Phosphorylation of the Multidrug Resistance Associated Protein Gene Encoded Protein P190†

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ABSTRACT: Recent studies suggest that multidrug resistance of HL60/ADR cells is related to an overexpression of the MRP (multidrug resistance associated protein) gene which encodes a 190-kDa ATP-binding membrane glycoprotein. In the present study we have further characterized P190 and have examined phosphorylation properties of the protein. The results demonstrate that P190 is highly phosphorylated and that the phosphate groups are metabolically active and undergo cycles of phosphorylation and dephosphorylation in the cell. Serine is the single amino acid phosphorylated in P190 and the phosphate groups are contained in nine tryptic peptides. Experiments have also been conducted to analyze the effect of various protein kinase inhibitors on phosphorylation levels of P190. The results show that H-7, staurosporine, and chelerythrine can reduce the phosphorylation of this protein. In the presence of both H-7 (200 μM) and staurosporine (200 nM) the phosphorylation of P190 is completely blocked. It has also been found that in the presence of these agents there is a major increase in drug accumulation and concomitant inhibition in drug efflux of resistant cells. These results therefore suggest the possibility that certain phosphate groups of protein P190 play an important role in modulating drug accumulation in resistant cells.

Previously we have isolated and characterized HL60 cells selected for resistance to adriamycin (HL60/ADR) (Marsh et al., 1986). These cells are multidrug-resistant and defective in the cellular accumulation of drug without detectable levels of P-glycoprotein (Center, 1993). Recent studies have shown that the MRP[1] gene (Cole et al., 1992) is overexpressed in HL60/ADR and that the level of expression of this gene is greatly reduced as resistant cells revert to drug sensitivity (Krishnamachary et al., 1994). Evidence that the MRP gene contributes to experimental drug resistance is provided by the finding that transfection of sensitive cells with a full-length MRP cDNA contained in an expression vector results in the formation of a drug-resistant phenotype (Grant et al., 1994; Kruh et al., 1994). Recent studies have also shown that many non-P-glycoprotein multidrug-resistant isolates contain overexpression of MRP (Cole et al., 1992; Slovak et al., 1993; Zaman et al., 1993; Schneider et al., 1994; Berrand et al., 1994). Evidence indicates that the MRP gene encodes a 190-kDa (P190) membrane glycoprotein which is capable of binding ATP (McGrath et al., 1989; Marquardt et al., 1990; Krishnamachary et al., 1993). Analysis of the deduced sequence of P190 suggests that this protein is a member of a superfamily of proteins which are involved in a variety of transport processes (Cole et al., 1992). At the present time, however, there is essentially no information available on the mechanism by which P190 contributes to multidrug resistance. It is interesting to note that P190 may play an important role in the normal physiology of certain cell types since it has been recently demonstrated that this protein can function in an ATP-dependent transport of glutathione S-conjugates such as leukotriene C_4 into isolated membrane vesicles (Jedlitschky et al., 1994). In view of the potential importance of P190 in drug resistance and possibly other biological functions, it becomes of interest to characterize this protein in detail. In the present work we have examined phosphorylation levels of P190 and the possible involvement of the phosphate groups in regulating drug resistance.

MATERIALS AND METHODS

Materials. [^{32}P]Orthophosphoric acid (8500−9000 Ci/mmol) was purchased from Dupont/New England Nuclear. Staurosporine was from Behring Diagnostics, and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) was from Sigma. Chelerythrine was obtained from LC Services Corp.

Cell Lines. HL60 cells isolated for resistance to adriamycin were prepared as described previously (Marsh et al., 1986). These cells exhibit an 80-fold increase in resistance to the selecting agent. A spontaneous revertant of HL60/ADR and designated HL60/ADR/R was obtained as previously described (Krishnamachary et al., 1994). The revertant exhibits about a 3-fold increase in resistance to adriamycin.

Preparation of an Antibody against a Peptide Containing the P190 Deduced Sequence. A peptide having the sequence QRGLFYSMAKDAGLV and corresponding to the deduced sequence of the extreme C-terminal end of MRP (Cole et al., 1992) was synthesized by Research Genetics, Hunstville, AL. Antiserum designated 6KQ and prepared against this peptide was as described previously (Krishnamachary et al., 1994).

Labeling Cells with $^{32}P_i$ and Immunoprecipitation of P190. Sensitive, resistant, or revertant HL60 cells growing in RPMI medium containing 10% fetal bovine serum were centrifuged

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[1] Abbreviations: MRP, multidrug resistance associated protein; SDS, sodium dodecyl sulfate; PBS, 0.01 M sodium phosphate (pH 7.5)−0.15 M NaCl; FBS, fetal bovine serum; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; TPCK, L-1-tosylamino-2-phenylethyl chloromethyl ketone; PKC, protein kinase C.

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and washed once with phosphate-free RPMI. The cells were suspended in phosphate-free RPMI containing 3% fetal bovine serum and incubated for 2 h at 37 °C in a CO_2 incubator. $^{32}P_i$ was added to a final concentration of 250 μCi/mL and the cells were incubated in phosphate-free RPMI containing 3% fetal bovine serum (FBS). To test the effect of a protein kinase inhibitor on P190 phosphorylation, various concentrations of the agents were added 1 h prior to the addition of $^{32}P_i$. $^{32}P_i$ was added and incubations were continued for an additional 2 h. At the end of this incubation P190 was immunoprecipitated from cell extracts according to the procedure described by Hamada et al. (1987). Thus, labeled cell pellets were washed once with PBS and thereafter suspended in 3 mL of buffer S containing 50 mM Tris-HCl (pH 8.0), 140 mM NaCl, 2 mM sodium vanadate, 0.2 mM PMSF, 4 mM EDTA, and 0.5% sodium deoxycholate. Cell lysates were prepared by incubating the cells on ice for 30–40. The lysates were clarified by centrifugation at 10000*g* for 30 min. Three milliliters of the supernate was mixed with the 6KQ antiserum (1:75 dilution) and the solution was placed on a platform and rocked for 18 h at 10 °C. At the end of this time period, 150 μL of protein A-Sepharose CL-4B suspension (15% v/v) in 10 mM Tris-HCl (pH 8.0) was added and the solution was rocked for an additional hour at 10 °C. The solution was thereafter centrifuged and the immunoprecipitates were washed five times with 1.5 mL of buffer S. The washed immunoprecipitates were mixed with 80 μL of a 2× sample buffer containing 100 mM Tris-HCl (pH 6.8), 10% SDS, and 30% glycerol and incubated for 20 min at room temperature. This solution was mixed with 60 μL of 10 mM Tris-HCl (pH 8.0) and 10 μL of 1 M dithiothreitol, and the proteins were separated in an SDS–7% polyacrylamide gel (Laemmli, 1970). Radioactively labeled proteins were detected after autoradiography.

Effect of Protein Kinase Inhibitors on Drug Accumulation and Efflux. Sensitive and resistant cells growing in complete RPMI medium were centrifuged and resuspended in phosphate-free RPMI containing 3% FBS at a concentration of 8×10^5/mL. For drug accumulation studies the cells were incubated in the absence or presence of protein kinase inhibitor for 30 min followed by the addition of [^{3}H]-daunomycin (600 cpm/ng). Incubation was continued for 50 min at 37 °C, during which time steady state is reached. The cells were thereafter pelleted by centrifugation. The cell pellet was suspended in 0.1 mL of water and the radioactivity was determined. For drug efflux studies, sensitive and resistant HL60 cells plated in phosphate-free RPMI containing 3% FBS were incubated in the absence or presence of protein kinase inhibitor for 30 min followed by the addition of [^{3}H]daunomycin for 50 min. The cells were centrifuged and suspended in phosphate-free RPMI containing 3% FBS in the absence or presence of protein kinase inhibitor. The cells were incubated at 37 °C, aliquots were removed at various time periods, and the radioactivity contained in the cell pellet was determined.

Phosphoamino Acid Analysis. P190 was immunoprecipitated from ^{32}P-labeled cell extracts and proteins were separated by SDS–polyacrylamide gel electrophoresis. ^{32}P-labeled P190 was detected by autoradiography of the unfixed gel. The gel slice containing P190 was excised and washed extensively with 5% 2-propanol for 1 h followed by water for 5 h. The gel slice was resuspended in 1.5 mL of 50 mM NH_4HCO_3 and incubated overnight with 30 μg/mL TPCK-treated trypsin. After incubation, the gel slice was removed and the supernatant was lyophilized twice. The dried sample was suspended in 6 N HCl and hydrolyzed in sealed tubes for 1.5 h at 110 °C. The hydrolysate was suspended in water containing marker phosphoamino acids. The amino acids were thereafter separated by one-dimensional electrophoresis on cellulose thin-layer plates in acetic acid–pyridine–water (100:10:1890). ^{32}P-Labeled amino acids were detected by autoradiography and markers by ninhydrin spray.

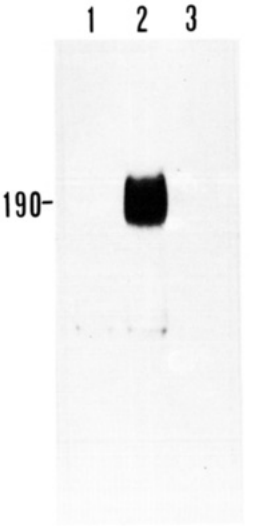

FIGURE 1: Immunoprecipitation of phosphorylated P190: sensitive (lane 1), resistant (lane 2), and revertant (lane 3). Cells were labeled with ^{32}P (250 μCi/mL) for 4 h at 37 °C and immunoprecipitation of proteins in cell extracts was carried out as described under Materials and Methods. Proteins contained in the immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis. After electrophoresis, radioactively labeled proteins were detected by autoradiography.

Analysis of Tryptic Peptides. ^{32}P-Labeled P190 prepared as described above was immunoprecipitated and thereafter electrophoresed in a SDS–7% polyacrylamide gel. The gel slice containing P190 as determined by autoradiography of the unfixed gel was washed extensively with 5% 2-propanol and thereafter with water. The gel slice was suspended in 1.5 mL of 50 mM NH_4HCO_3 and incubated overnight at 37 °C with 30 μg/mL TPCK-treated trypsin. After centrifugation the supernate was treated an additional 24 h at 37 °C with 30 μg/mL trypsin. After evaporation to dryness, the sample was suspended in water and spotted on a cellulose thin-layer plate. Two-dimensional tryptic peptide analysis was carried out using electrophoresis at pH 8.9 in 1% ammonium bicarbonate in the first dimension and chromatography in butanol–pyridine–acetic acid–water (15:10:3:12) in the second dimension. Labeled peptides were identified by autoradiography.

RESULTS

P190 Phosphorylation. Sensitive, resistant, and revertant cells were labeled with $^{32}P_i$ for 4 h and at the end of the labeling period cell extracts were prepared and immunoprecipitation in the presence of the 6KQ antiserum was carried out as described under Materials and Methods. Radioactively labeled proteins contained in the immunoprecipitates were analyzed after polyacrylamide gel electrophoresis and autoradiography. The results demonstrate that the antiserum

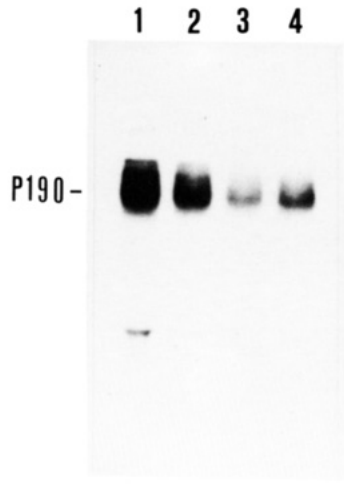

FIGURE 2: Pulse−chase analysis of phosphorylated P190. HL60/ADR cells were labeled with $^{32}P_i$ and pulse−chase analysis of phosphorylated P190 was conducted as described under Materials and Methods. Lanes 1−4 represent times 0, 20, 40, and 80 min, respectively, after the chase period.

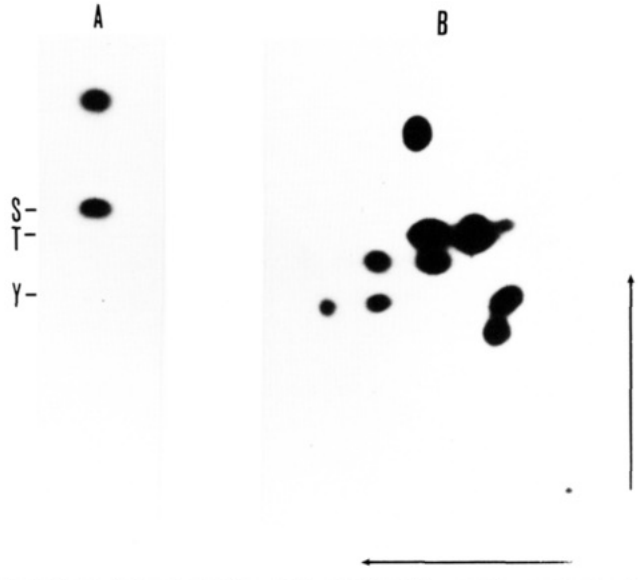

FIGURE 3: Phosphoamino acid and tryptic peptide analysis of phosphorylated P190. HL60/ADR cells were labeled with $^{32}P_i$ (250 μCi/mL) for 4 h at 37 °C and ^{32}P-labeled P190 was immunoprecipitated from cell extracts. Proteins contained in the immunoprecipitate were electrophoresed in an SDS−polyacrylamide gel, and after electrophoresis P190 was identified after autoradiography of the unfixed gel. The gel slice containing P190 was excised and phosphoamino acid (panel A) and tryptic peptide (pane B) analyses were carried out as described under Materials and Methods. In panel A the letters S, T, and Y refer to the positions to which serine, threonine, and tyrosine migrate, respectively. Radioactivity which migrates ahead of the amino acids represents free inorganic phosphate. In panel B the vertical arrow indicates the direction of electrophoresis and the horizontal arrow indicates the direction of thin-layer chromatography.

selectively immunoprecipitates a 190-kDa phosphorylated protein from extracts of HL60/ADR cells (Figure 1, lane 2). This protein is essentially absent in immunoprecipitation prepared from extracts of sensitive (lane 1) or revertant (lane 2) cells.

Pulse−Chase Analysis of P190 Phosphate Groups. In these experiments HL60/ADR cells were labeled with ^{32}P in low-phosphate medium for 2 h. The cells were centrifuged and thereafter suspended in complete RPMI (high phosphate) containing 10% FBS. An aliqout was taken for immunoprecipitation and the remaining cells were incubated at 37 °C. After various time periods, aliquots were taken, cell extracts were prepared, and immunoprecipitation was carried out in the presence of the 6KQ antiserum. The results clearly demonstrate that during the various chase periods in high-phosphate medium there is a major reduction in the levels of radioactively labeled phosphate groups in P190 (Figure 2). Western blot analysis of membrane proteins from unlabeled cells carried through the pulse−chase experiment demonstrate that during the course of this experiment there is no degradation of P190 (not shown).

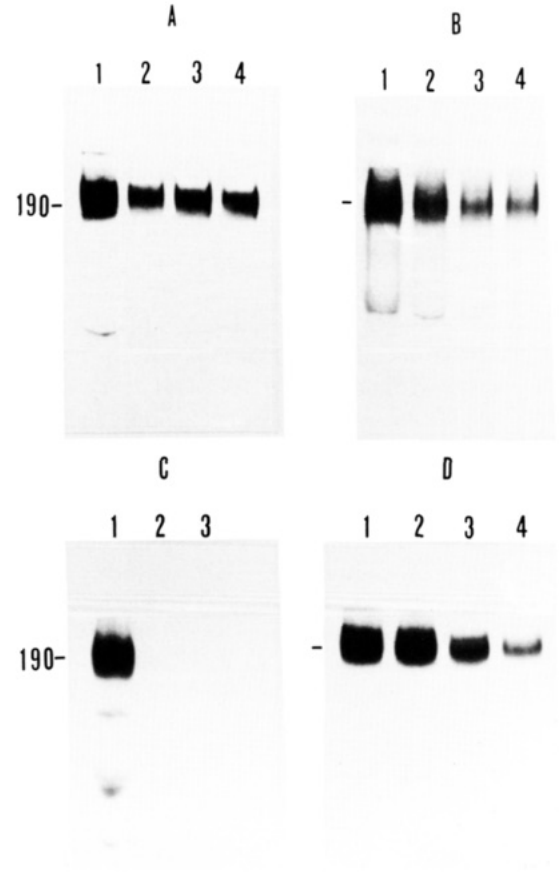

FIGURE 4: Effect of protein kinase inhibitors on P190 phosphorylation. HL60/ADR cells were centrifuged and suspended in fresh phosphate-free RPMI containing 3% FBS. The cells at a concentration of 8×10^5/mL were thereafter preincubated for 1 h with staurosporine, H-7, or chelerythrine. For certain experiments cells were preincubated with both staurosporine and H7 for 1 h. After the preincubation period, $^{32}P_i$ was added (250 μCi/mL) and the cells were incubated for an additional 2 h. Upon completion of the labeling period, cell extracts were prepared and ^{32}P-labeled P190 was immunoprecipitated as described under Materials and Methods. Radioactively labeled proteins were analyzed after electrophoresis in an SDS−7% polyacrylamide gel. (Panel A) Lanes 1−4: H-7 was used at concentrations of 0, 50, 100, and 200 μM, respectively. (Panel B) Lanes 1−4: Staurosporine was used at concentrations of 0, 50, 100, and 200 nM, respectively. (Panel C) Lane 1, no inhibitor; lane 2, 100 μM H7 and 100 nM staurosporine; lane 3, 200 μM H7 and 200 nM staurosporine. (Panel D) Lanes 1−4: chelerythrine was used at concentrations of 0, 5, 10, and 20 μM, respectively.

Analysis of P190 Phosphorylated Amino Acids and Tryptic Peptides. The identification of phosphoamino acids contained in P190 and phosphorylated tryptic peptides was carried out as described under Materials and Methods. The results demonstrate that P190 is phosphorylated exclusively on serine residues (Figure 3A). Further studies demonstrate

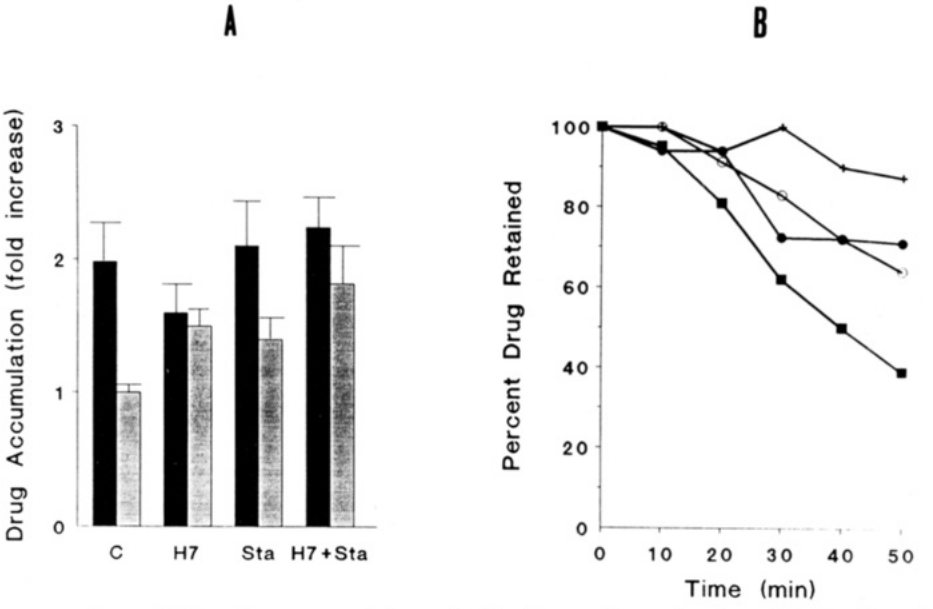

FIGURE 5: Effect of staurosporine and H-7 on drug accumulation and efflux in sensitive and resistant HL60 cells. (Panel A) Sensitive and resistant cells were preincubated 30 min in the absence of protein kinase inhibitor or in the presence of 200 μM H-7, 200 nM staurosporine, or 200 μM H-7 and 200 nM staurosporine. Upon completion of the preincubation period, the accumulation of [^{3}H]daunomycin was determined after a 50-min incubation period as described under Materials and Methods. In these experiments the control represents drug accumulation in sensitive or resistant cells in the absence of protein kinase inhibitor. This level of drug accumulation is compared to that occurring in the presence of various protein kinase inhibitors. Sensitive and resistant cells are represented by the darkly and lightly shaded bars, respectively. (Panel B) Resistant cells were preincubated in the absence or presence of 200 μM H-7, 200 nM staurosporine, or 200 μM H7 and 200 nM staurosporine and the effect of these agents on efflux of [^{3}H]daunomycin was determined as described under Materials and Methods. Symbols: ■, control; ●, H-7; ○, staurosporine; +, H-7 plus staurosporine.

that hydrolysis of ^{32}P-labeled P190 in the presence of trypsin results in the formation of nine tryptic peptides (Figure 3B).

Effect of Various Protein Kinase Inhibitors on P190 Phosphorylation. Detailed studies have been carried out to examine the effect of various protein kinase inhibitors on the phosphorylation of P190. In these experiments cells were preincubated with various concentrations of inhibitor for 1 h followed by the labeling of HL60/ADR for a 2-h time period. Cell extracts were prepared and P190 was immunoprecipitated as described under Materials and Methods. The results demonstrate that H-7 at concentrations of 50, 100, or 200 μM partially inhibits P190 phosphorylation (Figure 4A). Staurosporine at concentrations of 50, 100, or 200 nM also blocks P190 phosphorylation and the effect is somewhat greater than that seen with H-7 (Figure 4B). It was also observed, however, that incubation of HL60/ADR cells in the presence of both H-7 (100 μM) and staurosporine (100 nM) (Figure 4C, lane 2) or in the presence of H-7 and staurosporine at concentrations of 200 μM and 200 nM, respectively (Figure 4C, lane 3), results in essentially a complete block in P190 phosphorylation. Chelerythrine at concentrations of 5, 10, or 20 μM also inhibits the phosphorylation of P190 (Figure 4D).

Effect of Various Protein Kinase Inhibitors on Drug Accumulation and Efflux in Resistant Cells. Previous studies have shown that HL60/ADR cells are defective in drug accumulation and this may be related to an enhanced drug efflux system (Marsh et al., 1986). It has also been demonstrated that resistant cells which have reverted to drug sensitivity undergo a loss in the drug accumulation defect and a parallel loss in the levels of P190 (Krishnamachary et al., 1994). These results strongly suggest that protein P190 plays a role in modulating levels of drug accumulation in resistant cells. In view of this, studies were conducted to determine if the phosphorylation of P190 plays some role in regulating the biological function of the protein. In these experiments the protein kinase inhibitors capable of reducing P190 phosphorylation were also tested for their effect on drug accumulation. In the presence of H-7 (200 μM) or staurosporine (200 nM) the levels of drug accumulation increase but are still less than the levels of drug present in the control sensitive cell (Figure 5A). If, however, H-7 (200 μM) and staurosporine (200 nM) are mixed together, the two agents can further enhance drug accumulation to a level comparable to that found in control sensitive cells (Figure 5A). Additional experiments have been carried out to analyze the effect of staurosporine and H-7 on [^{3}H]daunomycin efflux from resistant cells. It was found that each agent alone could induce a slight decrease in the rate of drug efflux from the HL60/ADR isolate (Figure 5B). However, in the presence of both H-7 (200 μM) and staurosporine (200 nM) there is essentially a complete inhibition in the efflux of drug from resistant cells (Figure 5B). Similar types of experiments have been carried out in the presence of the protein kinase inhibitor chelerythrine (Figure 6). In the presence of this agent there is a concentration-dependent increase in the accumulation of [^{3}H]daunomycin in resistant cells (Figure 6A). This agent produces only a slight increase in the accumulation of drug in sensitive cells. Additional studies have also demonstrated that in the presence of 10 μM chelerythrine there is a major reduction in the rate of [^{3}H]daunomycin efflux from the resistant isolate (Figure 6B).

DISCUSSION

The results of the present study demonstrate that the MRP-encoded protein P190 is highly phosphorylated and that the phosphate groups are metabolically active in the cell. It was also observed that treatment of HL60/ADR cells with the

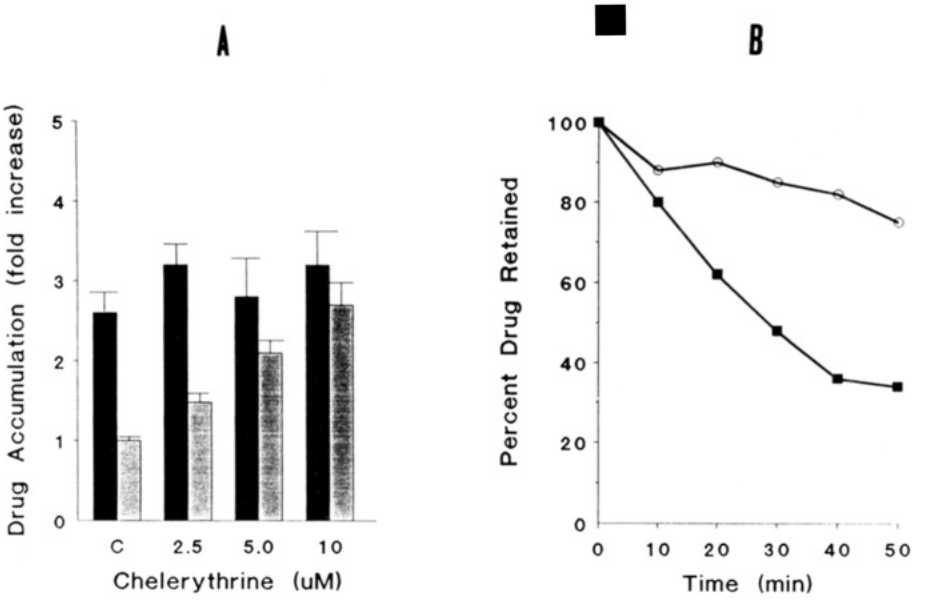

FIGURE 6: Effect of chelerythrine on drug accumulation and efflux. (Panel A) Sensitive and resistant cells were preincubated for 30 min in the absence or presence of the indicated concentrations of chelerythrine and the effect of this agent on the accumulation of [^{3}H]daunomycin was carried out as described under Materials and Methods. In these experiments the control represents drug accumulation in sensitive and resistant cells in the absence of chelerythrine. The effect of different concentrations of chelerythrine on this level of drug accumulation is indicated in the figure. Sensitive and resistant cells are represented by the darkly and lightly shaded bars, respectively. (Panel B) Resistant cells were preincubated with 10 μM chelerythrine for 30 min and the effect of this agent on [^{3}H]daunomycin efflux was determined as described under Materials and Methods. Symbols: ■, control; ○, plus chelerythrine.

protein kinase inhibitors H-7, staurosporine, and chelerythrine reduces the phosphorylation of P190 and also reverses the drug accumulation defect which is characteristic of this isolate (Marsh et al., 1986). These results therefore suggest the possibility that phosphorylation of P190 plays an important role in regulating the biological function of this protein. Previous studies have shown that an analysis of daunomycin distribution in cells overexpressing MRP by fluorescence microscopy suggest that some drug is sequestered into intracellular organelles away from cytotoxic targets (Gervasoni et al., 1991; Marquardt & Center, 1992; Barrand et al., 1993). At the present time the exact relationship between sequestration and pathways of drug efflux are not known. P190 may thus function to channel drugs into specific organelles for sequestration and/or efflux, and P190 phosphate groups may play a role in this process. Previous studies have shown that the cystic fibrosis transmembrane regulator which functions as a chloride channel is regulated by phosphorylation of a specific domain of the protein (Quinlon, 1990; Welsh, 1990). Recently it has also been shown that P190 is capable of transporting leukotriene–glutathione conjugates into membrane vesicles (Jedlitchky et al., 1994). Possibly P190 can transport glutathione drug conjugates and that phosphorylation is important in this process. It is interesting to note, however, that P-glycoprotein is phosphorylated (Carlsen et al., 1977; Garman et al., 1983; Hamada et al., 1987; Roy & Hurwitz, 1985), and studies with protein kinase inhibitors suggest that phosphorylation may regulate the drug transporter activity of this protein (Ma et al., 1991; Sato et al., 1990; Bates et al., 1993; Chambers et al., 1991). However, despite these results, there is still no direct evidence which indicates that phosphorylation is important in the biological function of this protein.

The protein kinase(s) which may be involved in the phosphorylation of P190 is not known. The protein is phosphorylated exclusively on serine residues and the phosphate groups are contained in nine tryptic peptides. Since multiple phosphorylation sites may be contained within these nine peptides, it seems indicated that the pattern of P190 phosphorylation is highly complex. Agents such as staurosporine and H-7, which are capable of reducing P190 phosphorylation, have been found to inhibit a variety of protein kinases, including protein kinase C and A, with about equal potency (Hidaka et al., 1984; Tamaoki et al., 1986). In contrast, chelerythrine seems to be more specific for PKC and inhibits this enzyme with a much greater potency than other kinases which have been tested (Herbert et al., 1990). PKC may thus be involved in phosphorylating P190, but it would be expected that other protein kinases are also involved in this event. Inspection of the deduced sequence of P190 indicates the presence of seven serine PKC sites and 16 serine casein kinase II sites. Whether any of these sites are actually involved in regulating drug accumulation must await more direct evidence which may be accrued by studying the effect of transfecting cells with MRP cDNA which has been mutagenized at sites that alter the pattern of P190 phosphorylation.

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