the observations that heavy metals are strong inducers of MT gene transcription (6, 7), and that Cd/Zn-MT is excreted in the kidney (8). Other detoxification roles ascribed to MT include hydroxyl radical scavenger (9), chelator of cis-platinum drugs (10, 11), and mediator of cellular resistance to the toxic effects of alkylating agents (12, 13), X-rays (14, 15) and ultraviolet radiation (16).

In contrast to its role as a detoxifying protein, it has also been proposed that MT is an essential component of intracellular copper, and probably zinc, metabolism (17, 18,

The abbreviations used are: GSH, reduced glutathione; GS-Cu, copper-glutathione complex; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MT, metallothionein; $M_r$, apparent molecular weight; PBS, phosphate buffered saline; FPLC, fast protein liquid chromatography.

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19). Metallothionein may help maintain copper homeostasis by serving as a temporary storage site for metal (17, 19). The copper complexed by MT could then be donated to other proteins, either following proteolytic degradation in lysosomes (20) or by exchange via glutathione complexation (21). These proposed mechanisms are contradicted by the observation that *in vitro*, Cu-MT does not donate metal to apo-forms of other copper proteins, such as superoxide dismutase and tyrosinase (22, 23), unless the metal is first released from the protein by the oxidation of copper and/or cysteine ligands.

In order to assess the role of MT in copper metabolism a series of hepatoma cell lines resistant to copper toxicity were utilized (24). These cells accumulate copper and produce MT at levels proportional to their cellular copper concentration (25). Resistant cells also have up to four times more glutathione than the parental wild type cell line. A majority of the cytoplasmic copper in both wild type and resistant cells was found as a GS-Cu complex (21). These observations, and the finding that *in vivo*, copper is complexed to GSH before it is bound to MT, suggested that GSH chelates cytoplasmic copper and then transfers the metal to MT for storage (21). In the present study, the reverse pathway is described; the *in vivo* transfer of copper from MT to GSH and superoxide dismutase. The results of this investigation further support the *in vivo* role of MT as a temporary depot for cellular copper.

## MATERIALS AND METHODS

*Materials*--All media and media supplements were obtained from Grand Island Biological Co. HEPES, Ultrol grade, was from Calbiochem. Cupric acetate (metal purity 99.99%) and EDTA were purchased from Mallinckrodt Chemical Works. Carrier free ($^{67}$Cu)cupric chloride, in 2 N HCl, was prepared at Los Alamos National Laboratory, as previously described (26). At the time of shipment the stock solution contained 0.4 $\mu$M $^{67}$Cu with a specific activity of 105 Ci/$\mu$mol.

*Cells and Tissue Culture*--The copper-resistant hepatoma cell line, $HAC_{600}$, was maintained in complete RPMI-1640 medium in 10.0 mM HEPES buffer, pH 7.4, supplemented with 10% fetal bovine serum, nonessential amino acids, 2.0 mM glutamine, penicillin G (10 units/ml), streptomycin (10 $\mu$g/ml), and 600 $\mu$M Cu(II)-(histidine)$_2$ (24). Under standard conditions, cells were incubated at 37 °C in a 5% $CO_2$:95% air atmosphere, in polystyrene tissue culture dishes.

*Intracellular Distribution of Copper*--The kinetics of the intracellular distribution of copper was determined by pulse-chase experiments. $HAC_{600}$ cells were grown on 35-mm plates for 24 h, under standard conditions. After this time, cells were washed twice with sterile, 37 °C PBS and then incubated for 2 h in the presence of 8 x $10^7$ cpm of $^{67}$Cu (~ 0.6 nmol) in RPMI-1640, containing 1% fetal bovine serum. At the end of this incubation the $^{67}$Cu-containing medium was removed and the cells were washed three times with 37 °C PBS-1.0 mM EDTA. 2-ml of complete medium, containing 600 $\mu$M Cu(II), was added and the cells were then incubated for 1 to 20 h under standard conditions. The medium was then removed, the cells were washed three times with ice cold PBS-EDTA, and 0.5 ml of 10.0 mM HEPES buffer, pH 7.4, was added. Cells were then removed by gentle pipetting, then transferred to a 1.5 ml microcentrifuge tube, and pelleted by centrifugation at 14,000 x g for 10 s. Cell pellets were suspended in 0.5 ml HEPES buffer and then lysed by sonication. The lysate was centrifuged for 15 min at 14,000 x g, and the supernatant was collected and stored at -70 °C.

To determine the intracellular distribution of copper, a 0.5 ml aliquot of the supernatant was fractionated under anaerobic conditions (21) by gel filtration FPLC using a 1.6 x 50 cm Superose 12B FPLC column equilibrated with 10.0 mM HEPES-0.15 M sodium chloride buffer, pH 7.4, purged for 30 min with ultra-high purity helium (Linde). Proteins were eluted at a flow rate of 1.5 ml/min, and 1.0 ml fractions were collected. The $^{67}$Cu content in each fraction was measured using an LKB Labgamma counter.

Chromatographic data were normalized to account for differences in the amounts of $^{67}$Cu and protein applied to the column. Protein concentration was determined by the method of Lowry, *et al* (27) using bovine serum albumin as a standard. The elution volumes for Cu/Zn-superoxide dismutase, Cu-MT and GS-Cu were identified as previously described (21). The level of $^{67}$Cu incorporation into these components was determined by integrating the area under the corresponding peaks in the normalized elution profile. The percent of $^{67}$Cu coeluting with these components was calculated by comparing the areas under the respective peaks to the total integrated area of the chromatogram.

## RESULTS AND DISCUSSION

In the model previously proposed outlining the pathways of cellular copper uptake and incorporation into proteins (21), it was suggested that copper taken up by cells is quickly chelated by GSH, forming a GS-Cu complex. This complex would function as an intracellular copper transport agent. The metal in GS-Cu would then be transferred to MT, where it is stored. It was also proposed that GSH-bound copper could be incorporated into other copper-proteins, such as superoxide dismutase and cytochrome oxidase. Previous results suggested that the transfer of copper between GSH and MT is a reversible reaction (21). Consequently, copper bound to MT should be available for incorporation into other copper proteins.

When copper-resistant cells, $HAC_{600}$, were labeled for 2 h with $^{67}$Cu, the majority (85%) of the radioactivity coeluted with Cu-MT (68-78 ml, peak $M_r$ = 13,000) (Fig. 1). Less than 10% of the $^{67}$Cu eluted in the void volume (32-40 ml, peak $M_r$ = 1,000,000) and complexed to GSH (78-89 ml, peak $M_r$ = 4,500). Less than 1% of the radioactivity coeluted with superoxide dismutase (58-62 ml, peak $M_r$ = 33,000). The distribution of radioactivity in the cell lysates in this experiment is comparable to that previously observed in hepatoma cells continuously labeled with $^{67}$Cu (21).

When resistant cells were removed from the $^{67}$Cu-containing medium and then incubated in medium containing nonradioactive copper, there was a small initial increase in $^{67}$Cu-labeled MT (Fig. 1). The level of $^{67}$Cu-MT then gradually decreased from 89% of the total $^{67}$Cu in the lysate, to 15% (Fig. 1). In contrast, the amount of $^{67}$Cu bound to GSH increased from 5% to 70%, a value similar to the steady-state level of GS-Cu previously observed in $HAC_{600}$ cells (21). These results suggest that MT-bound copper is released from the protein and is complexed by GSH. Further this indicates that the chelation of copper by MT from GS-Cu is a reversible process, as previously suggested (21).

In addition to an increase in the level of GS-$^{67}$Cu, there was a gradual increase in $^{67}$Cu-labeled superoxide dismutase (Fig. 2). The rate of $^{67}$Cu incorporation into superoxide

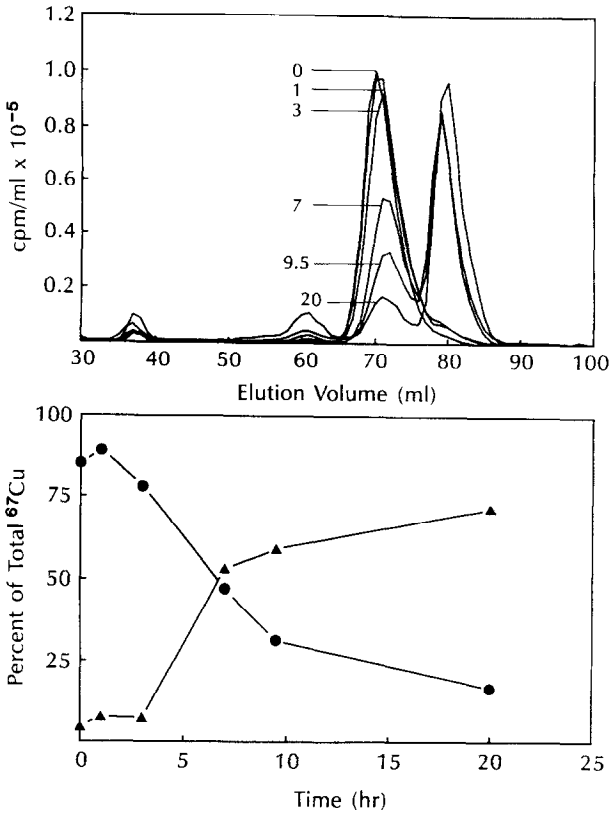


Figure 1. **Pulse-chase of $^{67}$Cu into subcellular pools in copper-resistant cells.** Resistant cells ($HAC_{600}$) were labeled with $^{67}$Cu for 2 h and then incubated in medium containing 600 μM nonradioactive copper for 0, 1, 3, 7, 9.5, and 20 h. Lysates were prepared and then fractionated by gel filtration FPLC in 10.0 mM HEPES-0.15 M sodium chloride buffer, pH 7.4, as described under "Materials and Methods." The chromatograms (upper panel) have been normalized to account for differences in the amount of $^{67}$Cu and protein applied to the column. Individual data points have been removed from the plot for clarity. The lower panel presents the kinetics of the distribution of $^{67}$Cu in Cu-MT (•) and GS-Cu (▲) as a function of incubation time in the presence of nonradioactive copper. The "percent of total $^{67}$Cu" was determined by comparing the integrated area under the peaks corresponding to GS-Cu and Cu-MT to the total integrated area of the chromatogram.

dismutase during the pulse-chase experiment was comparable to that previously observed for resistant cells incubated for up to 12 h in $^{67}$Cu containing medium (21). The slow production of $^{67}$Cu-labeled superoxide dismutase, compared to the increase in the level of GS-$^{67}$Cu or the loss of radioactivity from MT, may reflect the rate of superoxide dismutase synthesis in these cells. It may also be an indication of the rate of copper incorporation (presumably from the GS-Cu complex) into apo-superoxide dismutase.

The results of these pulse-chase experiments demonstrate that copper bound to MT is not permanently sequestered, but can be removed and then incorporated into other proteins. These observations contrast those obtained with liver supernatants of rats injected with $^{67}$Cu (28). In this study, when livers were isolated shortly after copper injection, the majority of the $^{67}$Cu was complexed to an 11 kDa, sulfhydryl-rich protein, now believed to

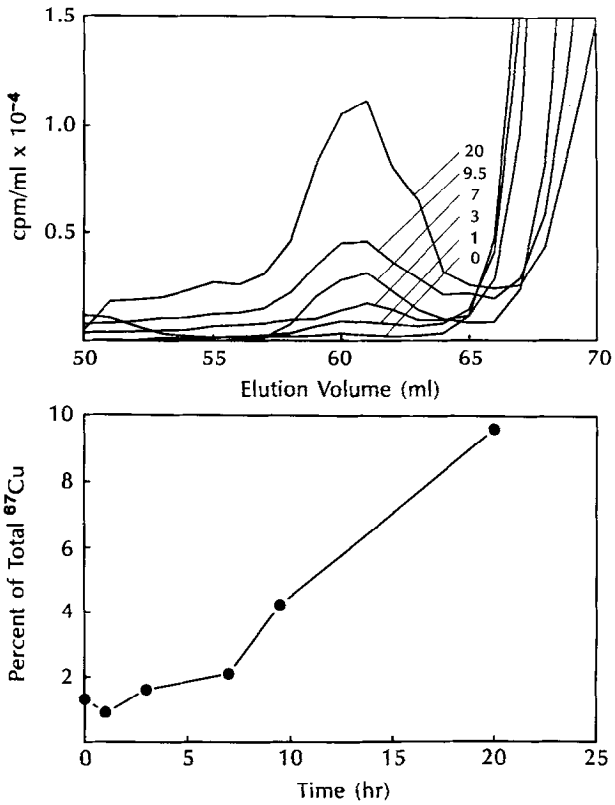


Figure 2. **Pulse-chase of $^{67}$Cu from Cu-MT into superoxide dismutase.** The figure (upper panel) presents an enlargement of the region of the chromatogram shown in Fig. 1 in which superoxide dismutase elutes (58 - 62 ml). Samples were prepared and chromatographed as described under "Materials and Methods." The effect of chase time on the amount of $^{67}$Cu incorporated in to superoxide dismutase is shown in the lower panel.

be MT. At longer incubation times, the level of $^{67}$Cu in the 11 kDa protein decreased with a concomitant increase in the radioactivity in a 31 kDa protein, presumably Cu/Zn-superoxide dismutase (28).[1]

Two mechanisms for the release of cellular copper from MT have been previously described, one where copper is released subsequent to Cu-MT incorporation and degradation in lysosomes (18, 20), and the other where the metal may be removed by direct chelation with GSH (21). In the pulse-chase experiment described above, both mechanisms may be operative. During the time course of the experiment, $^{67}$Cu-labeled MT may have become degraded in lysosomes, releasing $^{67}$Cu which could then be complexed by GSH and/or superoxide dismutase. However, there was a 50% decrease within 10 h in the level of $^{67}$Cu-MT in copper-resistant hepatoma cells, while the $t_{1/2}$ for Cu-MT has been shown to

[1]Under the isolation and fractionation conditions used in this study (i.e. aerobic and in Tris buffer) GS-$^{67}$Cu would not have been detected (21).

be greater than 18 h (20)[2]. These result suggest that in addition to proteolytic lysosomal digestion of $^{67}$Cu-MT another process is responsible for the release of $^{67}$Cu from the protein.

The ability of Cu-MT to donate copper to other copper proteins, *in vitro*, has been previously examined. The direct transfer of copper from yeast Cu-MT to laccase (29) and stellacyanin (30) was demonstrated. In contrast it was concluded in subsequent studies that the oxidation of copper-cysteine bonds in Cu-MT was required before the metal could be complexed by apo-tyrosinase and apo-hemocyanin (23). Similarly, Geller and Winge (22) showed that rat liver Cu-MT had to be oxidized in order to reconstitute apo-superoxide dismutase (22). The later results suggested that the copper bound to MT could not be directly transferred to other copper proteins, possibly due to the high stability of the metal-thiolate cluster (22). The pulse-chase experiments, however, showed that *in vivo*, $^{67}$Cu can be transferred from MT to GSH and superoxide dismutase (Fig. 1 and 2). Oxidation of the hepatoma $^{67}$Cu-MT during the preparation of cell lysates or chromatography would not account for this finding. If copper transfer were due to $^{67}$Cu-MT oxidation (i.e., an artifact of sample preparation) then the amounts of GS-$^{67}$Cu and $^{67}$Cu-superoxide dismutase detected would be independent of time the cells were incubated in nonradioactive copper.

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## REFERENCES

1. Kägi, J. H. R., and Kojima, Y. (1987) Exper. Suppl. (Basel) 52, 25-61
2. Lerch, K., Ammer, D., and Olafson, R. W. (1982) J. Biol. Chem. 257, 2420-2426
3. Lastowski-Perry, D., Otto, E., and Maroni, G. (1985) J. Biol. Chem. 260, 1527-1530
4. Webb, M. (1987) Exper. Suppl. (Basel) 52, 109-134
5. Nielson, K. B., Atkin, C. L., and Winge, D. R. (1985) J. Biol. Chem. 260, 5342-5350
6. Durnam, D. M., and Palmiter, R. D. (1981) J. Biol. Chem. 256, 5712-5716
7. Palmiter, R. D. (1987) Exper. Suppl. (Basel) 52, 63-80
8. Lee, Y. H., Shaikh, Z. A., and Tohyama, C. (1983) Toxicology 27, 337-345
9. Thornalley, P. J., and Vašák, M. (1985) Biochim. Biophys. Acta 827, 36-44
10. Kelly, S. L., Basu, A. Teicher, B. A., Hacker, M. P., Hamer, D. H., and Lazo, J. S. (1988) Science 241,1813-1815
11. Zelazowski, A. J., Garvey, J. S., and Hoeschele, J. D. (1984) Arch. Biochem. Biophys. 229, 246-252
12. Tobey, R. A., Enger, M. D., Griffith, J. K., and Hildebrand, C. E. (1982) Toxicol. Appl. Pharm. 64, 72-78
13. Endersen, L., and Rugstad, H. E. (1987) Exper. Suppl. (Basel) 52, 595-602
14. Matsubara, J., Tajima, Y., and Karasawa, M. (1987) Radiat. Res. 111, 267-275
15. Matsubara, J., Tajima, Y., and Karasawa, M. (1987) Environ. Res. 43, 66-74
16. Lieberman M. W., Beach, L. R., and Palmiter, R. D. Cell 35, 207-214

[2]The $t_{½}$ for Cu-MT in the copper-resistant hepatoma cells is greater than 24 h (J. H. Freedman, unpublished observation).

17. Cousins, R. J. (1985) Physiol. Rev. 65, 238-309
18. Bremner, I. (1987) J. Nutr. 117, 19-29
19. Bremner, I. (1987) Exper. Suppl. (Basel) 52, 81-107
20. Mehra, R. H., and Bremner, I. (1985) Biochem. J. 227, 903-908
21. Freedman, J. H., Ciriolo, M. R., and Peisach, J. (1989) J. Biol. Chem. 264, 5598-5605
22. Geller, B. L., and Winge, D. R. (1982) Arch. Biochem. Biophys. 213, 109-117
23. Beltramini, M., and Lerch, K. (1982) FEBS Lett. 142, 219-222
24. Freedman, J. H., Weiner, R. J., and Peisach, J. (1986) J. Biol. Chem. 261, 11840-11848
25. Freedman, J. H., and Peisach, J. (1989) Biochim. Biophys. Acta in press
26. Stockert, R. J., Grushoff, P. S., Morell, A. G., Bentley, G. E., O'Brien, H. A., Scheinberg, I. H., and Sternlieb, I. (1986) Hepatology 6, 60-64
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
28. Terao, T., and Owen, Jr., C. A. (1973) Am. J. Physio. 224, 682-686
29. Morpurgo, L., Hartmann, H.-J., Desideri, A., Weser, U., and Rotilio, G. (1983) Biochem. J. 211, 515-517
30. Hartmann, H.-J., Morpurgo, L., Desideri, A., Rotilio, G., and Weser, U. (1983) FEBS Lett. 152, 94-93