Mechanisms of Multidrug Resistance in HL60 Cells: Evidence That a Surface Membrane Protein Distinct from P-Glycoprotein Contributes to Reduced Cellular Accumulation of Drug

Tim McGrath and Melvin S. Center

Division of Biology, Kansas State University, Manhattan, Kansas 66506

ABSTRACT

HL60 cells exhibiting a 140-fold increase in resistance to vincristine contain three surface membrane proteins with molecular weights of 210,000 (P210), 180,000 (P180), and 150,000 (P150) which are highly phosphorylated in vivo and in an in vitro system in the presence of Mn2+ and [γ-32P]ATP. These phosphorylated proteins are either absent or present in very low levels in membranes of drug-sensitive cells. Growth of the vincristine-resistant isolate in the absence of drug results in a decrease in the level of resistance and a major reduction in the phosphorylation of P210 and P180. The phosphorylation of P150 is not altered in the revertant which still exhibits substantial levels of resistance. Further studies show that P210 and P180 are highly reactive with a monoclonal antibody against P-glycoprotein. These two proteins are present in only very low levels in revertant cells. The monoclonal antibody exhibits no reactivity with P150. In HL60 cells isolated for a 25-fold increase in vincristine resistance proteins reactive with P-glycoprotein monoclonal antibody are essentially absent. P150 is however highly phosphorylated in these cells. Additional experiments using lectin binding of 32P-labeled proteins demonstrate that P150 has properties distinct from P210 and P180. Analysis of drug uptake patterns in the vincristine-resistant isolates and the revertant shows that resistance is related to a reduced intracellular accumulation of drug. Reduced accumulation of vincristine is also found in HL60 cells isolated for resistance to Adriamycin. These cells are devoid of P-glycoprotein but contain phosphorylated P150.

These results suggest that proteins P150, P180, and P210 may contribute to multidrug resistance in HL60 cells through a mechanism which involves reduced cellular accumulation of drug. P180 and P210 are structurally related whereas P150 is distinct from these two proteins.

INTRODUCTION

Previous studies have provided evidence that experimental cell lines isolated for resistance to chemotherapeutic agents such as vinblastine, Adriamycin, or actinomycin D contain increased levels of a surface membrane phosphoglycoprotein with a molecular weight of 150,000–180,000 (P180) (1–14). This protein also referred to as the P-glycoprotein (1) is present in drug sensitive cells in only very low levels (1–4). Evidence that P-glycoprotein contributes to drug resistance is provided by the finding that the levels of this protein are reduced in isolates which have reverted to drug sensitivity (5, 6). Recent studies have also shown that transfer of the cDNA of the multidrug-resistant gene to sensitive cells converts these cells to a population exhibiting a multidrug-resistant phenotype (7). The exact function of P180 is not known although cells overexpressing this protein exhibit a reduced intracellular accumulation of drug (4, 8). The finding that P-glycoprotein can bind drug to which the cells are resistant (9, 10) supports a functional role of this protein in modulating cellular drug levels. Despite evidence that P-glycoprotein contributes to drug resistance in experimental cell lines the involvement of this protein in clinical drug resistance is not clear. Thus in many instances P-glycoprotein is not overexpressed in tumor cells from patients who have relapsed during chemotherapy (11, 12).

Recently it has been shown that HL60 cells isolated for resistance to Adriamycin do not contain detectable levels of P-glycoprotein (13, 14). These cells are however multidrug resistant and are defective in drug accumulation (14, 15). Analysis of these cells indicates that the resistant isolate contains a (P150) surface membrane protein with a molecular weight of 150,000 which is present as a modified (phosphorylated) form of a protein contained in drug-sensitive cells (14). It has thus been suggested that phosphorylation of P150 converts this protein to a form active in drug resistance.

In the present study we have examined surface membrane changes in HL60 cells isolated for resistance to vincristine. We find that this isolate contains increased levels of P-glycoprotein and in addition increased phosphorylation of P150. Studies with genetic revertants and low level resistant isolates provides further evidence however that P150 is distinct from P-glycoprotein.

MATERIALS AND METHODS

Materials

[3H]Vincristine (6.1 Ci/mmol) was obtained from Amersham. [γ-32P]ATP (2900 Ci/mmol) was from New England Nuclear. Bandeiraea simplicifolia BS-11 lectin (sugar specificity N-acetyl-d-glucosamine) insolubilized on 4% beaded agarose was purchased from Sigma.

Methods

Vincristine-resistant HL60 Cells. HL60 cells isolated for resistance to vincristine (HL60/vinc) or Adriamycin (HL60/Adr) were prepared as described previously (13, 15). Partial revertants of the HL60/vinc isolate were obtained after growing cells in the absence of drug for about 6 months. These cells are referred to as HL60/vinc/R. The HL60/Adr isolate is completely stable when grown for at least 2 years in the absence of drug. Dose-response curves for drug-resistant lines were determined by growing cells in increasing levels of drug and counting viable cells in a hemacytometer. Values of the concentration of drug resulting in a 50% loss in cell viability were determined from the dose-response curves. Some properties of the various isolates are given in Table 1.

In Vitro Phosphorylation of Proteins in Isolated Membranes. A membrane fraction containing both plasma membranes and endoplasmic reticulum was isolated as described previously (16). The membranes were suspended in 0.01 M Tris-HCl (pH 7.6)–0.125 M sucrose and stored on ice. For in vitro phosphorylation isolated membranes (25 μg of protein) were incubated in a 25-μl reaction mixture containing 0.02 M Tris-HCl (pH 7.6), 0.05 mM Mn2+, and 3 μCi of [γ-32P]ATP. Incubations were carried out for 15 min on ice. The reaction was stopped by the addition of 10 mM EDTA and the samples were thereafter electrophoresed in a 7.5% polyacrylamide gel (17). Labeled proteins were detected after autoradiography.

In Vivo Labeling of Phosphoproteins in Sensitive and Resistant Cells. Sensitive and resistant cells were labeled with 32P as previously de-
The supernate was recovered and the pellet was incubated as above was held on ice for 1 h and thereafter centrifuged at 10,000 x g for 20 min. The cell pellets were suspended in 0.2 ml of 1 N NaOH and incubated for 2 h at 55°C. An aliquot was taken for radioactivity concentration of 100 nM. The cells were incubated at 37°C in a CO2 atmosphere. The paper was thereafter treated with monoclonal antibody (C219) which is directed against P-glycoprotein (19). The monoclonal antibody was generously provided by Dr. Victor Ling. After a 5-h incubation period the paper was washed with 0.02 M phosphate buffer (pH 7.3) and membranes were prepared as described previously (16). The radioactively labeled proteins were analyzed after electrophoresis in a 7.5% polyacrylamide gel.

Immunoblot Analysis for P-Glycoprotein. Membranes from the drug-sensitive and -resistant HL60 cells were incubated in the in vitro phosphorylation system in the presence of Mn2+ and [γ-32P]ATP and the labeled proteins were analyzed after polyacrylamide gel electrophoresis. Membranes from the isolates HL60/Adr, HL60/vincCl, and HL60/vinc/R all contain a highly phosphorylated protein with a molecular weight of 150,000 (P150) (Fig. 1, lanes B–D). Phosphorylated P150 is essentially absent in membranes of drug sensitive cells (Fig. 1, lane A). Membranes from the HL60/vincCl isolate also contain 3 phosphorylated proteins with molecular weights of 210,000 (P210), 180,000 (P180), and 50,000 (P50) and these proteins are absent in membranes from sensitive cells (Fig. 1, lanes A and C). In membranes from revertant cells (HL60/vinc/R) the phosphorylation of proteins P210, P180, and P50 is greatly reduced whereas the phosphorylation of P150 is not altered (Fig. 1, lane D). Additional studies have been carried out to examine phosphorylation patterns in membranes from cells isolated for a low level of resistance to vincristine (HL60/vincC2). Thus in membranes from cells exhibiting a 25-fold increase in resistance to vincristine P150 is highly phosphorylated whereas there is only very slight phosphorylation of P210, P180, or P50 (Fig. 2, lane B). In contrast proteins P210, P180, P150, and P50 are highly phosphorylated in membranes from cells exhibiting a 140-fold increase in vincristine resistance (Fig. 2, lane C). As indicated in Table 1 the results of several experiments show that the

---

**Table 1** Some properties of multidrug-resistant HL60 cells

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Resistance*</th>
<th>Phosphorylated P150, resistant/sensitive (range)</th>
<th>P-glycoprotein (%)</th>
<th>Drug accumulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60/S</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>HL60/Adr</td>
<td>80</td>
<td>20</td>
<td>8–10</td>
<td>0</td>
</tr>
<tr>
<td>HL60/vincCl</td>
<td>15</td>
<td>140</td>
<td>8–10</td>
<td>100</td>
</tr>
<tr>
<td>HL60/vincC2</td>
<td>8</td>
<td>25</td>
<td>1</td>
<td>55</td>
</tr>
<tr>
<td>HL60/vinc/R*</td>
<td>8</td>
<td>20</td>
<td>4</td>
<td>55</td>
</tr>
</tbody>
</table>

* Resistance levels determined by dividing ID50 (dosage level producing 50% loss of cell viability) of resistant isolate by that of the sensitive cells.

**RESULTS**

In Vitro Phosphorylation of Membrane Proteins. Membranes were prepared from drug-sensitive and -resistant cells and from an isolate which had partially reverted to drug sensitivity. Some properties of these isolates are given in Table 1. The isolated membranes were incubated in the in vitro phosphorylation system in the presence of Mn2+ and [γ-32P]ATP and the labeled proteins were analyzed after polyacrylamide gel electrophoresis. Membranes from the isolates HL60/Adr, HL60/vincCl, and HL60/vinc/R all contain a highly phosphorylated protein with a molecular weight of 150,000 (P150) (Fig. 1, lanes B–D). Phosphorylated P150 is essentially absent in membranes of drug sensitive cells (Fig. 1, lane A). Membranes from the HL60/vincCl isolate also contain 3 phosphorylated proteins with molecular weights of 210,000 (P210), 180,000 (P180), and 50,000 (P50) and these proteins are absent in membranes from sensitive cells (Fig. 1, lanes A and C). In membranes from revertant cells (HL60/vinc/R) the phosphorylation of proteins P210, P180, and P50 is greatly reduced whereas the phosphorylation of P150 is not altered (Fig. 1, lane D). Additional studies have been carried out to examine phosphorylation patterns in membranes from cells isolated for a low level of resistance to vincristine (HL60/vincC2). Thus in membranes from cells exhibiting a 25-fold increase in resistance to vincristine P150 is highly phosphorylated whereas there is only very slight phosphorylation of P210, P180, or P50 (Fig. 2, lane B). In contrast proteins P210, P180, P150, and P50 are highly phosphorylated in membranes from cells exhibiting a 140-fold increase in vincristine resistance (Fig. 2, lane C). As indicated in Table 1 the results of several experiments show that the
levels of P150 phosphorylation are about the same in membranes of all the isolates. Thus there is an 8- to 10-fold increase in P150 phosphorylation in resistant membranes as compared to the phosphorylation of this protein in membranes from sensitive cells.

Analysis of in Vitro 32Pi-labeled Proteins. Sensitive, vincristine-resistant and revertant cells were labeled with 32Pi as described in “Materials and Methods.” TPA was added during the labeling period since it has been shown that this agent enhances the phosphorylation of P-glycoprotein (21). In the absence of TPA it has been difficult to detect P-glycoprotein phosphorylation in drug-resistant HL60 cells. During the course of these studies we also found that under the conditions of these experiments TPA slightly enhances the phosphorylation of P150. This is in contrast to previous studies where it has been shown that prior incubation of HL60/Adr cells with TPA for prolonged periods (15 h) greatly reduces P150 phosphorylation (22). Thus in the presence of TPA P210, P180, and P150 are phosphorylated in the HL60/vincC2 isolate (Fig. 3, lane B). In contrast in the revertant there is a major reduction in phosphorylated P180 and P210 whereas the phosphorylation levels of P150 are not altered (Fig. 3, lane C). The phosphorylated forms of these proteins are essentially absent in sensitive cells (Fig. 3, lane A).

Analysis of Cells for P-Glycoprotein. Membranes from drug-resistant cells were examined for the presence of P-glycoprotein using immunoblot analysis as described in “Materials and Methods.” The C219 antibody used in these studies is directed against P-glycoprotein and is species independent (19). In agreement with our previous results membranes from the HL60/Adr isolate do not contain detectable levels of P-glycoprotein (Fig. 4, lane A). In contrast the HL60/vincC2 isolate contains two membrane proteins with molecular weights of 210,000 and 180,000 which are highly reactive with the C219 monoclonal antibody (Fig. 4, lane B) (13). These two proteins are essentially absent in membranes prepared from the HL60/vinc/R isolate (Fig. 4, lane C). Analysis of a densitometric scan of the autoradiogram shows that in the revertant there has been a 96% reduction in the levels of P210 and P180. Additional studies have been carried out to examine P-glycoprotein levels in the HL60/vincC2 isolate which exhibits a 25-fold increase in vincristine resistance. As determined by immunoblot analysis this isolate contains only trace amounts of P-glycoprotein (not shown). The P-glycoprotein level is about 1% of that contained in the HL60/vincC1 isolate (Table 1).

Lectin Binding Experiments. Previous studies have provided evidence that P150 (14, 23) and P180 (1–4) are glycoproteins. We have therefore carried out experiments to determine if these proteins can be distinguished by their lectin binding properties. Thus in these experiments membrane proteins labeled in the in vitro system with 32Pi were solubilized and thereafter bound to BS-11 lectin. Proteins were eluted with various concentrations of N-acetylglucosamine and analyzed after polyacrylamide gel electrophoresis. Proteins P210 and P180 are eluted from the lectin at a NAG concentration of 0.1 M whereas under these conditions only very low levels of P150 are eluted from the lectin (Fig. 5, lane A). In contrast P150 is found in the eluates obtained after incubation of the lectin with 0.2 M or 0.5 M NAG (Fig. 5, lanes D and F). P210 and P180 are not detected in the eluates obtained at the higher concentrations of NAG (Fig. 5, lanes D and F). Proteins P150, P180, and P210 are essentially absent in eluates of detergent extracts prepared from drug sensitive cells (Fig. 5, lanes A, C, and E).
Patterns of Cellular Drug Accumulation in Sensitive and Resistant Cells. Sensitive or resistant cells were incubated with \[^{3}H\]vincristine for various time periods and the levels of drug accumulation were determined as described in "Materials and Methods." The HL60/vincC1 isolate was found to be highly defective in drug uptake and only very low levels of vincristine were cell associated after a 150-min incubation period (Fig. 6A). As these cells revert to drug sensitivity there is a corresponding increase in cellular accumulation of drug (Fig. 6A). Drug uptake in the HL60/vinc/R is still however, considerably less than that found for the sensitive cells. Similar experiments have also been carried out with HL60 cells isolated for resistance to Adriamycin (Table 1). This isolate which does not contain detectable levels of P-glycoprotein (13, 14) is highly defective in the cellular accumulation of vincristine (Fig. 6B).

Cross-Resistance Patterns. Previous studies have shown that the HL60/Adr isolate is cross-resistant to a number of other agents (14). Similar studies show that the HL60/vincC1 and HL60/vincC2 isolates in addition to their resistance to Adriamycin are also resistant to daunomycin, colchicine, and actinomycin D.

**DISCUSSION**

Previous studies have provided evidence that a surface membrane protein with molecular weight 150,000–160,000 (P150) is associated with Adriamycin resistance in HL60 cells (14, 15, 23). Thus it has been shown that in resistant cells P150 can be labeled in vivo with \[^{32}P\]Pi and in an in vitro system in the presence of [\(\gamma\)-\[^{32}P\]]ATP (14, 15). Under these labeling conditions the protein is not detected in sensitive cells (14, 15). P150 can also be identified in resistant but not sensitive cells by using surface membrane labeling techniques (15, 23). Recent studies have provided evidence that P150 may be contained in sensitive cells but in a nonphosphorylated form (14). Thus it has been found that sensitive and resistant cells contain a surface membrane glycoprotein with a molecular weight of 150,000 and the proteins in the two cell types are structurally related (14). Further-
more the glycosylated and phosphorylated P150 of resistant cells appears to be the same (14). At the present time there is no evidence that P150 is overexpressed in drug-resistant cells. In view of these findings it was of interest to examine the phosphorylation of P150 in HL60 cells isolated for resistance to other chemotherapeutic agents and to further analyze the relationship of P150 to P-glycoprotein.

In the present study we have examined phosphorylation patterns of surface membrane proteins in cells isolated for resistance to vincristine. The results show that in cells exhibiting a 140-fold increase in vincristine resistance there is a major increase in the phosphorylation of three proteins, P150, P180, and P210. These phosphorylated proteins are not detected in drug-sensitive cells. Monoclonal antibody against P-glycoprotein is highly reactive with P210 and P180 whereas there is no reactivity with P150. Studies with other drug-resistant isolates have generally demonstrated a single protein with a molecular weight 170,000-180,000 which is reactive with the C219 monoclonal antibody (19). At the present time the exact relationship between P210 and P180 is not known. It is interesting to note however that previous studies have suggested that a protein with a molecular weight of 220,000 may have a role in drug resistance in other cell lines (24-26). Further studies also demonstrate that as the vincristine-resistant isolate undergoes partial reversion to drug sensitivity there is a parallel loss in the levels of P210 and P180. In contrast reversion does not affect the levels of P150 phosphorylation. Additional studies have also shown that in cells exhibiting a 25-fold increase in resistance to vincristine P150 is highly phosphorylated whereas phosphorylated P210 and P180 are essentially absent. P150 has also been found to have distinct lectin binding properties from P210 and P180. These results taken together indicate that P150 is not structurally related to proteins P210 or P180.

Certain lines of evidence indicates that P150 may contribute to multidrug resistance in HL60 cells. Thus it has been found that HL60 cells isolated for resistance to Adriamycin are devoid of P-glycoprotein but contain P150 in a phosphorylated form (13-15). These cells are multidrug resistant and are defective in the cellular accumulation of drug (14, 15). The present studies also show that reverants of cells isolated for vincristine resistance contain phosphorylated P150 but only very low levels of P-glycoprotein. Furthermore, cells exhibiting a 25-fold increase in resistance to vincristine P150 is highly phosphorylated whereas phosphorylated P210 and P180 are essentially absent. P150 has also been found to have distinct lectin binding properties from P210 and P180. These results taken together indicate that P150 is not structurally related to proteins P210 or P180.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Victor Ling for providing the C219 monoclonal antibody used in these studies.

REFERENCES

Mechanisms of Multidrug Resistance in HL60 Cells: Evidence That a Surface Membrane Protein Distinct from P-Glycoprotein Contributes to Reduced Cellular Accumulation of Drug

Tim McGrath and Melvin S. Center


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/14/3959

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.