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Dimethylsulfoxide, Retinoic Acid and 12-O-tetradecanoylphorbol-13-acetate Induce a Selective Decrease in the Phosphorylation of P150, a Surface Membrane Phosphoprotein of HL60 Cells Resistant to Adriamycin

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Summary: Studies have been carried out to analyze protein phosphorylation in membranes isolated from adriamycin resistant HL60 cells which have been grown for various time periods in the presence of dimethylsulfoxide (DMSO), retinoic acid (RA) or 12-O-tetradecanoylphorbol-13-acetate (TPA). The results show that membranes isolated from cells treated with these agents are defective in the phosphorylation of P150, a membrane phosphoprotein associated with drug resistance in HL60 cells. This response is highly selective since only a few membrane proteins show decreased phosphorylation levels under these conditions. Magnesium dependent protein kinase activity in membranes from cells treated with DMSO, RA or TPA is not altered relative to untreated membranes under conditions where there is a major decrease in P150 phosphorylation. Additional studies also show that treatment of resistant cells with TPA results in a major decrease in the in vivo phosphorylation of P150. These results thus demonstrate that agents capable of inducing differentiation in HL60 cells can selectively modulate the phosphorylation of P150. This system should be of value in clarifying mechanisms involved in the phosphorylation of this protein.

Introduction: Recent studies have demonstrated that adriamycin resistance in the human leukemia cell line HL60 is associated with the presence of a surface membrane protein of 150-160 kilodaltons (P150) (1,2). Additional studies also show that P150 can be phosphorylated in vivo and in isolated membrane vesicles incubated in the presence of Mg^{2+} and [γ-^{32}P]ATP (2). This finding together with reports which show that drug resistant associated proteins in other cell lines are also phosphorylated (3,4) suggests that levels of phosphorylation may have an important regulatory function in drug resistance. This is consistent with previous studies which indicate that the biological activity of proteins involved in adriamycin resistance in Chinese hamster lung cells

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is regulated by phosphorylation (5-7). In view of these findings we have carried out studies to examine in greater detail mechanisms involved in the phosphorylation of P150. In the present study we have analyzed P150 phosphorylation in membranes isolated from resistant cells grown in the presence of certain inducers of HL60 cellular differentiation. The results show that growth of cells in the presence of these agents results in a selective inactivation of the P150 phosphorylation system.

Materials and Methods

Chemicals. Nitrobluetetrazolium, TPA, RA and DMSO were purchased from Sigma Chemical Co. [γ-^{32}P]ATP (3,000 Ci/mmol) was from New England Nuclear.

Isolation of adriamycin resistant HL60 cells. Human promyelocytic leukemic cells (HL60) (8), were isolated for adriamycin resistance as described previously (2). Using this same procedure three additional independent drug resistant isolates have been obtained. All isolates exhibit a 10-15 fold increase in adriamycin resistance.

Isolation of cell membranes. A membrane fraction containing both plasma membranes and endoplasmic reticulum was prepared from sensitive and resistant HL60 cells as previously described (3). The isolated membrane fraction was suspended in 0.01 M Tris-HCl (pH 7.6) - 0.125 M sucrose and stored on ice.

In vitro protein phosphorylation. Isolated membranes (25 µg of protein) were incubated in a 25 µl reaction mixture containing 0.05 M Tris-HCl (pH 7.6) - 2 mM β-mercaptoethanol - 5 mM Mg^{2+} and 2 µCi of [γ-^{32}P]ATP. Incubations were carried out for 20 minutes at room temperature and the reaction was stopped by the addition of 10 mM EDTA. Samples were thereafter electrophoresed in a 7% polyacrylamide gel (9) and the phosphorylated proteins were detected by autoradiography.

Growth of cells in the presence of DMSO, RA, or TPA. Sensitive or resistant HL60 cells were seeded at a density of 7 x 10^5/ml in RPMI media containing 10% fetal calf serum. Cultures were thereafter grown in the absence or presence of 1.8% DMSO, 2 µM RA or 8 x 10^{-10} M of TPA for various time periods. At the end of the growth period the cells were harvested and membranes were prepared (3).

Assessment of cellular differentiation. HL60 cells grown in the presence of DMSO or RA were assessed for differentiation by measuring the formation of cells capable of reducing nitrobluetetrazolium (NBT) (10). The NBT positive cells were examined in a light microscope. Differentiation of cells in the presence of TPA was determined by visualizing the extent of cell adherence to the culture flasks.

Results

In vitro phosphorylation in membranes from adriamycin resistant HL60 cells. Membranes were prepared from four independent drug resistant isolates of HL60 cells. All isolates have a 10-15 fold increase in resistance to adriamycin. The isolated membranes were incubated in the *in vitro*

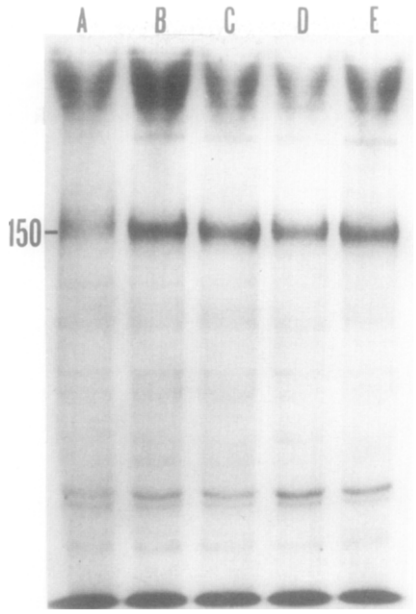
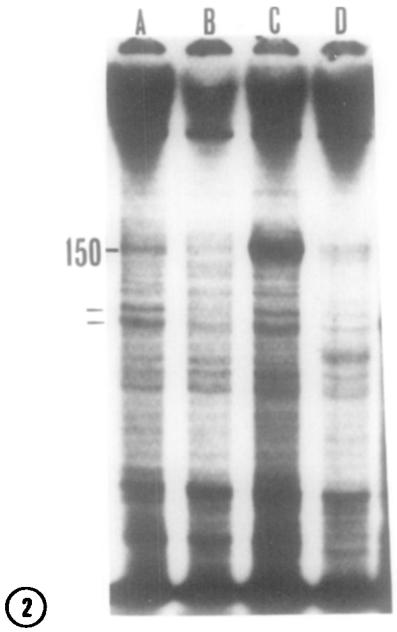


Figure 1: Proteins phosphorylated in membranes prepared from four independent adriamycin resistant isolates. Independent drug resistant isolates of HL60 cells were obtained as described in Materials and Methods. Membranes were isolated and thereafter incubated in the in vitro phosphorylation system for 20 min at room temperature. The phosphorylated proteins were analyzed after electrophoresis in a 7% polyacrylamide gel (9). The phosphorylated proteins were detected by autoradiography. Lane A, membranes from sensitive cells; Lanes B-E, membranes from resistant isolates RX1-4 respectively.

phosphorylation system and the labeled proteins were analyzed after polyacrylamide gel electrophoresis. Membranes from all four isolates were found to contain a highly phosphorylated protein of 150 kilodaltons (P150) (Figure 1, B-E). A protein of similar molecular mass is present in only very low levels in drug sensitive cells (Figure 1, A). These results extend previous findings (2) and further suggest a close correlation between the presence of P150 and the drug resistant phenotype. Previously we had also found that a minor 120 kilodalton protein (P120) could be phosphorylated in vitro in drug resistant but not sensitive membranes (2). In subsequent studies we find that this protein is not consistently phosphorylated with each membrane preparation from resistant cells. The reason for this is not known.

Phosphorylation in membranes from HL60 cells grown in the presence of DMSO, RA or TPA. HL60 cells were grown for 48 hours in the presence of DMSO (1.8%) and cell membranes were prepared and incubated in the in vitro



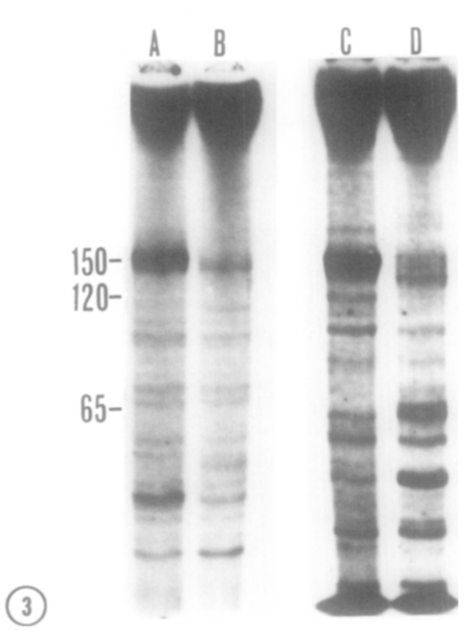


Figure 2. Proteins phosphorylated in membranes isolated from sensitive and resistant HL60 cells treated with DMSO. Membranes were prepared from sensitive and resistant cells grown in the absence or presence of 1.8% DMSO for 48 hours. The isolated membranes were incubated in the in vitro phosphorylation system for 20 min at room temperature. The phosphorylated proteins were thereafter analyzed as described in the legend to figure 1. Lanes A and B, membranes from untreated and treated sensitive cells respectively; Lanes C and D membranes from untreated and treated resistant cells respectively.

Figure 3. Protein phosphorylation in membranes from resistant cells treated with RA or TPA. Drug resistant HL60 cells were grown in the absence or presence of either 2 μM RA for 48 hours or 8×10^{-10} M TPA for 24 hours. The phosphorylated proteins were analyzed as described in the legend to figure 1. Lanes A and B, membranes from untreated and RA treated resistant cells respectively; Lanes C and D, membranes from untreated and TPA treated resistant cells respectively.

phosphorylation reaction. Analysis of phosphorylated proteins from drug resistant cells shows that DMSO treatment results in a major decrease in the phosphorylation of P150 (Figure 2, C and D). Membranes from drug sensitive cells also contain a minor 150 kilodalton protein whose phosphorylation level is reduced after growth in the presence of DMSO (Figure 2, A and B). As shown in Figure 2, two lower molecular weight proteins of about 100 and 92 kilodaltons also have reduced levels of phosphorylation in membranes from both sensitive and resistant cells treated with DMSO. In several experiments of

this type P150, P100, and P92 are the only proteins which exhibit decreased phosphorylation after DMSO treatment.

Studies have also been carried out in which protein phosphorylation was examined in membranes prepared from resistant cells grown for 48 hours in the absence or presence of 2 μM RA. The results demonstrate that membranes from treated cells have a major decrease in the in vitro phosphorylation of P150 (Figure 3, A and B). This response is also highly selective since P150 is the only membrane protein which consistently shows reduced phosphorylation levels after treatment with RA.

Additional studies have been carried out in which protein phosphorylation was examined in membranes from resistant cells grown for 24 hours in the presence of 8×10^{-10} M TPA. The results demonstrate that TPA like DMSO and RA induces a cellular change which results in a major decrease in the in vitro phosphorylation of P150 (Figure 3, C and D). A second protein of 120 kilodaltons (P120) also has reduced levels of phosphorylation but no other membrane protein shows this pattern (Figure 3, C and D). As indicated P120 seems to be present in resistant but not sensitive membranes but the phosphorylation of this protein is not always observed. A 65 kilodalton protein (P65) is found to be present in membranes from treated but not untreated cells (Figure 3, D). A P65 is also present in membranes from sensitive cells treated with TPA.

Protein kinase activity in membranes from HL60 cells grown in the presence of DMSO, RA or TPA. Detailed studies have been carried out to analyze Mg^{2+} dependent protein kinase activity in membranes from resistant cells grown in the absence or presence of DMSO, RA or TPA. The results have clearly demonstrated that under conditions where these agents induce a major decrease in the phosphorylation of P150 the levels of protein kinase in membranes from untreated and treated cells are essentially the same (not shown).

Effect of TPA on the in vivo phosphorylation of P150. Previous studies have shown that incubation of adriamycin resistant HL60 cells with ^{32}Pi

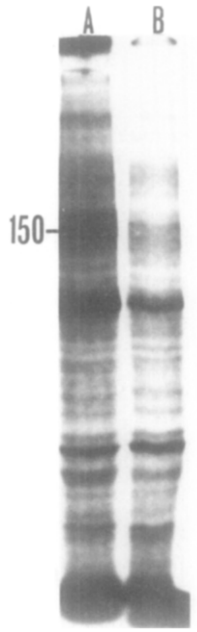


Figure 4. Effect of TPA on the in vivo phosphorylation of P150. Drug resistant cells were grown in the absence or presence of TPA (8×10^{-10} M) for 24 hours and the cells were labeled with ^{32}Pi as described (2). Proteins contained in membranes were analyzed after polyacrylamide gel electrophoresis. Lane A, phosphoproteins in membranes from untreated cells; lane B, phosphoproteins in membranes from cells treated with TPA.

results in the labeling of P150 (2). This protein is essentially absent in drug sensitive cells treated under identical conditions. In the present study resistant cells were incubated in the absence or presence of 8×10^{-10} M TPA for 24 hours and the cells were thereafter labeled with ^{32}Pi (2). The phosphorylated proteins contained in isolated membranes were analyzed after polyacrylamide gel electrophoresis. The results show that cells treated with TPA are highly defective in the phosphorylation of P150 (Figure 4, A and B). This in vivo response is also highly selective since only a few other membrane proteins have reduced phosphorylation levels.

Discussion: Previous studies have shown that HL60 cells grown in the presence of DMSO or RA undergo a program of terminal differentiation which results in the formation of cells with morphologic and biochemical properties similar to granulocytes (11,12). TPA induces a program of differentiation which leads to

the formation of macrophage like cells (13). It has also been found that during differentiation a number of surface membrane changes occur (14-16). In the present study we have analyzed *in vitro* phosphorylation in membranes from adriamycin resistant HL60 cells which have been grown in the presence of DMSO, RA or TPA. The results demonstrate that treatment of cells with these agents leads to a cellular change(s) which results in a major decrease in the *in vitro* phosphorylation of P150 a surface membrane protein (1,2) which may have a major role in adriamycin resistance in HL60 cells. We have also shown that P150 phosphorylation is reduced *in vivo* in cells treated with TPA. Similar *in vivo* studies with DMSO and RA are in progress. Of particular interest in the present study is the finding that of many membrane proteins phosphorylated P150 is one of very few proteins which shows reduced phosphorylation under the treatment conditions used. Since protein phosphorylation may have a role in regulating adriamycin (5-7) resistance, the present system may provide insight into strategies that could be used to selectively inactivate P150. The inactivation of this protein could possibly result in the conversion of a resistant cell to one exhibiting a drug sensitive phenotype.

The mechanism by which DMSO, RA and TPA induce a decrease in P150 phosphorylation is not known. This effect does not appear to be due to a major loss in Mg^{2+} dependent protein kinase activity since this activity is essentially identical in membranes from treated and untreated cells under conditions where the phosphorylation of P150 is greatly reduced. It is possible however that a minor protein kinase activity which selectively phosphorylates P150 is inactive in membranes from treated cells. If this is the case it may be possible to utilize the *in vitro* system to establish a complementation assay for isolating proteins involved in P150 phosphorylation.

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