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Evidence That Adriamycin Resistance In Chinese Hamster Lung Cells Is Regulated By Phosphorylation Of A Plasma Membrane Glycoprotein

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Summary: Incubation of adriamycin resistant Chinese hamster lung cells with low levels of N-ethylmaleimide (NEM) results in a major increase in the cellular accumulation of drug. When resistant cells are prelabeled with [^{32}Pi] and thereafter treated with NEM there also occurs a selective superphosphorylation of an 180K plasma membrane glycoprotein (P-180). This phosphorylation reaction occurs at both serine and threonine residues. In similar experiments with drug sensitive cells only minor levels of this protein can be detected. Detailed studies have established that in cells which have reverted to drug sensitivity there is a parallel loss in the presence of phosphorylated P-180. Also in cells which have undergone partial reversion to drug sensitivity there is a correlation between levels of superphosphorylated P-180 and adriamycin resistance. These results provide evidence that adriamycin resistance is dependent on the presence of P-180. The results also suggest that the biological activity of this protein is highly regulated by phosphorylation and that in the superphosphorylated state P-180 is inactive and under these conditions the resistant cell is converted to a drug sensitive phenotype.

Introduction: Adriamycin is an effective chemotherapeutic agent which is used in the treatment of a variety of human neoplasms (1). It has been found however that during chemotherapy cells emerge which have acquired a high degree of resistance to the drug. The basis of this cellular change is unknown but studies with a variety of cell lines isolated for resistance suggest that the defect is due both to a membrane restriction to drug uptake (2,3) and an enhanced efflux mechanism which extrudes drug from the cell (3,4). Despite extensive studies on cellular changes occurring in drug resistant cells there has been essentially no information obtained on biochemical mechanisms that may be involved in regulating the adriamycin resistant phenotype. Previous studies have shown however that this phenotype can be modulated since it has been observed that treatment of cells with various metabolic inhibitors (3-5), calcium antagonists or calmodulin inhibitors (6,7) converts

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the resistant cell to a drug sensitive phenotype. Recently we have shown that treatment of adriamycin resistant cells with N-ethylmaleimide results in a major increase in the cellular accumulation of drug and a parallel super-phosphorylation of a plasma membrane glycoprotein (8). This protein which has a molecular weight of about 180,000 (P-180) has previously been shown to be present in drug resistant but not sensitive cells (9).

In the present study we have analyzed P-180 phosphorylation in isolates of cells which have reverted to drug sensitivity. Our results provide evidence that this protein plays a major role in drug resistance and that phosphorylation of P-180 is involved in regulating the adriamycin resistant phenotype.

Materials and Methods

Drugs. Adriamycin was provided by the Developmental Therapeutics Program of the Division of Cancer Treatment, NCI.

Cells. Chinese hamster lung cells (HT-1) resistant to adriamycin were isolated as described previously (9). The isolate R3R is a spontaneous revertant obtained after growing R3 resistant cells for eight months in culture. Detailed analysis using drug cytotoxity studies has shown that R3R has reverted completely to the drug sensitive phenotype. Revertants have also been obtained from a second isolate R24C4 which had been cloned in soft agar. We have found that if this isolate is cloned a second time in soft agar, cells can be obtained which have undergone a partial reversion to drug sensitivity. Cells obtained from two clones designated R24C4C5 and R24C4C12 were used in this study. Both sensitive and resistant cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

Plasma Membranes. Plasma membranes were prepared and analyzed for purity as described previously (8). The endoplasmic reticulum was also isolated for use in these studies.

Phosphorylation of P-180. Sensitive and resistant cells were grown in 100 mm dishes in DMEM containing 10% fetal calf serum for 48 hours. The media was thereafter removed and 1 ml of TM media containing 50 μCi of [^{32}Pi] was added to each dish. TM media contains 0.05 M Tris-HCl (pH 7.6), 0.15 M NaCl, 0.005 M KCl, 0.0055 M D-glucose, and 1X MEM amino acids and vitamins. After a 1 hr labeling period at 37°C, the media was removed and the cells washed once with glucose free TM. To each dish 3 ml of glucose free media was added followed by NEM which was added to all dishes except the control cells. After incubations at 37°C for various time periods the cells were collected and membranes were prepared. The phosphorylated proteins were analyzed after electrophoresis (10) in polyacrylamide gels. Labeled proteins were detected by autoradiography. Autoradiograms in some instances were traced with a Joyce-Loebl densiometer.

Phosphoamino acid analysis. Plasma membranes and endoplasmic reticulum containing [^{32}Pi] labeled proteins were electrophoresed in 7% polyacrylamide gels. [^{32}P] labeled P-180 was eluted from the gel, lyophilized and thereafter hydrolyzed in 6 N HCl for 2 hours at 105°C. The hydrolysate was lyophilized and thereafter electrophoresed on cellulose thin-layer plates in 7.8% acetic acid, 2.5% formic acid for 2 hours at 1,000 V. Markers were visualized with ninhydrin spray and [^{32}P] labeled phosphoamino acids by autoradiography.

Results. Previously we have shown that adriamycin resistant cells contain a cell surface glycoprotein of 180,000 molecular weight (P-180) (9). Further analysis of this protein shows that it is a phosphoprotein which is undergoing cycles of phosphorylation and dephosphorylation in the cell (8). Recently we have observed that when adriamycin resistant cells are treated with N-ethylmaleimide there is a major increase in the cellular accumulation of drug (8). We have also observed that under these conditions there is a parallel superphosphorylation of P-180 (8). A typical result of this type of experiment is shown in Figure 1. In the endoplasmic reticulum (lane B) and plasma membranes (lane D) from prelabeled cells incubated in the absence of NEM only low levels of phosphorylated P-180 can be detected. However in the membrane preparations from cells treated with NEM (lanes A and C) there is a major increase in the phosphorylation levels of this protein. Of particular interest is the finding that P-180 is the

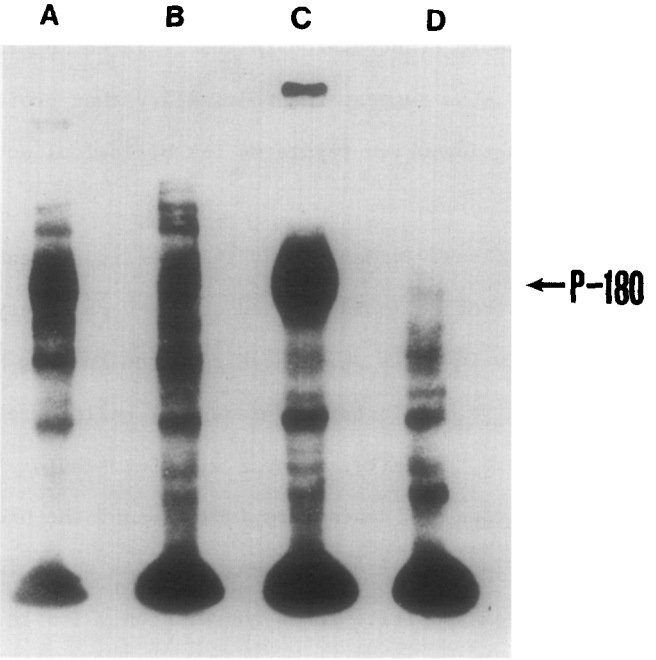

Figure 1: Superphosphorylation of P-180 in drug resistant cells. Adriamycin resistant cells were prelabeled with [^{32}Pi] and thereafter incubated in the absence (lanes B and D) or presence (lanes A and C) of 5 mM NEM for 20 min. Labeling conditions were as described in Methods. At the end of the labeling period plasma membranes (lanes Cand D) and endoplasmic reticulum (lanes A and B) were prepared and phosphoproteins analyzed after electrophoresis in a 5% polyacrylamide gel.

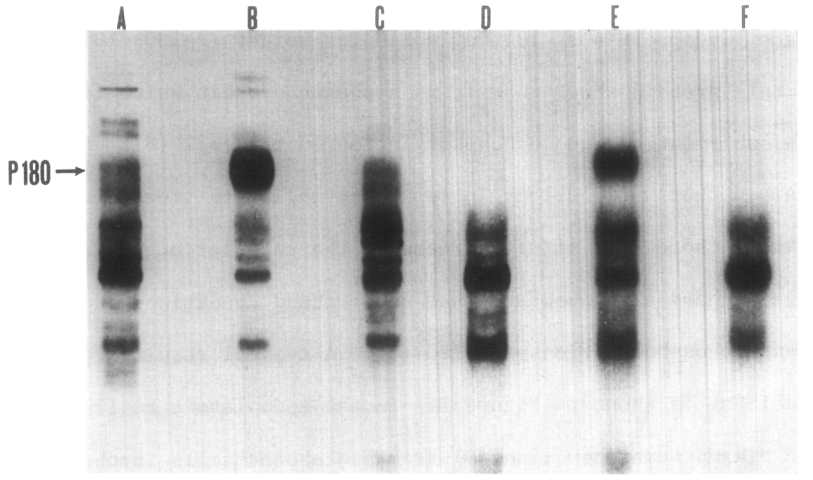

Figure 2: P-180 phosphorylation in drug sensitive, resistant and revertant cells. Drug sensitive, resistant (R3) and revertant (R3R) cells were prelabeled with [^{32}Pi] and thereafter incubated for 20 min. with 5 mM NEM under conditions described in Methods. Endoplasmic reticulum (lanes A, B, and C) and plasma membranes (lanes D, E, and F) were prepared and proteins analyzed after electrophoresis in a 7% polyacrylamide gel. Lanes A and D, B and E, and C and F show the results obtained with membranes isolated from sensitive, resistant and revertant cells respectively.

singular protein which is superphosphorylated in the isolated membrane preparations. These results thus suggest the possibility that protein phosphorylation is a key reaction which regulates the biological activity of P-180.

In order to obtain additional evidence that the NEM induced enhancement of drug uptake in resistant cells is related to P-180 phosphorylation we have examined these events in cells which have reverted to drug sensitivity. In these experiments sensitive, resistant and revertant cells were prelabeled with [^{32}Pi] and the cells were thereafter treated with 5mM NEM for 20 min. Endoplasmic reticulum and plasma membranes were isolated and the proteins were analyzed after polyacrylamide gel electrophoresis. As shown in Figure 2,A the endoplasmic reticulum from sensitive cells contains only low levels of P-180. In similar membrane preparations from resistant cells this protein is present in a superphosphorylated form (Figure 2, B). Studies with revertants which have regained drug sensitivity shows that the levels of P-180 in the endoplasmic reticulum is essentially identical to that found for sensitive cells (Figure 2, C).

Additional studies show that P-180 is present in a highly phosphorylated form in plasma membranes from resistant cells (Figure 2, E) whereas in similar fractions from sensitive and revertant cells the protein is essentially absent (Figure 2, D and F). These results thus demonstrate that as the resistant cell reverts to drug sensitivity there is a parallel loss in the presence of phosphorylated P-180. Additional studies have been carried out with a second revertant obtained from an independent drug resistant isolate. These revertant cells are of interest since we have obtained isolates which have undergone partial reversion to drug sensitivity. Thus it has been possible to correlate various levels of drug resistance with levels of phosphorylated P-180. In these studies crude membranes (endoplasmic reticulum plus plasma membranes) were isolated from[^{32}Pi] prelabeled cells treated with 5 mM NEM for 20 minutes and the proteins were analyzed after gel electrophoresis. As shown in Figure 3 cells with varying degrees of resistance have different levels of phosphorylated P-180. The highly resistant parent cell contains P-180 in a highly phosphorylated form (Figure 3A). Relative to the sensitive cell there is about an 18-fold increase in the phosphorylation of this protein. Analysis of partial revertants reveals that as the degree of resistance decreases there is a resulting decrease in the levels of phosphorylated P-180 (Figure 3, B, C). In drug sensitive cells P-180 is present in only low levels (Figure 3, D).

We have also carried out experiments to identify the phosphoamino acids of superphosphorylated P-180. As shown in Figure 4,A P-180 of plasma membranes contains predominantly phosphoserine but also significant levels of phosphothreonine. P-180 of endoplasmic reticulum contains both phosphoserine and phosphothreonine in high levels (Figure 4, B). The significance of the difference in phosphothreonine of P-180 in plasma membranes and endoplasmic reticulum remains to be determined. In parallel experiments with plasma membranes or endoplasmic reticulum from drug sensitive cells only negligible amounts of phosphoamino acids could be detected.

Discussion: Previously we have shown that N-ethylmaleimide is capable of inducing a major increase in drug uptake in resistant cells (8). Similar

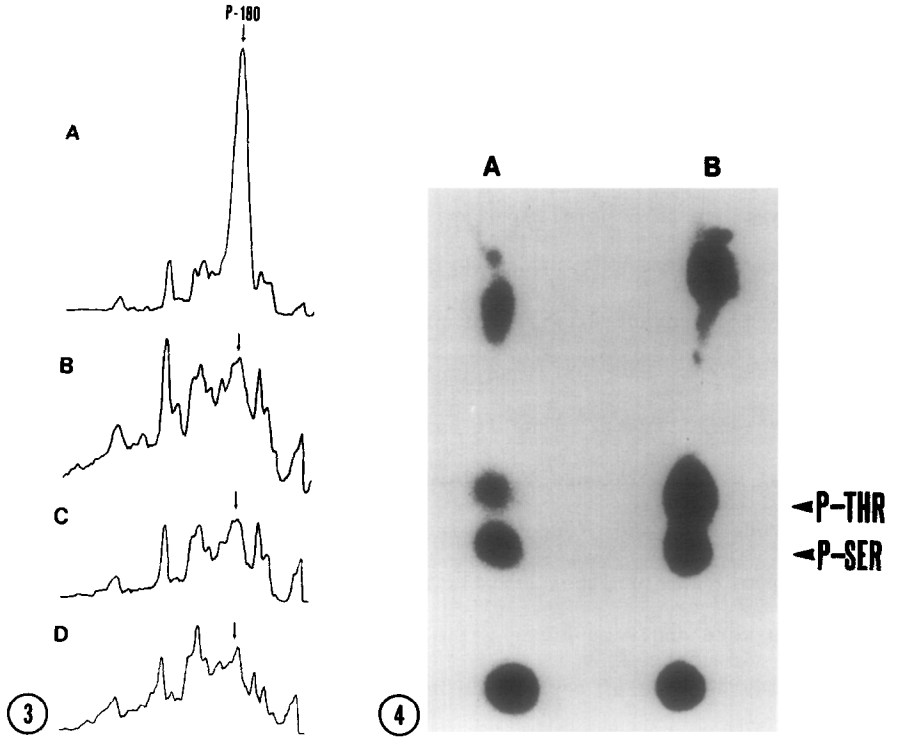

Figure 3: P-180 phosphorylation in drug sensitive and resistant cells and partial revertants. Experiment was carried out as described in the legend to Figure 2 except that the cells used were R24C4, (panel A), partial revertant, R24C4C5, (panel B), partial revertant, R24C4C12, (panel C), and sensitive, (panel D). The IC_{50} values (concentration of drug that inhibits cell growth by 50%) relative to sensitive cells were 200, (R24C4), 35 (R24C4C5) and 15 (R24C4C12). The phosphoproteins contained in crude membranes (endoplasmic reticulum plus plasma membranes) were analyzed after electrophoresis in a 7% polyacrylamide gel. Autoradiograms was traced with a Joyce-Loebl densitometer. The migration of proteins is from right to left.

Figure 4: Phosphoamino acids of superphosphorylated P-180. The phosphoamino acids of [^{32}P] labeled P-180 of plasma membranes (lane A) and endoplasmic reticulum (lane B) were determined as described in Methods.

findings using a variety of metabolic inhibitors have been described previously (3-5). We have also observed that when resistant cells are prelabeled with [^{32}Pi] and incubated for brief time periods in NEM, there is a selective superphosphorylation of an 180K plasma membrane glycoprotein. Several lines of evidence indicate that drug uptake in NEM treated resistant cells is related to P-180 phosphorylation. Thus we have found that in cells which have reverted to drug sensitivity there is a parallel loss in the NEM induced superphosphorylation of P-180. Furthermore in studies with partial revertants

there is a correlation between levels of superphosphorylated P-180 and degree of adriamycin resistance. We have also observed that there is a close correlation between the induction of P-180 superphosphorylation and the time and concentration of NEM required for increasing drug uptake. Finally, in recent studies we have found that the calmodulin inhibitor trifluoperazine (11) induces a major increase in drug uptake and a parallel superphosphorylation of P-180 in resistant cells (manuscript in preparation).

These results taken together strongly suggest that the modulation of adriamycin resistance by NEM or trifluoperazine is mediated through a mechanism which involves the phosphorylation of P-180. It is suggested that this protein in the active form contains low levels of phosphate and in this state can act to prevent drug accumulation in the cell. However if this protein is superphosphorylated it becomes inactive and the cell now assumes a drug sensitive phenotype. Understanding the details of this reaction should provide considerable insight into the basis of adriamycin resistance.

It is also of interest that a protein of similar size as P-180 is present in plasma membranes of cells selected for resistance to colchicine (12), Vinblastine (13) and actinomycin D (14) and that these isolates are cross-resistant to adriamycin. It would thus be indicated that regulation of resistance in these cells is also mediated by phosphorylation of the P-180 cell surface protein.

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