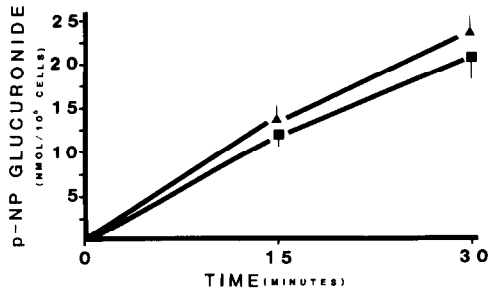

Fig. 1. Effects of urethane (■) and diethyl ether (▲) on the glucuronidation of *p*-NP in isolated hepatocytes. Data represent the mean ± S.E.M. of four determinations. Student's *t*-test was performed to determine statistical significance.

Table 1. Effects of urethane and diethyl ether on UDPGA levels in isolated hepatocytes

Treatment	UDPGA (nmoles/10^6 cells) 0 min	15 min	30 min
Urethane	2.14 ± 0.13	2.25 ± 0.18	3.10 ± 0.24
Ether	2.17 ± 0.24	1.99 ± 0.04	3.20 ± 0.21

Values represent the mean ± S.E.M. of four experiments.

urethane. Because ether anesthesia drastically reduces hepatic UDPGA levels while urethane does not [3, 5], this trend may be due to "overshoot" of the synthetic enzymes attempting to restore control levels while UDPGA is also being utilized to glucuronidate *p*-NP. Watkins and Klaassen [5] also observed a small increase in UDPGA levels over control values in animals allowed to recover from ether anesthesia.

Inasmuch as the concentration of UDPGA can be an important determinant of the rates of glucuronidation and xenobiotic elimination, the use of diethyl ether as an anesthetic must be carefully considered in some experiments. However, when diethyl ether anesthesia is used in the preparation of isolated hepatocytes, its effects on glucuronidation are not of consequence.

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In vitro phosphorylation and the identification of multiple protein changes in membranes of Chinese hamster lung cells resistant to adriamycin

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Previous studies have shown that plasma membranes of cells resistant to the antitumor agent adriamycin contain a phosphorylated glycoprotein (P180) which is not detected in drug sensitive cells [1, 2]. It has also been observed that certain isolates of resistant cells revert to drug sensitivity during growth in culture and that during this reversion process there is a loss of P180 from the cell surface [3]. These results suggest that this protein may play an important role in the cellular changes which contribute to drug resistance. Recent studies also show that, under conditions in which the level of phosphorylation of this protein is enhanced, there is a rapid conversion of the resistant cell to a drug sensitive phenotype [3, 4]. Thus, phosphorylation of protein P180 may be a means of regulating cell resistance to adriamycin. Additional studies also suggest that adriamycin resistance is related to changes occurring in the plasma membrane. Thus, it has been shown that resistance is due to a membrane restriction to drug uptake [5, 6] and/

or an enhanced efflux mechanism which extrudes drug from the cell [7–9]. This process, however, is quite complex since cells isolated for adriamycin resistance are also cross-resistant to a number of different compounds such as actinomycin D, colchicine and vincristine [9–11]. In view of the complexity of this process, it seems possible that proteins in addition to P180 may have a role in restricting the cellular accumulation of drug. However, no other protein change has been detected previously in cells isolated for resistance to adriamycin.

In the present study we have analyzed the *in vitro* phosphorylation of proteins in membranes isolated from drug sensitive, resistant and revertant cells. Our results provide evidence that membranes of resistant cells contain protein alterations in addition to those previously described and that these changes may be related to the drug resistant phenotype.

Experimental procedures

Materials. [γ-^{32}P]ATP (2900 Ci/mmole) was purchased from New England Nuclear.

Cells. Chinese hamster lung cells (HT1) resistant to adriamycin were isolated as previously described [1]. The resistant cell isolate R-PC4 was cloned in soft agar before use in these studies. The revertant cell isolate PC4C12 was obtained as described previously [3].

Membranes. Membrane preparations consisting of both plasma membranes and endoplasmic reticulum were prepared as previously described [2]. The isolated membranes were suspended in 0.025 M Tris–HCl (pH 7.6), 0.125 M sucrose and stored on ice.

In vitro *protein phosphorylation.* Isolated membranes were incubated in a reaction mixture containing 0.05 M Tris–HCl (pH 7.6), 2 mM β-mercaptoethanol, either 5 mM Mn^{2+} or Mg^{2+}, and 2 μCi of [γ-^{32}P]ATP. The total volume of the reaction mixture was 25 μl. Incubations were carried out for various time periods at room temperature, and the reaction was stopped by the addition of 10 mM EDTA. Samples were thereafter electrophoresed in a 7.5% polyacrylamide gel according to the method of Laemmli [12]. After electrophoresis the phosphorylated proteins were detected by autoradiography.

Results

In vitro *phosphorylation in isolated membranes.* Membranes were isolated from drug sensitive, resistant or revertant cells and thereafter incubated in the standard reaction mixture containing either 5 mM Mn^{2+} or Mg^{2+}. The proteins phosphorylated in the *in vitro* system were analyzed after polyacrylamide gel electrophoresis. When incubations were carried out in the presence of Mg^{2+}, two proteins of 180 kilodaltons (P180) and 220 kilodaltons (P220) were highly phosphorylated in resistant membranes but were essentially absent in membranes from drug sensitive or revertant cells (Fig. 1, D–F). When incubations were carried out in the presence of Mn^{2+}, there was a marked increase in the phosphorylation levels of several membrane proteins including P180 and P220 (Fig. 1, A–C). Also,

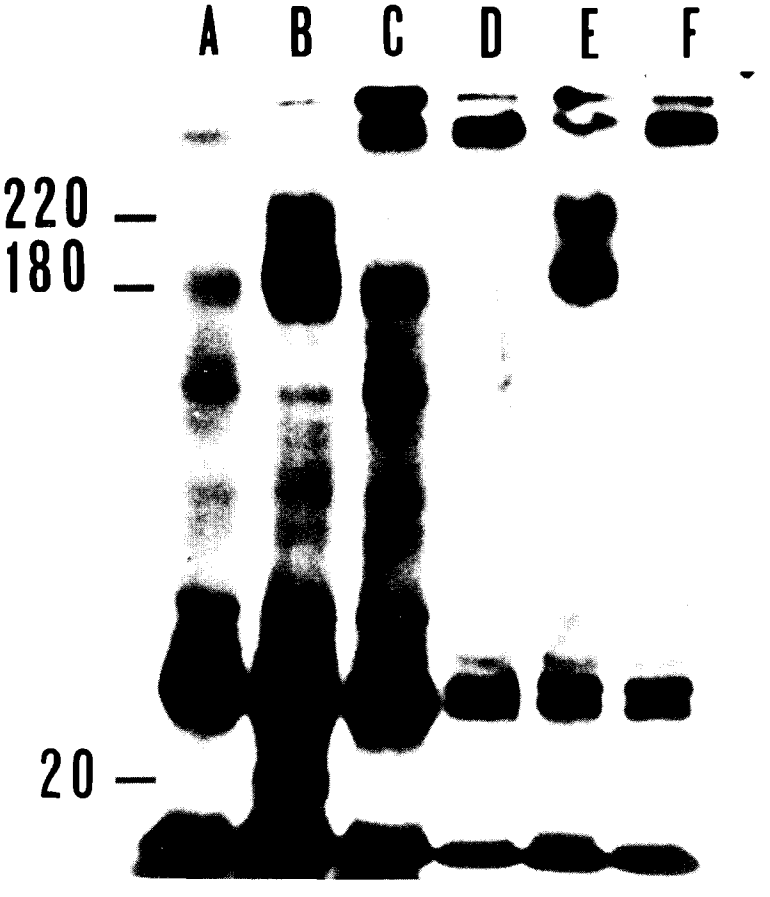

Fig. 1. *In vitro* phosphorylation in sensitive, resistant and revertant membranes. Isolated membranes were incubated in the standard reaction mixture containing either 5 mM Mn^{2+} (lanes A–C) or 5 mM Mg^{2+} (lanes D–F). After a 20-min incubation period, the phosphorylated proteins were analyzed after polyacrylamide gel electrophoresis. Lanes A and D, sensitive membranes; lanes B and E, resistant membranes; lanes C and F revertant membranes.

under these assay conditions a protein having a molecular weight of 20 kilodaltons (P20) was highly phosphorylated in membranes from drug resistant cells (Fig. 1B). This protein was present in only very low levels in membranes from drug sensitive or revertant cells (Fig. 1, A and C). In studies carried out thus far, P20 has not been detected when *in vitro* phosphorylation reactions were carried out in the presence of Mg^{2+} (Fig. 1, D–F).

Protein phosphorylation in the presence of Zn^{2+}. In these studies, sensitive or resistant membranes were incubated in the standard reaction mixture containing 5 mM Mn^{2+} in either the absence or presence of 0.2 or 1 mM Zn^{2+}. In reactions carried out in the absence of Zn^{2+}, resistant membranes contained phosphorylated P20, P180 and P220 (Fig. 2D) and proteins of similar molecular weights were either absent or present in very low levels in membranes from drug sensitive cells (Fig. 2A). In reactions carried out in the presence of Mn^{2+} and either 1.0 or 0.2 mM Zn^{2+}, there was essentially a complete loss in the phosphorylation of either P180 or P220 in drug resistant membranes (Fig. 2, E and F). The phosphorylation of a protein of 180K was also inhibited in sensitive membranes incubated in the presence of 1 mM Zn^{2+} (Fig. 2B). In contrast to the effect of Zn^{2+} on P180 and P220 phosphorylation, there was essentially no inhibition of P20 phosphorylation in resistant membranes incubated with either 1 mM or 0.2 mM Zn^{2+} (Fig. 2, E and F). Additional studies also showed that, in the presence of Zn^{2-} alone, there was essentially no phosphorylation of any membrane protein (data not shown). These results thus suggest that P20 is not a degradation product of P180 or P220 and that this protein represents a separate entity which is independently phosphorylated.

Phosphoamino acid analysis. Studies have been carried out to identify the phosphoamino acids of *in vitro* phosphorylated P180 and P220. In these experiments, membranes were incubated in the standard reaction mixture containing Mg^{2-}, and the proteins P180 and P220 were isolated after gel electrophoresis. Phosphoamino acid analysis of the isolated proteins demonstrated that both P220 and P180 were phosphorylated only at serine residues (Fig. 3, A and B). Additional studies have also shown that P20 was phosphorylated exclusively at serine. We have shown previously that P180 labelled with ^{32}Pi *in vivo* in the presence of *N*-ethylmaleimide is phosphorylated at both serine and threonine [3]. The basis of the threonine phosphorylation in the presence of *N*-ethylmaleimide is not known.

Discussion

In the present study we analyzed the *in vitro* phosphorylation of proteins in membranes isolated from Chinese hamster lung cells resistant to adriamycin. The results demonstrate that in this *in vitro* system three pro-

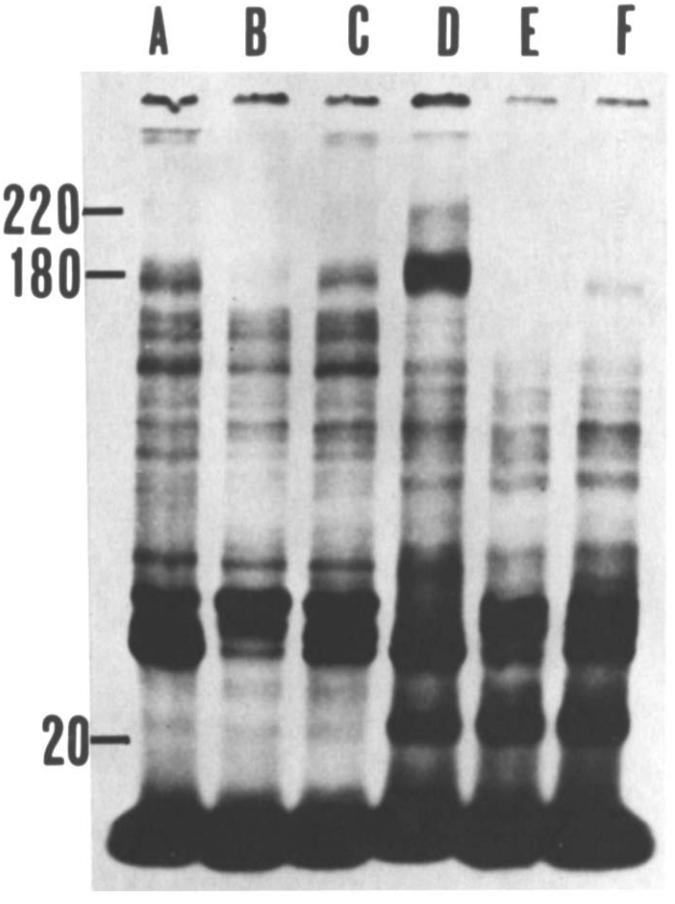

Fig. 2. Effect of Zn^{2-} on protein phosphorylation in isolated membranes. Membranes isolated from drug sensitive and resistant cells were incubated in the standard phosphorylation reaction mixture containing 5 mM Mn^{2-} in either the absence (lanes A and D) or presence of either 1.0 mM Zn^{2-} (lanes B and E) or 0.2 mM Zn^{2+} (lanes C and F). Incubations were for 30 min, and the proteins phosphorylated were analyzed after polyacrylamide gel electrophoresis. Lanes A–C, sensitive membranes; lanes D–F, resistant membranes.

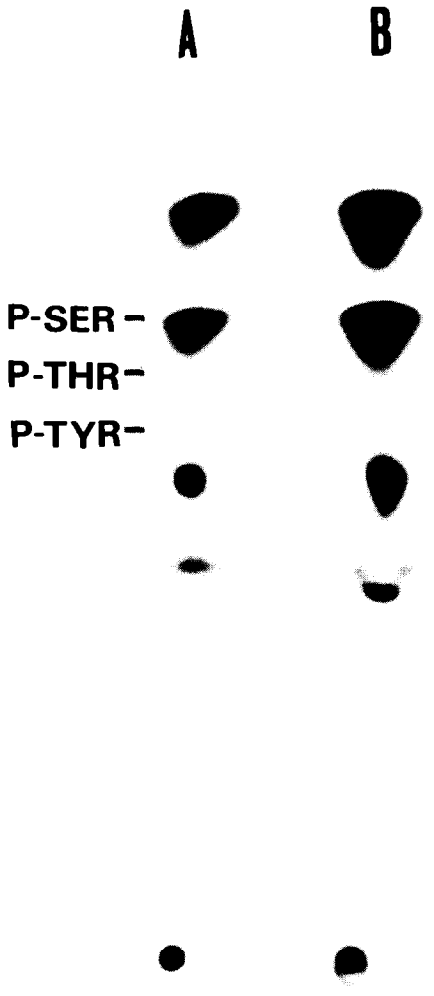

Fig. 3. Phosphoamino acid analysis of *in vitro* phosphorylated P180 and P220. Isolated membranes from drug resistant cells were incubated in the standard *in vitro* phosphorylation system in the presence of Mg^{2+}. P180 and P220 were isolated after polyacrylamide gel electrophoresis, lyophilized and thereafter hydrolyzed in 6 N HCl for 2 hr at 105°. The samples were evaporated to dryness, dissolved in 10 μl of a solution containing marker phosphoamino acids, and electrophoresed on cellulose thin-layer plates in acetic acid–pyridine–water, 50:5:945 (pH 3.5). Markers were visualized with ninhydrin spray and ^{32}P-labeled phosphoamino acids by autoradiography. (A) P220; (B) P180.

teins of 20, 180 and 220 kilodaltons were highly phosphorylated in resistant membranes but were either absent or present in very low levels in membranes from drug sensitive cells. The finding that these proteins were not phosphorylated in membranes from cells which have reverted to drug sensitivity suggests a correlation between their presence and the drug resistant phenotype. These results thus suggest that adriamycin resistance in Chinese hamster lung cells may require the involvement of multiple protein components.

Previous studies have demonstrated that cell lines isolated for resistance to a variety of chemical agents contain proteins which have molecular weights similar to those identified by *in vitro* phosphorylation of proteins in membranes of adriamycin resistant cells. Thus, it has been observed that cells resistant to vincristine contain two proteins having molecular weights of 150K [13] and 20K [14, 15] which are not detected in wild-type cells. It has also been observed that plasma membranes of cells isolated for resistance to colchicine [16], vinblastine [17], actinomycin D [18], daunomycin [13, 19] and adriamycin [1, 2] contain a 170–180K protein which is not detected in cells sensitive to drug. Recent studies have also shown that aclacinomycin resistant cells contain a 230K protein which is absent in drug sensitive cells [20]. A 200K protein has also been observed in colchicine resistant cells using *in vitro* phosphorylation techniques [21]. It is of particular interest that the drug resistant cell lines mentioned above are also cross-resistant to adriamycin [9–11]. It thus seems possible that these various drug resistant cell lines contain P20, P180 and P220 and that these proteins may have a major role in the drug resistant phenotype. It is thus suggested that *in vitro* phosphorylation using high specific activity [γ-^{32}P] ATP may be capable of detecting drug resistant related proteins which are difficult to detect with *in vivo* labeling techniques.

In summary, membranes isolated from Chinese hamster lung cells resistant to adriamycin were incubated in an *in vitro* protein phosphorylation system containing [γ-^{32}P] ATP, 2-mercaptoethanol and divalent metal ions. The proteins phosphorylated in this system were thereafter analyzed by polyacrylamide gel electrophoresis. The results demonstrate that when incubations were carried out in the presence of Mn^{2+} three proteins having molecular weights of 220 kilodaltons (P220), 180 kilodaltons (P180) and 20 kilodaltons (P20) were highly phosphorylated in resistant membranes but were present in only very low levels in membranes from drug sensitive cells. Similar results were obtained in the presence of Mg^{2+} except that there was only a very low level of P20 phosphorylation. When incubations were carried out in the presence of Mn^{2+} and low levels of Zn^{2+}, the phosphorylation of P180 and P220 was inhibited while the phosphorylation of P20 was not affected, thus suggesting that the latter protein does not result from degradation of P220 or P180. Phosphoamino acid analysis demonstrated that P20, P180, and P220 were phosphorylated exclusively at serine residues. P220, P180 and P20 were not phosphorylated in membranes from cells that had reverted to drug sensitivity, suggesting that these three proteins may have an important role in adriamycin resistance.

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Effect of aflatoxin B_1 on translation in isolated hepatocytes

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Aflatoxin B_1 (AFB_1), a potent hepatocarcinogen, inhibits protein synthesis [1–4]. Investigators working with intact rats suggest that the inhibition of liver protein synthesis by the toxin is a consequence of blockage at elongation and termination [5, 6], while others working with cultured rat liver cells propose that the ribosomal site of AFB_1 inhibition is the initiation step [7]. Thus, there is still uncertainty as to the locus of AFB_1 inhibition of translation. We have approached the problem using isolated rat hepatocytes as an alternate to whole animal or cell culture studies, comparing cytoplasmic ribonucleoprotein complex (cRPC) content, proportion of polysome size classes, nascent polypeptides released from polysomes, and formation of initiation complexes in untreated and AFB_1-treated hepatocytes.

Materials and methods

Hepatocytes were prepared from male Wistar rats (250–300 g) using the procedure of Berry and Friend [8] with minor modifications [9]. The cells (1 to 1.5×10^6 cells/ml) were preincubated without or with AFB_1 (5 or 50 μM) for 15 min before [^{3}H]leucine (5 μCi/ml, Amersham Corp., Arlington Heights, IL) was added. At 60 min, hepatocytes were collected and subcellular fractions were obtained by differential centrifugation. The cRPC were isolated by the method of Palmiter [10], and various size classes of polysomes were separated [11]. Incorporation of [^{35}S]methionine (1345 μCi/mmol, Amersham Corp.) into methionyl-tRNA was determined by preincubation of hepatocytes without or with AFB_1 (50 μM) for 65 min before addition of 50 μCi [^{35}S]methionine. After 10-min labeling, 40S and 80S initiation complexes were isolated from the cRPC, and [^{35}S]methionyl-tRNA was precipitated by cetyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO). Peptidylpuromycin was prepared by incubation of 20–25 A_{260} units of cRPC with puromycin (0.5 mM) at 37° for 30 min. Peptidylpuromycin of $M_r > 8000$ was purified as described elsewhere [11] and separated on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate [12]. Cell viability was checked by trypan blue exclusion (0.27%). Protein was assayed by the method of Lowry *et al.* [13]. Radioactivity of trichloroacetic acid or cetyltrimethylammonium bromide insoluble material collected on glass fiber filters (Whatman GF/C) and gel slices was determined by counting in a Beckman liquid scintillation spectrometer. When counting [^{3}H], cpm were converted to dpm, while [^{35}S] was counted under a wide [^{14}C] window, and cpm were recorded. Counting errors were ±5%.

Results and discussion

Previous studies report that at 180 min of incubation, inhibition of protein synthesis measured by [^{3}H]leucine incorporation is dependent on the dose of AFB_1. The lowest dose to show significant inhibition is 5 μM, and the most inhibition is obtained with 50 μM AFB_1 [14]. Since 50 μM AFB_1 causes significant decreases in cell viability at 120 and 180 min of incubation, experiments were terminated at 60 min. This time allows the toxin to enter and act on the cells but avoids changes due to alteration of the cellular membrane. As shown in Table 1, 50 μM AFB_1 significantly inhibited [^{3}H]leucine incorporation into subcellular fractions. Specific radioactivities of cell lysate, nuclei, and cytosol were 10, 23 and 14% of the control respectively. The greater inhibition of [^{3}H]leucine incorporation into proteins of cell lysate (90%) and cytosol (84%) compared to cRPC (71%) may have been due to impairment of posttranslational processes. Since there was no change in the content of the cRPC, the decreased specific radioactivity in treated cells reflected a decrease in the rate of protein synthesis.

To determine the ribosomal site or sites affected by AFB_1, polysome size classes were examined (Table 2). In the presence of 5 μM AFB_1 there was a slight increase in the concentration of monomers accompanied by a decrease in the polysome/monomer ratio compared to the control. The changes were not significant. In the presence of 50 μM AFB_1, however, the increased concentration of monomers and decreased polysome/monomer ratio became significant. According to the scheme of Pestka [15], the accumulation of monomers suggested that the toxin blocked the initiation stage of the ribosomal cycle. Lack of complete polysome breakdown in treated cells indicated that initiation may have been only partially inhibited. To assess the extent of involvement of initiation, initiation complexes were examined (Table 3). [^{35}S]Methionine in untreated cells was associated with both 40S and 80S initiation complexes. The 80S/40S ratio of 3.3 suggested that methionyl-tRNA was present in the 40S initiation complex, allowing subsequent formation of the 80S initiation complex. The content of the complexes was similar in untreated and treated cells, but there was a greater decline in radioactivity in the 80S initiation complex in AFB_1-treated cells. The 80S/40S ratio was decreased to 1.8, similar to the inhibition of the initiation step produced by actinomycin D in CHO cells [16]. The decreased 80S/40S (Table 3) and polysome/monomer ratios (Table 2), although significant, were not sufficient to account for the 71% decrease in overall protein synthesis seen in the cRPC from AFB_1-treated cells (Table 1). The elongation stage of the ribosomal cycle was then considered as an additional target for AFB_1 action.

Analysis of [^{3}H]leucine incorporation into peptidylpuromycin (Fig. 1) indicated that labeled polypeptides of various molecular weights (M_r 10,000–160,000) were found