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IDENTIFICATION AND CHARACTERIZATION OF A PLASMA MEMBRANE PHOSPHOPROTEIN WHICH IS PRESENT IN CHINESE HAMSTER LUNG CELLS RESISTANT TO ADRIAMYCIN

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Abstract—Studies have been carried out to analyze the phosphoprotein composition of plasma membranes from Chinese hamster lung cells resistant to the action of adriamycin. Gel electrophoretic analysis of [$^{32}P_i$]-labeled proteins revealed that plasma membranes from resistant cells contain a phosphoprotein of 180,000 molecular weight (P180) which is not detected in drug sensitive cells. Protein P180 can also be identified after phosphorylation of resistant plasma membranes in an *in vitro* protein kinase system. Pulse-chase experiments indicated that the P180 was metabolically active and underwent cycles of phosporylation and dephosphorylation in the cell. Additional studies showed that, in the presence of *N*-ethylmaleimide (NEM), there was a major increase in the uptake of adriamycin in resistant cells. A similar effect was observed with KCN but not with sodium azide. When resistant cells were grown in the presence of [$^{32}P_i$] and then incubated in the presence of NEM, there was a considerable increase in the phosphorylation of P180. In contrast, many other plasma membrane proteins were dephosphorylated under these incubation conditions. The results suggest the possibility that, as P180 was hyperphosphorylated, the protein was inactivated and this contributed to the ability of resistant cells to accumulate adriamycin.

Previous studies have demonstrated that a variety of cell lines isolated for resistance to adriamycin or daunomycin contain a defect which restricts cellular accumulation of drug. Evidence indicates that this defect consists primarily of two components, a restriction to drug uptake [1–6] and the presence of a highly active efflux mechanism which rapidly extrudes drug from the cell [3,4,6,7]. It has also been observed that, when resistant cells are incubated in the presence of various metabolic inhibitors in the absence of glucose, there is a major uptake and retention of drug [3,4,8,9]. Evidence indicates that the metabolic inhibitors block drug efflux [3,4,8], but the agents may also have the effect of enhancing uptake as well.

Previously we have shown that adriamycin resistant cells contain a 180,000 molecular weight plasma membrane glycoprotein (P180) which is not detected in cells sensitive to drug [5]. A protein of similar size has also been detected on the surface of cells resistant to colchicine [10], vinblastine [11] and actinomycin D [12]. Many of these cell types are also cross-resistant to adriamycin and daunomycin [1,4,13]. These results taken together indicate a strong correlation between the presence of P180 and the drug resistant phenotype.

In the present study, evidence is presented that protein P180 is modified by phosphorylation and that the phosphoprotein is metabolically active in the cell. Evidence has also been obtained that, in the presence of certain metabolic inhibitors which increase drug accumulation in the cell, there is a considerable enhancement of P180 phosphorylation. This phosphorylation event may be a means of regulating the activity of P180 in drug resistant cells.

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 [5]. 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 from sensitive and resistant cells were isolated using a minor modification of the method described by Esko *et al.* [14]. The crude cell membrane preparation was applied to a discontinuous sucrose gradient (16–60% sucrose) and thereafter centrifuged for 2 hr at 35,000 rpm in a Spinco SW50.1 rotor. The respective fractions containing plasma membranes and endoplasmic reticulum were collected, diluted in 0.01 M Tris–HCl (pH 7.6), and pelleted by centrifugation for 1 hr at 35,000 rpm in a Spinco SW50.1 rotor. The membrane preparations were suspended in 0.01 M Tris–HCl (pH 7.6) and stored at −70°. The isolated plasma membranes are enriched 10-fold for 5′nucleotidase activity [15] and are essentially free of succinic dehydrogenase [16] and NADPH–cytochrome *c* reductase [16]. Analysis of thin sections of plasma membranes in the electron microscope reveals the presence of typical membrane vesicles which are essentially devoid of contaminating cell organelles.

Labeling cells with $^{32}P_i$. Sensitive and resistant

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HT-1 cells were grown in 100 mm dishes in DMEM plus 10% fetal calf serum for 48 hr. The medium was then removed, and 1 ml of TG media containing 50 μCI of $^{32}P_i$ was added to each dish. After incubation at 37° for various time periods, plasma membranes were prepared. TG media contained 0.05 M Tris–HCl (pH 7.6), 0.15 M NaCl, 0.005 M KCl, 0.0055 M D-glucose, 1X MEM amino acids and vitamins. For pulse-chase experiments, the cells were incubated with $^{32}P_i$ for 1 hr and then washed with 0.01 M sodium phosphate (pH 7.6)–0.15 M NaCl. DMEM containing 10% serum was added, and at various time periods the cells were collected and membranes were prepared.

Effect of N-ethylmaldeimide (NEM) and sodium azide on cellular drug accumulation. Sensitive and resistant cells were grown in standard media in 60 mm dishes. The cells were washed once with glucose-free DMEM. Four millimeters of this same media containing 5% fetal calf serum was added followed by the addition of sodium azide or NEM to a final concentration of 0.01 M. Control cells were incubated in DMEM containing 0.025 M glucose and 5% fetal calf serum in the absence of inhibitor. After a 10-min incubation at 37°, adriamycin was added to a concentration of 10 μg/ml. At various times after the addition of the drug, the medium was removed, and the cells were washed once with 0.01 M sodium phosphate (pH 7.4)–0.15 M NaCl (PBS). The cells were scraped into 1 ml of PBS and centrifuged. The drug contained in the cell pellet was extracted with 0.3 N HCl–50% ethanol [17], and the fluorescence of the supernatant fraction was determined (excitation, 470 nm; fluorescence, 585 nm).

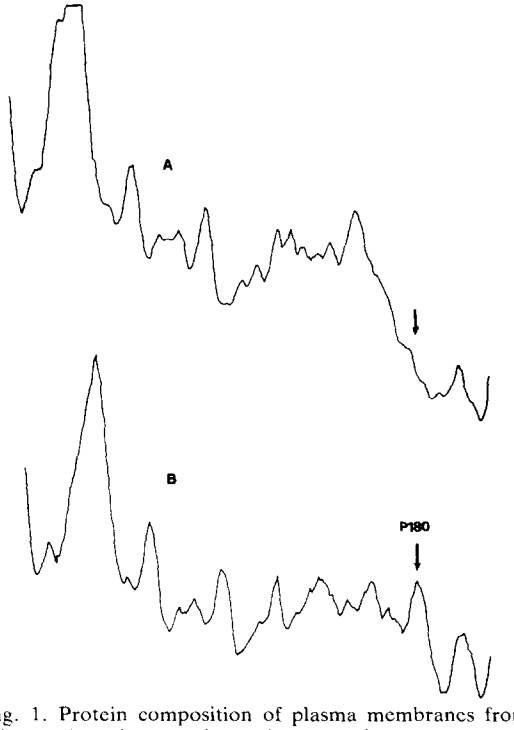

Fig. 1. Protein composition of plasma membranes from adriamycin resistant and sensitive cells. Plasma membranes from sensitive and resistant cells were dissolved in 0.5% sodium dodecyl sulfate and the proteins were labeled with ^{125}I in the chloramine-T reaction. The labeled proteins from sensitive (A) or resistant (B) membranes were then analyzed after electrophoresis in a 7% polyacrylamide gel. In this figure and others which show the results of gel electrophoretic procedures, the migration of the protein material is from right to left. The results are presented as densitometric scans of the autoradiogram.

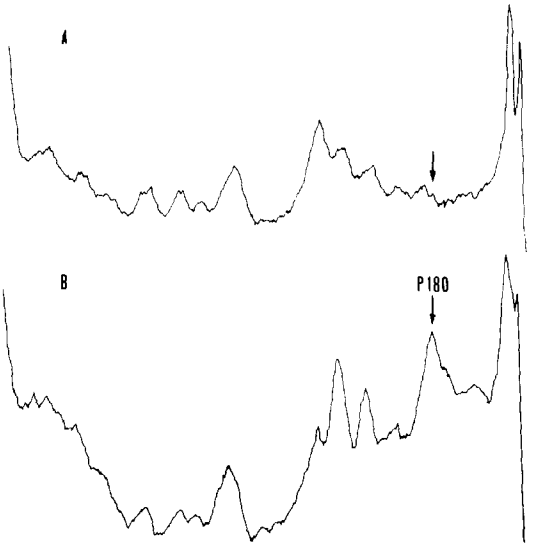

Fig. 2. Phosphoprotein composition of plasma membranes of sensitive and resistant cells. Cells were labeled for 1 hr with $^{32}P_i$ as described in Materials and Methods. Plasma membranes were isolated and the protein from sensitive (A) or resistant (B) cells was analyzed after electrophoresis in a 7% polyacrylamide gel.

In vitro *phosphorylation of isolated plasma membranes.* Plasma membranes isolated from sensitive and resistant cells were incubated in a reaction mixture containing 0.01 M Tris–HCl (pH 7.6), 0.015 M $MgCl_2$, 0.001 M β-mercaptoethanol, and 0.2 μM [γ-^{32}P]ATP. Incubations were carried out on ice for various time periods, EDTA was added to 0.01 M, and the phosphorylated proteins were analyzed after electrophoresis in a 7% polyacrylamide gel.

Gel electrophoresis. Polyacrylamide gel electrophoresis was carried out as described previously [5]. Autoradiograms were traced with a Joyce-Loebl densitometer.

Labeling plasma membrane proteins with ^{125}I. Plasma membrane proteins were labeled with ^{125}I in the presence of chloramine-T according to the procedure described by Klinman and Taylor [18].

Protein. Protein was determined according to the method of Peterson [19].

RESULTS

Protein composition of plasma membranes from resistant cells. Isolated plasma membranes from sensitive and resistant cells were dissolved in sodium dodecyl sulfate, and the total proteins were labeled with ^{125}I in the chloramine-T procedure [18]. The labeled proteins were analyzed after polyacrylamide gel electrophoresis (Fig. 1). The results of several different experiments demonstrated that the plasma membranes of resistant cells contain a protein of 180,000 molecule weight (P180) which was not detected in cells sensitive to adriamycin (Fig. 1). In

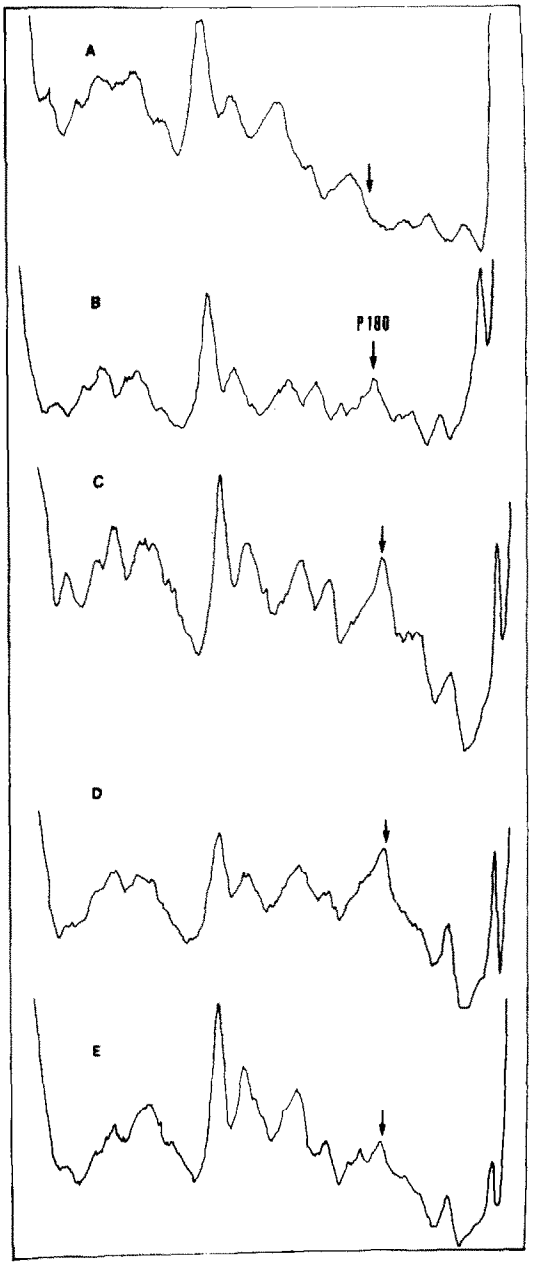

Fig. 3. Pulse-chase experiment of membrane phosphorylated proteins. The pulse-chase experiment was carried out as described in Materials and Methods. The phosphorylated proteins were analyzed in crude membrane preparations (plasma membranes plus endoplasmic reticulum) in order to minimize membrane manipulations and avoid differential loss of protein material. Pulse periods with 50 μCi/ml of $^{32}P_i$ were for 1 hr. Chase periods were for 20, 40 and 60 min. Panel A: sensitive cell membranes, 1 hr pulse with $^{32}P_i$. Panel B: resistant cell membranes, 1 hr pulse with $^{32}P_i$. Panel C–E: resistant membranes chase period, 20, 40 and 60 min respectively.

many experiments of this type no other protein alteration has been noted in resistant plasma membranes. In studies carried out thus far using ^{125}I-labeling, we have not been able to detect the presence of P180 in isolated endoplasmic reticulum.

Plasma membrane phosphoproteins in resistant cells. In these experiments, sensitive and resistant cells were incubated in media containing $^{32}P_i$. The plasma membranes of labeled cells were then isolated, and the proteins were analyzed after polyacrylamide gel electrophoresis. The results of these studies clearly demonstrate that resistant cell membranes contain a major phosphorylated protein of 180,000 molecular weight which was not detected in membranes isolated from sensitive cells (Fig. 2). This protein was found to co-migrate with the 180K material labeled with ^{125}I in the chloramine-T procedure. The results of these experiments and those described previously [5] strongly suggest that a 180K protein which is both glycosylated and phosphorylated is uniquely present in plasma membranes of resistant cells.

Additional studies have been carried out in which the phosphorylation pattern of P180 was examined in pulse-chase experiments. In these studies cells were labeled for 1 hr with $^{32}P_i$ in low phosphate media and thereafter incubated in high phosphate media for various time periods. After the pulse-labeling period, P180 was detected in resistant but not sensitive cell membranes (Fig. 3, A and B). Incubation of resistant cells in high phosphate media for a 20-min period resulted in about a 2-fold increase in the $^{32}P_i$-labeled phosphate of P180 (Fig. 3C). During an additional 20 min chase period, the phosphate level of the protein declined only slightly (Fig. 3D). However, after a third 20 min chase there was considerable loss of labeled phosphate from P180 and only minor levels of the phosphorylated protein was detected.

In vitro *labeling of* P180 *in isolated plasma membranes.* Experiments have also been carried out in

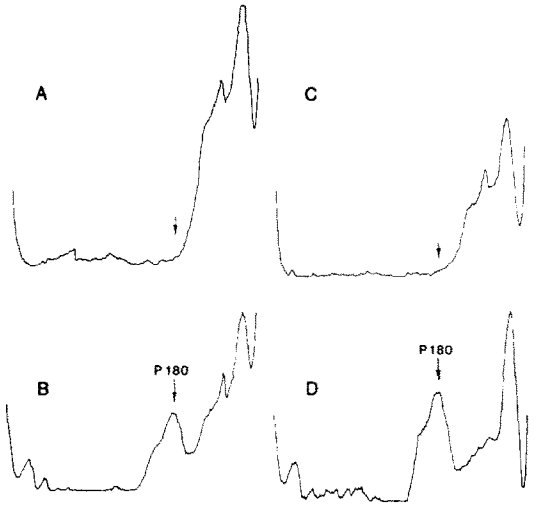

Fig. 4. *In vitro* phosphorylation of plasma membrane proteins. *In vitro* phosphorylation of plasma membrane proteins was carried out as described in Materials and Methods. Incubations were carried out on ice for 1 min (panels A and B) or 5 min (panels C and D). At the end of the incubation period, EDTA was added to 0.01 M, and the phosphorylated proteins were analyzed after electrophoresis in a 7% polyacrylamide gel. Panels A and C are incubations with sensitive plasma membranes; panels B and D are incubations with resistant plasma membranes.

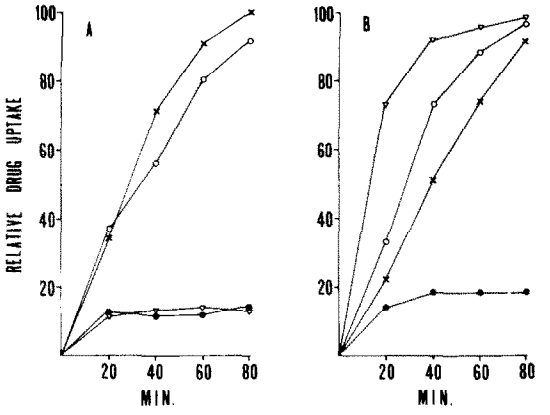

Fig. 5. Effects of sodium azide and NEM on the uptake of adriamycin. Uptake experiments with sensitive and resistant cells were carried out as described in Materials and Methods. Panel A: effect of sodium azide; panel B: effect of NEM on drug uptake. Symbols: (●——●), resistant cells without inhibitor; (▽——▽) resistant cells with inhibitor; (×——×) sensitive cells without inhibitor; and (○——○) sensitive cells with inhibitor.

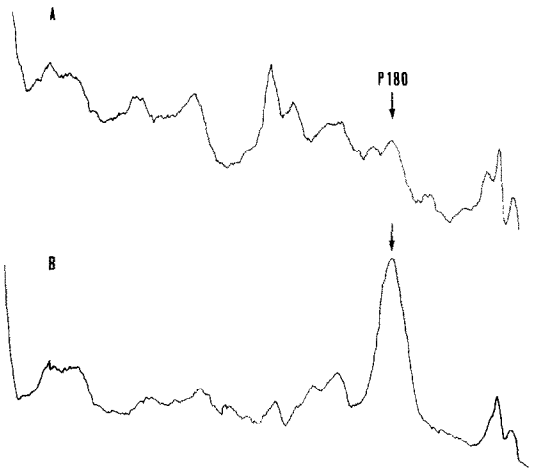

Fig. 6. Effect of NEM on pre-labeled membrane phosphoproteins. Adriamycin resistant cells were labeled with 40 μCi/ml of $^{32}P_i$ for 1 hr as described in Materials and Methods. At the end of the labeling period, the cells were washed twice with 0.01 M Tris–HCl (pH 7.6). Low phosphate medium without glucose was added followed by the addition of NEM to one set of cells to a final concentration of 0.01 M. After a 30-min incubation period, the cells were collected and membranes were prepared. The phosphorylated proteins were analyzed after electrophoresis in a 7% polyacrylamide gel. An equal amount of radioactivity from cells incubated in the absence (A) or presence (B) of NEM was applied to the gel.

which isolated plasma membranes from resistant and sensitive cells were incubated for brief time periods in the presence of Mg^{2+} and [γ-^{32}P]ATP. The proteins labeled in the *in vitro* reaction were examined after polyacrylamide gel electrophoresis. During a 1-min incubation, resistant plasma membranes contained a major phosphorylated protein of 180,000 molecular weight (Fig. 4B). This protein was not detected when plasma membranes from sensitive cells were incubated under identical conditions (Fig. 4A). If the incubation period was continued for 5 min, the level of phosphorylated P180 was considerably increased, and there was still no evidence for the labeling of a similar molecular weight protein in sensitive cells (Fig. 4, C and D). Of particular interest is the finding that, under the incubation conditions used, P180 appeared to be selectively labeled and other membrane proteins, despite being phosphorylated *in vivo*, were labeled to only a minor extent in the *in vitro* system.

Effect of metabolic inhibitors on adriamycin accumulation in resistant cells. Previous studies from several laboratories have shown that incubation of adriamycin resistant cells with various metabolic inhibitors results in a considerable increase in cellular accumulation of drug [3,4,8]. The basis of this finding appears to be related to the ability of these agents to inhibit the drug efflux mechanism which is highly active in resistant cells [3,4,6–9]. Since the efflux mechanism appears to be energy dependent and possibly related to P180 phosphorylation, we have examined the effects of various metabolic inhibitors on drug accumulation in HT-1 cells resistant to adriamycin. As shown in Fig. 5A, sodium azide had essentially no effect on drug accumulation in HT-1 resistant or sensitive cells. This is in contrast to results obtained with a variety of other cell lines [4,9] but accounts for our previous inability to detect an energy-dependent uptake and retention of drug in HT-1 resistant cells [5]. In contrast to sodium azide, NEM had a major effect on cellular drug accumulation. Incubation in glucose-free media in the presence of 0.01 M NEM resulted in a rapid uptake and retention of drug (Fig. 5B). The cellular drug content was always equal to or slightly greater than that for sensitive cells treated under identical conditions. We also consistently observed that NEM caused a slight increase in drug accumulation in sensitive cells (Fig. 5B). In addition we found that KCN had an effect on adriamycin uptake and retention identical to that observed for NEM.

Effect of NEM on the metabolism of pre-labeled membrane phosphoproteins. In these experiments, we examined the effect of NEM on pre-labeled phosphoproteins of crude and plasma membranes isolated from resistant and sensitive cells. The cells were labeled with $^{32}P_i$ and, after removal of the media, were incubated in glucose-free media containing 0.01 M NEM. After a 30-min incubation period, membranes were isolated and the proteins were analyzed after polyacrylamide gel electrophoresis. The crude membrane preparation (plasma membranes plus endoplasmic reticulum) prepared from resistant cells grown in glucose-free media for 30 min contained multiple components including P180 (Fig. 6A). When the resistant cells were incubated under identical conditions in the presence of NEM, many of the protein components were dephosphorylated while certain other proteins underwent essentially no change in $^{32}P_i$-labeled phosphate content (Fig. 6B). In contrast to these proteins the $^{32}P_i$-labeled phosphate of P180 increased several-fold (Fig. 6B). A significant increase in P180 phosphorylation can also be observed after a 20-min incubation in the presence of NEM. Additional studies have been

carried out in which the plasma membranes were isolated, and the phosphoprotein pattern was analyzed. Those membranes isolated from NEM-treated cells contained as the major phosphorylated protein P180. The level of $^{32}P_i$-labeled phosphate of this protein was found to be 6-fold greater than that for P180 contained in plasma membranes isolated from cells incubated in the absence of inhibitor. In drug sensitive cells labeled under similar conditions in the absence of NEM, P180 was not detected in the crude membrane preparation. However, in the presence of NEM, a minor protein of 180K was identified after gel electrophoresis. We have not, however, observed the presence of this protein in isolated plasma membranes. Additional studies have been carried out in which cells pre-labeled with $^{32}P_i$ were incubated in the presence of sodium azide in glucose-free media. Under these conditions, there was no increase in the phosphorylation pattern of P180.

DISCUSSION

Previous studies have shown that cells resistant to colchicine [10], vinblastine [11], actinomycin D [12] and adriamycin [5] contain a unique cell surface protein of about 180,000 molecular weight. Certain lines of evidence indicate that this protein may be required for a cell to exhibit a drug resistant phenotype. This is based on the finding that there is a correlation between cellular levels of P180 and drug resistance [10,11]. In addition, it has also been observed that revertants of colchicine resistant cells contain only low levels of this protein [20].

In the present study, we have shown that P180 was phosphorylated and that the labeled phosphate groups were metabolically active in the cell. Thus, during pulse experiments the protein was labeled with $^{32}P_i$ and within a 60-min chase period most of the labeled phosphate was removed. Other work has shown that P180 is a glycoprotein [5,10–12], and at least a portion of the molecule is exposed on the cell surface [5]. The finding that P180 was phosphorylated is of particular interest since previous studies and those of the present work show that various metabolic inhibitors are capable of inducing a major accumulation of drug in resistant cells [3,4,6–9].

The results of the present study suggest a mechanism whereby agents such as NEM act to enhance drug accumulation in resistant cells. It is suggested that P180 in resistant cells is phosphorylated at critical sites and, as such, is functional and is required for the drug exclusion mechanism. In the presence of NEM, P180 becomes phosphorylated at additional sites. This results in the inactivation of the protein and the cell is thus capable of drug uptake and retention. Of particular interest is the finding that in NEM-treated cells P180 was selectively phosphorylated. Under the conditions of the experiment, most membrane proteins were dephosphorylated, while some were not changed in phosphate content. It is also of interest to note that P180 underwent a highly selective phosphorylation in a system in which plasma membranes were incubated in the presence of Mg^{2+} and [γ-^{32}P]ATP. The mechanism by which a single membrane protein becomes highly phosphorylated after treatment of cells with certain metabolic inhibitors is completely unknown. Possibly the topological arrangement of the protein in the lipid bilayer and/or certain structural features make it highly accessible for phosphorylation. P180 could also be a protein kinase which is capable of undergoing a rapid autophosphorylation. In any case, understanding these events should provide greater insight into the basis of adriamycin resistance and also the function and mechanisms of protein phosphorylation in plasma membranes.

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