The MRP Gene Associated with a Non-P-glycoprotein Multidrug Resistance Encodes a 190-kDa Membrane Bound Glycoprotein

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Abstract

HL60 cells isolated for resistance to Adriamycin (HL60/ADR) overexpress a 190-kDa ATP binding protein which has a minor sequence homology with P-glycoprotein. It has also been observed that HL60/ADR overexpress the MRP gene which was first identified as a component of a non-P-glycoprotein mediated multidrug resistance of H69/ADR cells [Cole et al., Science (Washington DC), 258:1650, 1992]. A complementary DNA of MRP has been cloned and based on the deduced sequence encodes a member of the superfamly of proteins which bind ATP and function in various transport processes [Cole et al., Science (Washington DC), 258:1650, 1992]. In view of this it was of interest to identify the protein encoded by MRP and determine if it may be related to p190. In the present study we have prepared antisera against three synthetic peptides which correspond to the deduced sequence of the MRP protein. Proteins reactive with the antisera have been examined in HL60/ADR cells using Western blot analysis. All antisera react with a 190 kDa protein contained in membranes of resistant but not sensitive cells. One antisera used for further studies is not reactive with P-glycoprotein contained in membranes of HL60 cells isolated for resistance to vincristine. Analysis of subcellular fractions demonstrates that p190 is present primarily in the endoplasmic reticulum with lower levels also present in plasma membranes. Treatment of HL60/ADR cells with tunicamycin results in the appearance of a 165-kDa resistance associated protein which reacts with the antipeptide serum. The results of this study therefore demonstrate that the MRP gene encodes a 190-kDa membrane bound glycoprotein.

Introduction

Previously we have isolated and characterized HL60 cells selected for resistance to Adriamycin (1). These cells are multidrug resistant and definitive in the cellular accumulation of drug (1). Despite the absence of detectable P-glycoprotein (2) reduced drug accumulation appears to be related to enhanced levels of a drug efflux pump (1). It has also been found that HL60/ADR cells overexpress a membrane 190-kDa ATP binding protein which has a minor sequence homology with P-glycoprotein (3, 4). This is based on the finding that an antiserum prepared against a P-glycoprotein peptide reacts with both P-glycoprotein and p190 (4).

Recently Cole et al. (5) have identified a gene designated MRP (multidrug resistance associated protein) which is overexpressed in the non-P-glycoprotein multidrug resistant isolate (H69/AR) derived from human small cell lung cancer cells (6). A cDNA of this gene has now been cloned and the nucleotide sequence has been determined (5, 7). Based on this sequence the MRP gene encodes a protein of 1531 amino acids (7) which is a member of the superfamily of ATP binding transmembrane transport proteins (5). Recently we have observed that the MRP gene is also highly overexpressed in the HL60/ADR isolate. In view of this we have examined HL60/ADR cells for proteins reactive with antisera prepared against synthetic peptides which correspond to the deduced sequence of the MRP protein. The results demonstrate that the MRP gene encodes a membrane glycoprotein with a molecular mass of 190 kDa.

Materials and Methods

Cell Lines. HL60 cells isolated for resistance to Adriamycin (HL60/ADR) and HL60 cells isolated for resistance to vincristine (HL60/Vinc) were as described previously (1, 3). HL60/ADR and HL60/Vinc exhibit an 80- and 140-fold increase, respectively, to the selecting agent.

Peptide Synthesis and Immunization. Peptides were synthesized according to the deduced sequence of the MRP protein (7) by Research Genetics, Huntsville, AL. Three peptides having the following sequences were prepared: peptide 1, KEDTSEQVVPVLVK (amino acids 246–260); peptide 2, KSDKNRIKLMNEILN (amino acids 496–510); peptide 3, EAKKEETWKLMEADK (amino acids 836–850). The synthetic peptides were conjugated by means of glutaraldehyde to keyhole limpet hemocyanin, emulsified with Freund’s complete adjuvant, and thereafter injected s.c. into a New Zealand White rabbit. After 3 and 6 weeks a second and third injection of conjugated material in incomplete adjuvant was made and after an additional 3 weeks the rabbit was bled. Antisera ASP45 and ASP14 were prepared against the P-glycoprotein peptides TKVGDKGTQLSGGQKQ (amino acids 1166–1181) and GTQLSGGQQRQIA (amino acids 1173–1187), respectively, as described previously (4).

Immunoblots. Membrane proteins (75 µg) were separated by electrophoresis in a 7.5% sodium dodecyl sulfate-polyacrylamide gel and the proteins were transferred to nitrocellulose paper as described by Towbin et al. (8). The paper was incubated in PBNT for 2 h at 37°C and thereafter incubated with antiserum diluted in PBNT for 15 h at room temperature. After a washing with PBNT the nitrocellulose was incubated with 125I-labeled protein A for 2 h at room temperature and thereafter washed extensively with PBNT. The paper was dried and immunoreactive proteins were detected by autoradiography. Molecular weight standards containing myosin (M, 212,000), β-galactosidase (M, 116,000), phosphorylase b (M, 97,000), bovine serum albumin (M, 66,200), catalase (M, 57,500), and aldolase (M, 40,000) were used to calculate the apparent molecular size of proteins reactive with the antipeptide sera.

Cell Membranes. A crude membrane fraction was isolated from sensitive and resistant cells as described previously (9). For certain experiments plasma membranes and endoplasmic reticulum were isolated after centrifugation of the crude membrane fraction in a discontinuous sucrose gradient (9). The membrane subfractions have been previously characterized enzymatically and by electron microscopy (9).

Results

Western Blot Analysis with Antisera Prepared against Peptides of the Deduced MRP Protein Sequence. Using an oligonucleotide probe based on the sequence of a cloned cDNA of the MRP gene (5) we have found that this sequence is highly overexpressed in HL60 cells isolated for resistance to Adriamycin. The oligonucleotide reacts with a single major mRNA of about 6 kilobases. In order to identify the protein encoded by MRP antisera were prepared against peptides synthesized according to the deduced sequence of the MRP protein (5). Western blot analysis with antisemum designated, ASPKE prepared against peptide 1 (see “Materials and Methods”) demon-
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Fig. 1. Western blot analysis of proteins reactive with an antiserum against a synthetic peptide of the Mrp protein. Crude membranes were prepared from sensitive and resistant cells and Western blot analysis using the ASPKE antiserum was carried out as described in "Materials and Methods." Lane 1. HL60/S; Lane 2. HL60/ADR; Lane 3. HL60/Vinc.

Fig. 2. Western blot analysis of membrane subfractions. Plasma membranes and endoplasmic reticulum were prepared from sensitive and HL60/ADR cells as described in "Materials and Methods" and used in Western blots with the following antisera. A. ASPKE; B. ASP45; C. ASP14. The sequence of peptide used to generate the various antisera is given in "Materials and Methods." Lanes 1 and 2 show the results obtained with endoplasmic reticulum of HL60/ADR and HL60/Vinc, respectively. The nature of the lower molecular weight proteins recognized by ASP45 (Fig. 3B) and ASP14 (C, Lane 1) is not known. P-gp indicates the position to which P-glycoprotein migrates.

Fig. 3. Western blot analysis of endoplasmic reticulum of HL60/ADR and HL60/Vinc cells. Endoplasmic reticulum from the resistant isolates was prepared as described in "Materials and Methods" and Western blot analysis was carried out using the ASPKE antiserum. Lanes 1 and 3. plasma membranes and endoplasmic reticulum, respectively, of sensitive cells. Lanes 2 and 4. plasma membranes and endoplasmic reticulum, respectively, of resistant cells.

Stratifies the presence of a 190-kDa protein contained in membranes of resistant cells (Fig. 1, Lane 2) but absent in membranes prepared from parental HL60 cells (Fig. 1, Lane 1). Essentially identical results have been obtained with antisera against peptides 2 and 3 (see "Materials and Methods") except that reactivity against p190 was less than that found with anti serum ASPKE (not shown). Parallel experiments have been carried out with membranes prepared from HL60 cells isolated for resistance to vincristine (3). These cells exhibit a multidrug resistance which is related to the presence of P-glycoprotein (3). The results show that ASPKE does not react with P-glycoprotein. This would be expected since the peptide sequence used to prepare ASPKE is not contained in the P-glycoprotein molecule (5). Western blot analysis has also been conducted with plasma membranes and endoplasmic reticulum prepared from sensitive and resistant HL60 cells. p190 detected with ASPKE is contained primarily in the endoplasmic reticulum (Fig. 2, Lane 4) with lower levels also found in the plasma membrane fraction (Fig. 2, Lane 2). p190 is not detectable in plasma membranes or endoplasmic reticulum prepared from sensitive cells (Fig. 2, Lanes 1 and 3). Other studies have also demonstrated that the cytoplasmic fraction of HL60/ADR cells does not contain any resistance associated protein reactive with the ASPKE antiserum (not shown). Additional studies have been carried out in which endoplasmic reticulum of HL60/ADR and HL60/Vinc cells were examined in parallel by Western blot analysis using antisera ASPKE (Fig. 3A) and
glycosylated form of p190 has a molecular mass of about 165 kDa. Based on the deduced sequence of a full length cDNA of MRP the gene encodes a protein containing 1531 amino acids (5, 7). Thus p165 has an apparent size close to that expected for the protein encoded by MRP. Previously, a 190-kDa protein was detected in HL60/ADR cells using an antiserum (ASP14) prepared against a synthetic peptide which corresponds to the deduced sequence of P-glycoprotein (4). This peptide has the sequence GTQLSGGKQKRIA (4) and contains some homology with the deduced sequence GVNLSG-GKQKQRSLA contained in the MRP protein (5, 7). This latter sequence contained in the MRP protein was presumably recognized by the ASP14 serum in studies with HL60/ADR cells (4). It is thus indicated that the ASP14 and ASPKE antisera recognize the same p190 protein contained in the HL60/ADR isolate. It is also of interest that the ASP14 peptide and the homologous sequence in MRP represent a domain which may be involved in nucleotide binding (5). This is consistent with previous studies which have shown that p190 is an ATP binding protein and is capable of reacting with the photoactive agent 8-azido[32P]ATP (3).

Previous studies have shown that the independent anthracycline resistant isolate HL60/AR (10) also contains overexpression of p190 as determined in Western blots using the ASP14 antiserum (4). More recently using antiserum against the ASP14 peptide p190 has been found to be overexpressed in the non-P-glycoprotein mediated resistant isolates CORL23/R (11) derived from a large cell lung cancer cell line (12), GLC4/R (13), derived from a small cell lung tumor cell line (14) and MOR/R derived from adenocarcinoma cells (13). Using appropriate MRP probes it has also been observed that this gene is highly overexpressed in HL60/AR,4 CORL23/R and MOR/R (15), and GLC4/R (16). These results thus demonstrate a close correlation between the levels of MRP expression and the levels of the p190 protein. Also the finding that MRP is overexpressed in many independent resistant isolates provides further support for an involvement of this gene in drug resistance. Future studies using the ASPKE antiserum should provide insight into the importance of the MRP protein in clinical drug resistance.

The results of this study demonstrate that the p190 protein of HL60/ADR cells detected with ASPKE and ASP14 has essentially the same electrophoretic mobility relative to the 180-kDa P-glycoprotein (Fig. 3). Experiments have also been conducted in which resistant cells were incubated in the absence or presence of tunicamycin for 15 h and endoplasmic reticulum was prepared and proteins reactive with ASPKE were examined by Western blot analysis. Treatment of cells with 2.5 or 5.0 µg/ml of tunicamycin results in the appearance of a resistance associated protein with a molecular mass of 165 kDa which is reactive with the antipeptide serum (Fig. 4, Lanes 2 and J). P165 is not present in cells grown in the absence of tunicamycin (Fig. 4, Lane J).

Discussion

The present study demonstrates that the MRP gene encodes a 190-kDa glycoprotein which is highly overexpressed in HL60 cells isolated for resistance to Adriamycin. The protein is present primarily in the endoplasmic reticulum of resistant cells but lower levels are also contained in the plasma membrane fraction. Studies using tunicamycin to block N-linked carbohydrate addition suggests that a reduced protein. Also the finding that MRP is overexpressed in many independent resistant isolates provides further support for an involvement of this gene in drug resistance. Future studies using the ASPKE antiserum should provide insight into the importance of the MRP protein in clinical drug resistance.

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