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GLUTATHIONE CONJUGATION AND INDUCTION OF A 32,000 DALTON STRESS PROTEIN*

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Abstract—Chinese hamster ovary cells were exposed to various concentrations of diethylmaleate in order to produce various levels of intracellular glutathione (GSH) depletion. Exposure to a 20 μM concentration or more of diethylmaleate depleted the intracellular glutathione concentration by 80% or more and resulted in enhanced synthesis of two 32 kDa proteins which exhibited a pI of about 6.5. Exposure of cells to 50 μM buthionine sulfoximine for 24 hr reduced GSH levels by 95% but did not enhance the synthesis of this protein. Addition of diethylmaleate to buthionine sulfoximine-treated cells resulted in enhanced synthesis of the 32 kDa protein however. Exposure to 0.4 mM diamide triggered the synthesis of several heat shock proteins but did not induce the synthesis of the 32 kDa protein. These results indicated that enhanced synthesis of the 32 kDa protein occurred only after glutathione depletion exceeded 80% and required formation of a glutathione conjugate.

Exposure of eukaryotic cells to heat shock induces or enhances the synthesis of four stress proteins having molecular weights of 110, 90, 70, and 22 kDa [for review see Ref. 1]. Exposure to thiol binding species such as copper, cadmium, zinc, mercury, sodium arsenite, iodoacetamide or *p*-chloromercuribenzoate also enhances the synthesis of the 110, 90 and 70 kDa proteins as well as induces the synthesis of a 30–34 kDa protein [2–4]. Glucose starvation strongly enhances the synthesis of a set of proteins known as the glucose-regulated proteins (GRP)‖ having molecular weights of 97 and 76 kDa, while weakly enhancing the synthesis of a 175 and a 34 kDa protein [5]. The ionophore A23107 enhances the synthesis of the 97 and 76 kDa glucose-regulated proteins but not the 30–34 kDa ones [4]. Caltabiano *et al.* [4] have shown that the 30–34 kDa protein induced by sodium arsenite fails to incorporate [^{3}H]mannase or [^{35}C]cysteine. The synthesis of this protein is inhibited by exposure to actinomycin D or cycloheximide. Low concentrations of sodium arsenite (e.g. 6 μM) induce this protein but not HSP 90 or 70. Higher concentrations of arsenite which induce HSP 90 or 70 (e.g. 100 μM) fail to induce the 30–40 kDa protein. The cadmium-induced 30–34 kDa protein has an observed pI between pH 6.0 and 6.3 as measured by isoelectric focusing [6].

Shelton *et al.* [7] have investigated the induction of a 30–34 kDa stress protein which they called SH-30. They found that it is induced by 1-chloro-2,4-dinitrobenzene or diethylmaleate (DEM) but not by buthionine sulfoximine (BSO) or *N*-ethylmaleimide (NEM). These results were interpreted to indicate that glutathione (GSH) conjugation may be the induction signal but that the level of GSH depletion was not important. Whether the SH-30 protein is the and 6) or in leucine-free medium containing 20 μCi/ starvation has yet to be established, however.

This present investigation will provide evidence that two criteria have to be met before synthesis of the SH protein is enhanced. The first is that GSH depletion had to exceed 80% and the second was the formation of a GSH conjugate.

MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells, growing exponentially in monolayer cultures, were maintained at 37° and at pH 7.4 in McCoy's 5A medium supplemented with 10% fetal bovine serum, sodium bicarbonate (2.2 g/liter), penicillin G sodium (100 units/ml), and streptomycin sulfate (100 mg/ml).

DEM, greater than 99.9% pure, was dissolved in DMSO. The DEM/DMSO solution was then added to the flasks containing growth medium. The final concentration was 100 μM DEM and 0.5 mM DMSO. Buthionine sulfoximine and diamide were both dissolved in Dulbecco's saline. DMSO (0.5 mM) was added to some of the flasks as a vehicle control. The pH of the medium after drug addition was 7.2 to 7.4.

Glutathione was measured from cells rinsed twice with Dulbecco's saline and lysed in 10% perchloric

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‖ Abbreviations: BSO, buthionine sulfoximine; CHO, Chinese hamster ovary; DEM, diethylmaleate; DMSO, dimethyl sulfoxide; GSH, glutathione; GRP, glucose-regulated proteins; HSP, heat shock protein; and NEM, *N*-ethylmaleimide.

acid. Glutathione concentrations were determined using high performance liquid chromatography [8].

Protein synthesis was measured by labeling the cells (4×10^6 per flask) in 3 ml of methionine-free medium containing 20 μCi/ml of [^{35}S]methionine (sp. act. approximately 1100 Ci/mmol; Figs. 2, 3, 4, and 6) or in leucine-free medium containing 20 μCi/ml [^{3}H]leucine (sp. act. approximately 144 Ci/mmol). After the labeling period, the cells were rinsed twice with 0.15 M NaCl, 10 mM 3-morpholinepropanesulfonic acid (pH 7.4) and scraped into 1 ml of the saline solution. A replicate was used for protein determination. For one-dimensional electrophoresis, cellular protein was dissolved in sample buffer (62 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol and 0.0025% bromphenol blue) and heated at 95° for 5 min. Proteins were electrophoretically separated using 0.75 mm thick, 9.5% polyacrylamide gel slabs with a 4% acrylamide stacking gel in the discontinuous buffer system of Laemmli [9]. Protein molecular weight was determined by comparison to molecular weight standards (myosin, 205,000; β-galactosidase, 116,000; phosphorylase *b*, 97,400; bovine serum albumin, 66,000; egg albumin, 45,000; and carbonic anhydrase, 29,000). After electrophoresis, the gels were fixed (50% methanol, 10% acetic acid), stained with Coomassie brilliant blue, destained, treated for fluorography using EnHance (New England Nuclear, Boston, MA), dried, and exposed to Kodak SB-5 X-ray film at −70°.

Two-dimensional electrophoresis was performed using the technique of O'Farrell [10] as described by Pollard [11]. A rinsed cell pellet was resuspended in 100 μl of sonication buffer (4°) and then sonicated. Solid urea was added (1 mg/ml) and then an equal volume of lysis buffer. Cellular protein was loaded onto prefocused gels and run at 400 V, constant voltage for 18 hr at 20°. The pH was measured from slices obtained from a replicate gel. Proteins were separated by molecular weight using 1.5-mm thick, 9.5% polyacrylamide slab gels with a 5% stacking gel. Samples were electrophoretically separated at 30 mA/gel, constant current at 20°. After electrophoresis, the slabs were processed for fluorography as described above.

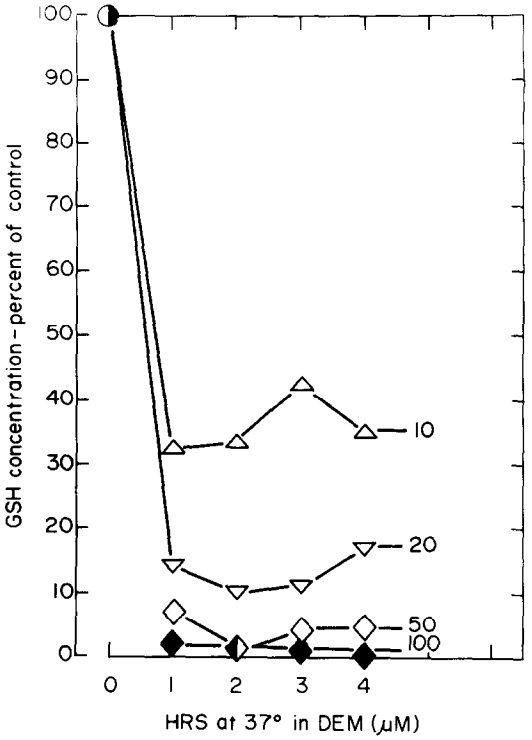

Fig. 1. Intracellular GSH concentration as a function of DEM concentration. Cells were exposed to the indicated concentration of DEM/DMSO at time zero and then incubated at 37°. The GSH concentration was measured at 1, 2, 3 or 4 hr by addition of 10% ice-cold perchloric acid to cells washed twice with PBS. Key: (○) % of GSH in cells exposed to 0.5 mM DMSO for 4 hr; (●) % of GSH in untreated controls; (△) cells exposed to 10 μM DEM; (▽) cells exposed to 20 μM DEM; (◇) cells exposed to 50 μM DEM; and (◆) cells exposed to 100 μM DEM. The GSH concentration in untreated controls was 69 ± 5 nmol/mg protein (mean ± SE, N = 3). This figure was derived from two experiments. In each experiment, there were two independent measurements per point.

RESULTS

Figure 1 illustrates that exposure to various concentrations of DEM produced various levels of GSH depletion. Furthermore, the degree of depletion remained relatively constant over the experimental time course.

Figure 2 represents the pattern of protein synthesis measured in CHO cells after exposure to 100 μM DEM for up to 5 hr. For this experiment cells were incubated at 37° for the indicated durations; then the DEM was washed out and the cell was labeled for 1 hr at 37° with [^{35}S]methionine. After 2 hr of exposure to DEM, the synthesis of a 32 kDa protein was observed. No other changes in the pattern of protein synthesis were apparent. Lanes (5 + BSO) and (5′ + BSO) represent protein synthesis in cells simultaneously exposed to 100 μM DEM and 50 μM BSO for 5 hr at 37°. The DEM/BSO was then washed out. In lane (5′ + BSO) fresh BSO (50 μM) was added back. Cells were then labeled for 1 hr with [^{35}S]methionine. This experiment was interpreted to mean that addition of DEM induced the synthesis of a protein having an apparent molecular weight of 32 kDa (p 32) and that addition of BSO did not prevent its synthesis.

Next, cells were exposed for 3 hr to various concentrations of DEM ranging from 10 to 100 μM. Figure 3 represents the pattern of protein synthesis. As can be seen, exposure to 100, 75, or 50 μM induced the synthesis of a p 32 protein. A 20 μM concentration of DEM produced a weak induction and 10 μM did not affect synthesis. Comparison of Figs. 3 and 1 indicates that synthesis was induced only after 80% or more of the intracellular GSH had been depleted. Figure 4 examines this point in greater detail. Lane C_0 represents protein synthesis in control cells. Lane 1 illustrates synthesis of cells exposed to 50 μM BSO for 24 hr. This lowered GSH levels to 5% or less of control values. The BSO was then washed out and the cells were labeled for 1 hr.

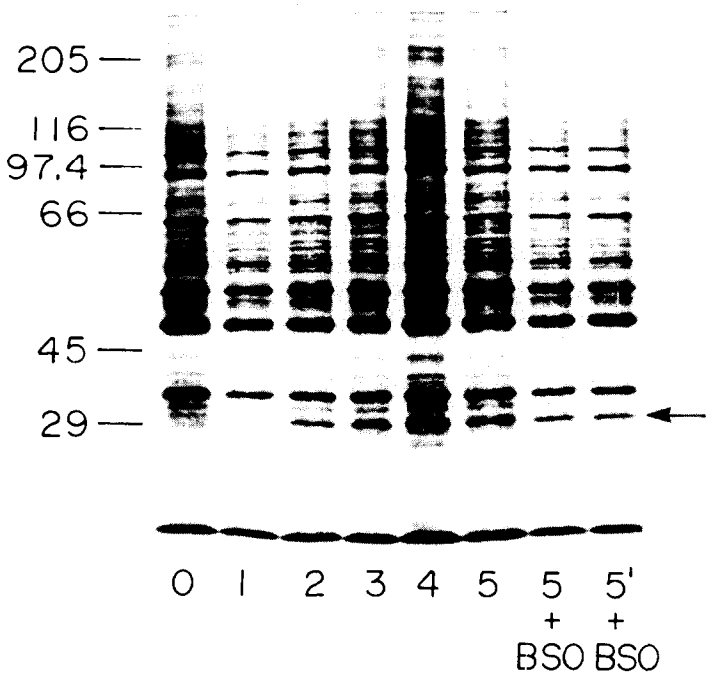

Fig. 2. Fluorograph of protein synthesis pattern after 100 μM DEM exposure. CHO cells were exposed to 100 μM DEM for 0, 1, 2, 3, 4, or 5 hr at 37°. The DEM was then washed out and the cells were labeled for 1 hr at 37° with [^{35}S]methionine. Lanes (5 + BSO) and (5' + BSO) represent protein from cells simultaneously exposed to 100 μM DEM and 50 μM BSO for 5 hr. Cells were then either labeled in the absence of the DEM/BSO (5 + BSO) or in the presence of BSO (5' + BSO). In this and in all other fluorographs the gels were loaded on an equal protein basis. The plating efficiency of the cells was unaffected by any of the treatments.

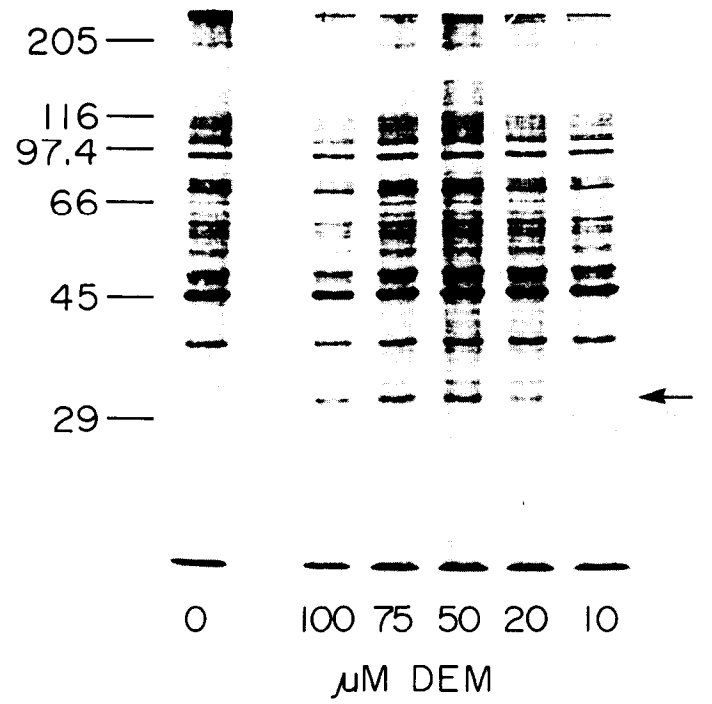

Fig. 3. Fluorograph of cell protein from cells exposed to 0, 10, 20, 50, 75, or 100 μm DEM for 3 hr at 37°. The DEM was then washed out, and the cells were labeled for 1 hr.

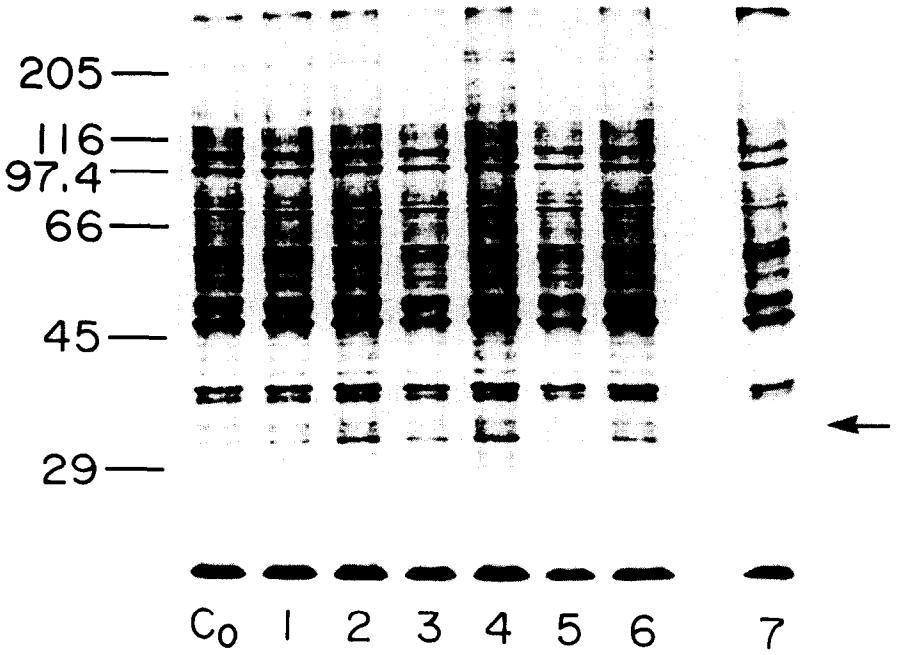

Fig. 4. Fluorograph of cell protein from cells exposed to DEM and/or BSO. Lane C_0: untreated control cells. Lane 1: cells exposed to 50 μM BSO for 24 hr. The BSO was washed out and the cells were labeled for 1 hr. Lane 2: cells exposed to 50 μM BSO for 24 hr. DEM (100 μM) was added for the last 4 hr. Lane 3: cells exposed to 50 μM BSO for 24 hr. The cells were also exposed to 100 μM DEM between hours 20 and 21. Lane 4: cells exposed to 100 μM DEM for 4 hr at 37°. Lane 5: cells exposed to 50 μM BSO for 4 hr at 37°. Lane 6: cells exposed to 100 μM DEM and 50 μM BSO for 1 hr and then to 50 μM BSO alone for 3 hr at 37°. Lane 7: cells exposed to 100 μM DEM for 1 hr at 37°.

This protocol did not induce the synthesis of the p 32 protein. Lane 2 represents synthesis from cells exposed to 50 μM BSO for 24 hr. DEM (100 μM) was added during the last 4 hr. Hence, induction of the p 32 protein is apparent. Less synthesis occurred in the DEM exposure was reduced to 1 hr (lane 3). Lane 4 represents cells exposed to 100 μM DEM for 4 hr with no BSO exposure. Lane 5 represents cells exposed to 50 μM BSO for 4 hr, resulting in depression of GSH levels to 30% of control values. No induction was apparent. Lane 6 represents a 4-hr exposure to BSO. During the first hour cells were also exposed to 100 μM DEM. Again, p 32 synthesis was observed. Lane 7 represents exposure to 100 μM DEM for only 1 hr with no BSO exposure.

Figure 5 illustrates cellular protein separated by two-dimensional electrophoresis. Cells were exposed to either 0.5 mM DMSO or 100 μM DEM for 5 hr and the cells were labeled for 1 hr. The fluorograph shows 2 proteins that were induced by the DEM exposure. They appear to have molecular weights of approximately 30 kDa and focus at about pH 6.5.

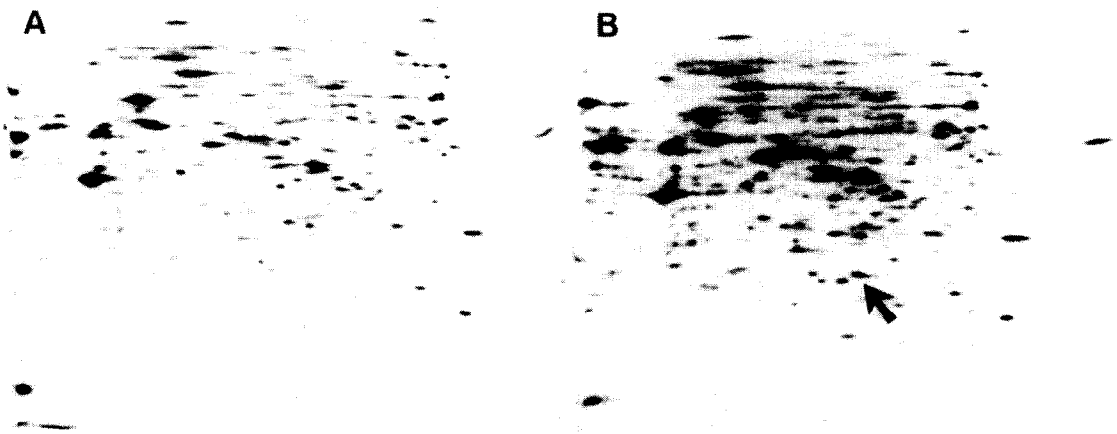

Fig. 5. Two-dimensional fluorograph from cells exposed to 0.5 mM DMSO for 5 hr or to 100 μM DEM for 5 hr. The DMSO/DEM was washed out, and the cells were labeled for 1 hr.

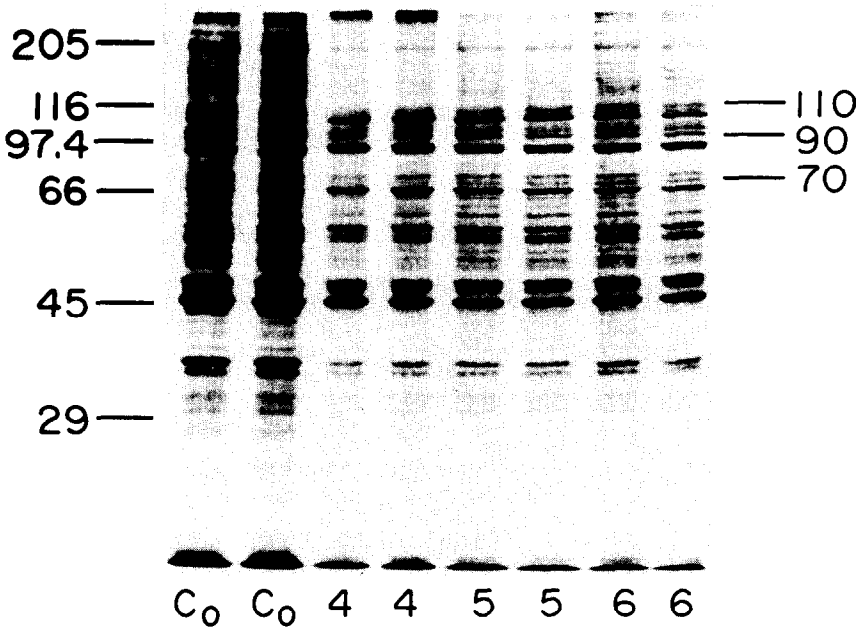

Fig. 6. Fluorograph of cell protein from cells exposed to diamide. Cells were exposed to either no (C_0) or 0.4 mM diamide for 1 hr at 37°. All cells were then washed twice and incubated in diamide-free medium for 4, 5, or 6 hr at 37° prior to being labeled for 1 hr.

The enhanced synthesis of the p 32 protein was not observed when CHO cells were exposed to 0.4 mM diamide which induced the synthesis of HSP (Fig. 6). The diamide exposure lowered GSH levels by about 50%. Greater concentrations could not be used due to extensive inhibition of protein synthesis. A heat shock consisting of 43° for 15, 30, or 45 min did not induce p 32 synthesis either (data not shown).

DISCUSSION

This current investigation has shown that exposure to DEM resulted in enhanced synthesis of a 32 kDa protein which focused at a pH of approximately 6.5. Induction occurred only after two criteria were met. The first was that depletion of intracellular GSH had to exceed 80% or more. The second was that synthesis was triggered by formation of a GSH conjugate rather than by GSH depletion occurring via metabolic turnover. The data presented in Fig. 3 illustrate that the synthesis of the 32 kDa protein was not enhanced by exposure to 10 μM DEM for 3 hr. This concentration of DEM lowered GSH levels by 60–70%. Further, the data in Fig. 1 show that induction occurred after a 2-hr exposure to 100 μM DEM, indicating that the failure to observe induction when 10 μM was used was not due to an insufficient exposure interval. When the cells were exposed to 20 μM DEM which lowered GSH concentration by 80–90% (Fig. 1), then a weak level of induction was observed (Fig. 3). Induction was most pronounced when the DEM concentration was equal to or exceeded 50 μM, which lowered GSH levels by 90% or more. In cells exposed to 50 μM BSO for 24 hr, GSH levels fell by 90% or more, yet induction of the 32 kDa protein was not observed. Addition of DEM to BSO-treated cells resulted in enhanced synthesis of the 32 kDa protein. These results were interpreted to mean that the BSO exposure resulted in the first criterion being met, namely the occurrence of GSH depletion, but failed to meet the second criterion. Addition of DEM resulted in GSH conjugation and rapid efflux during conditions of GSH depletion, thus fulfilling the second criterion. Thus, extensive GSH depletion is a necessary, but not sufficient, condition for 32 kDa protein induction.

Shelton *et al.* [6] have shown that chlorodinitrobenzene, as well as DEM, induces the synthesis of this protein; however, *N*-ethylmaleimide does not. These investigators pointed out, though, that the results with NEM are complicated by inhibition of protein synthesis. Finally, exposure to diamide failed to enhance the synthesis of the 32 kDa protein, indicating that induction did not depend upon the thiol oxidation. Although GSH depletion and thiol oxidation are observed with diamide, no conjugate is formed (excluding the reducible GSH homoconjugate, oxidized GSH). The question of whether the DEM-induced protein is the same as that induced by exposure to such heavy metals as cadmium or other soft Lewis acids and sulfhydral modifying reagents was not addressed in this study. Preliminary experiments have shown that a 1-hr exposure to 50 μM sodium arsenite or 10 μM $CdCl_2$ induced a 32 kDa protein. When protein from $CdCl_2$-treated cells was mixed with protein from DEM-treated cells and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the induced proteins were resolved as one band at 32 kDa. Experiments are underway to determine if they are the same protein. If they are the same, then induction

of the p 32 protein may be a general cellular response triggered by GSH conjugation and efflux.

Note added in proof: The $CdCl_2$ and DEM p 32 proteins focused at different isoelectric points when analyzed by two-dimensional electrophoresis.

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