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MECHANISMS REGULATING CELL RESISTANCE TO ADRIAMYCIN

EVIDENCE THAT DRUG ACCUMULATION IN RESISTANT CELLS IS MODULATED BY PHOSPHORYLATION OF A PLASMA MEMBRANE GLYCOPROTEIN

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Abstract—Incubation of adriamycin resistant Chinese hamster lung cells with the calmodulin inhibitor trifluoperazine (TFP) resulted in a significant increase in the cellular accumulation of drug. When resistant cells were prelabeled with $^{32}P_i$ and then treated with TFP, a major increase also occurred in the phosphorylation of a plasma membrane glycoprotein (P-180). The concentration of TFP required for inducing the superphosphorylation of this protein correlated well with the TFP concentration required for inducing an increase in drug accumulation in resistant cells. In addition to TFP, the Ca^{2+} channel blocker verapamil also induced drug uptake and enhanced the phosphorylation level of P-180. Additional studies showed that, when resistant cells reverted to drug sensitivity, there was a parallel loss in the TFP-induced P-180 phosphorylation. The results of this study indicate that the trifluoperazine-induced uptake of drug in resistant cells is mediated by a mechanism which involves an enhanced phosphorylation of P-180. It is suggested that, when this protein is superphosphorylated, it becomes biologically inactive, and that this results in the conversion of the resistant cell to one having a drug sensitive phenotype.

Previous studies have shown that cells resistant to the antitumor agent adriamycin are highly defective in the cellular accumulation of this drug [1–6]. This defect appears to be due to an impaired drug transport into the cell [4, 5] and/or a major enhancement of a drug efflux mechanism [2, 3, 5, 6]. These findings seem to indicate that plasma membrane alterations may make a significant contribution to the drug resistant phenotype. Consistent with this is the finding that plasma membranes of adriamycin resistant cells contain a phosphorylated glycoprotein (P-180) that is not detected in cells sensitive to drug [1]. A similar type of protein has also been detected in plasma membranes of cells selected for resistance to colchicine [7], vinblastine [8], vincristine [9], actinomycin D [10] and daunomycin [9, 11]. These isolates are cross resistant to adriamycin [5, 12, 13], and recent studies show that the surface glycoproteins of cells isolated for resistance to colchicine, actinomycin D, daunorubicin and vinblastine are cross-reactive immunologically [14]. It has also been shown that, when resistant cells revert to drug sensitivity, there is a parallel loss in the cell surface glycoprotein [7, 9, 15]. Furthermore, an analysis of partial revertants has also demonstrated a direct correlation between the degree of resistance and the level of this protein [15]. These results taken together strongly suggest that the plasma membrane glycoprotein plays a role in the multi-drug resistant phenotype.

Previously we have shown that treatment of adriamycin resistant cells with certain metabolic inhibitors such as *N*-ethylmaleimide (NEM*) produces a significant increase in drug uptake and an enhanced phosphorylation of P-180 [15, 16]. Analysis of these events in genetic revertants provided evidence that phosphorylation of this protein plays a major role in regulating the adriamycin resistant phenotype [15].

Recently, evidence has been obtained that a variety of Ca^{2+} channel blockers [17–19] and calmodulin inhibitors [17, 18, 20] are capable of reversing adriamycin resistance by inducing a significant increase in the cellular accumulation of drug. In view of our previous findings, it was of interest to determine if these agents were also capable of altering the level of protein phosphorylation in drug resistant cells. The results of this study provide evidence that the calmodulin inhibitor trifluoperazine [21] modulates adriamycin resistance by a mechanism that involves the phosphorylation of P-180.

MATERIALS AND METHODS

Chemicals. Trifluoperazine and prenylamine were from Sigma. Nimodipine and verapamil were supplied by Miles Laboratories and Knoll Pharmaceutical respectively. $^{32}P_i$ was from ICN. [^{3}H]-Daunomycin, 2.5 Ci/mmole, was obtained from New England Nuclear.

Cells. Chinese hamster lung cells (HT-1), resistant to adriamycin, were isolated as described previously [1]. The isolate R3R is a spontaneous revertant

* Abbreviations: NEM, *N*-ethylmaleimide; and TFP, trifluoperazine.

obtained after growing R3 resistant cells for 8 months in culture. Both sensitive and revertant cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

Effect of trifluoperazine on cellular drug accumulation. Sensitive and resistant cells were grown in standard medium in 60 mm dishes. TFP was added followed by the addition of 5 μM [^{3}H]daunomycin. Control cells were incubated with [^{3}H]daunomycin in the absence of TFP. After various time periods the medium was removed and the cells were washed once with PBS [0.01 M sodium phosphate (pH 7.4), 0.15 M NaCl]. The cells were scraped into 1 ml of PBS and centrifuged. The cell pellet was suspended in 0.2 ml of 1 N NaOH and thereafter incubated for 1 hr at 50°. An aliquot was taken for radioactivity and protein [22] determination.

Effect of trifluoperazine on P-180 phosphorylation. Sensitive and resistant cells were grown in 100 mm dishes in DMEM containing 10% fetal calf serum for 48 hr. The medium was thereafter removed, and 1 ml of TG medium containing 50 μCi of $^{32}P_i$ was

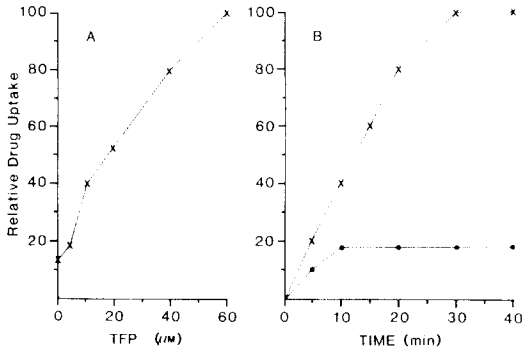

Fig. 1. Effect of trifluoperazine on drug uptake in resistant cells. Analysis of the cellular uptake of [^{3}H]daunomycin was carried out as described in Materials and Methods. Panel A: effect of various concentrations of TFP on drug uptake. Cells were incubated for 20 min at 37° prior to determining uptake of [^{3}H]daunomycin. Panel B: drug uptake as a function of time of cells incubated in the absence (●) and presence (×) of 40 μM TFP. Drug uptake values are plotted relative to maximum drug uptake which is set at 100%.

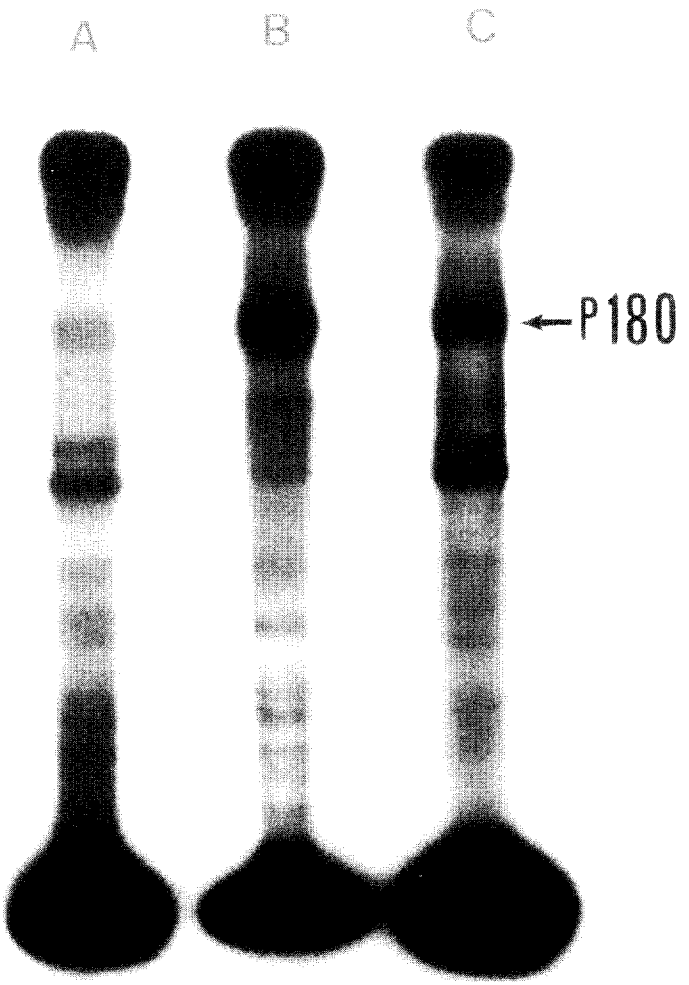

Fig. 2. Effects of TFP and NEM on P-180 phosphorylation in crude membrane preparations. Adriamycin resistant cells were prelabeled with $^{32}P_i$ and then incubated in the absence or presence of 2 mM NEM or 40 μM TFP for 20 min. Membranes containing endoplasmic reticulum and plasma membranes were prepared as described in Materials and Methods. The phosphoproteins contained in the membrane fraction were analyzed after electrophoresis in a 7% polyacrylamide gel. Lanes A, B and C show the results obtained with cells incubated in the absence of agent, the presence of NEM, or the presence of TFP respectively.

added to each dish. TG medium contains 0.05 M Tris–HCl (pH 7.6), 0.15 M NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM D-glucose and 1 × MEM amino acids and vitamins. After a 1-hr labeling period at 37°, the medium was removed and the cells were washed once with TG. To each dish 3 ml of TG medium was added followed by the addition of trifluoperazine which was added to all dishes except to the control cells. The cells were incubated for 20 min after which time cell membranes were prepared as described previously [16]. The effect of verapamil on P-180 phosphorylation was carried out in an identical manner. Incubation of prelabeled cells with NEM was carried out under conditions in which glucose was omitted from the TG medium. The phosphorylated proteins in the membrane fraction were analyzed after electrophoresis [23] in polyacrylamide gels. Prior to gel electrophoresis all samples were equalized for radioactivity. Labeled proteins were detected by autoradiography. Autoradiograms in some instances were traced with a Joyce–Loebl densitometer. All densitometric tracings for a particular experiment were performed at the same setting.

RESULTS

Effect of trifluoperazine on drug accumulation in resistant cells. Previous studies by Tsuruo *et al.* [17, 18] and Ganapathi and Grabowski [20] have shown that TFP induces a significant increase in drug accumulation in P388 mouse leukemia cells resistant to adriamycin. Similar results were obtained with adriamycin resistant Chinese hamster lung cells. As shown in Fig. 1, increased levels of [^{3}H]daunomycin were detected in resistant cells incubated with 10 μM TFP. As the concentration of TFP increased, there was a corresponding increase in cellular drug accumulation. In the presence of 40 μM TFP, increased uptake of drug was detected after a 5-min incubation period (Fig. 1B). The amount of drug contained in resistant cells increased almost linearly with time through a 30-min incubation period. In identical studies carried out with drug sensitive cells, TFP (40 μM) produced only a 1.3-fold increase in drug uptake during a 30-min incubation period. Under identical conditions with drug resistant cells, TFP induced a 4-fold increase in cellular drug accumulation (Fig. 1B). It should be indicated that studies carried out in our laboratory have shown that the adriamycin resistant Chinese hamster cells are also highly resistant to daunomycin. In additional studies, TFP (40 μM) did not induce any change in the cellular uptake of radioactivity labeled thymidine, deoxyglucose, lysine and glucosamine. These results taken together suggest that the TFP-induced uptake of [^{3}H]daunomycin in resistant cells is not due to non-specific membrane changes.

TFP-induced phosphorylation of P-180. Previously we have shown that P-180 in drug resistant cells is present in a phosphorylated form and that phosphorylation of this protein may provide a mechanism for modulating cellular accumulation of drug [15, 16]. In view of the finding that TFP is capable of inducing cellular uptake of drug [17, 18, 20], studies were carried out to examine the effect of this agent on the level of phosphorylation of P-180. In these experiments drug resistant cells were prelabeled with $^{32}P_i$ and thereafter incubated with 40 μM TFP or 2 mM *N*-ethylmaleimide (NEM) for 20 min. At the end of the incubation period, a membrane fraction containing both endoplasmic reticulum and plasma membranes was prepared, and the phosphoproteins were analyzed after polyacrylamide gel electrophoresis. Membranes isolated from cells incubated in the absence of TFP or NEM contained multiple phosphorylated proteins including P-180 (Fig. 2A). However, analysis of membrane proteins from cells treated with TFP revealed that the P-180 now exists in a highly superphosphorylated form (Fig. 2C). Parallel studies also showed that the level of phosphorylation of P-180 induced by TFP was comparable to that which occurred when resistant cells were incubated in the presence of NEM (Fig. 2B). Thus, both TFP and NEM were capable of inducing drug uptake in resistant cells and enhancement of P-180 phosphorylation.

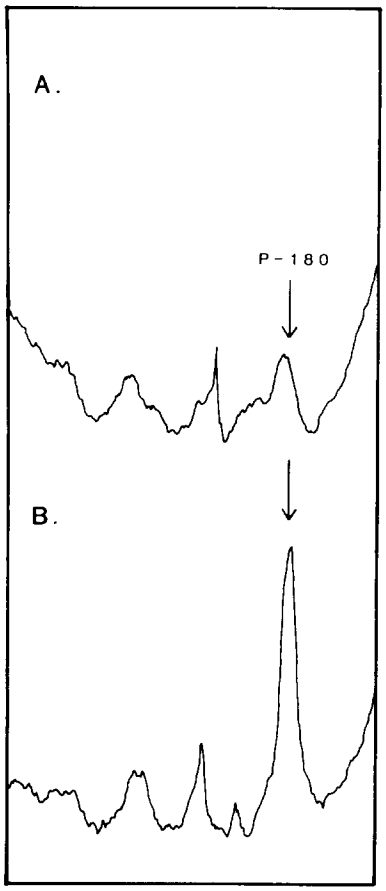

Fig. 3. TFP-induced phosphorylation of P-180 in plasma membranes. Adriamycin resistant cells were prelabeled with $^{32}P_i$ and then incubated in the absence (A) or presence (B) of 40 μM TFP for 20 min as described in Materials and Methods. Plasma membranes were isolated [16], and the phosphoproteins were analyzed after electrophoresis in a 7% polyacrylamide gel. The results are presented as densitometer scans of the autoradiogram. The migration of proteins is from right to left.

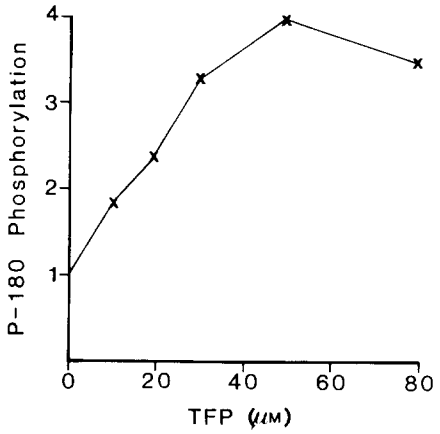

Fig. 4. P-180 phosphorylation in cells treated with various concentrations of TFP. Adriamycin resistant cells prelabeled with $^{32}P_i$ were incubated for 20 min in the absence or presence of the indicated concentrations of TFP. A membrane fraction containing endoplasmic reticulum and plasma membranes was prepared, and the proteins were electrophoresed in a 7% polyacrylamide gel. Autoradiograms were prepared and used to locate the region of the gel containing P-180. This section of the gel was cut out, and the radioactivity in this fraction was determined. A portion of the gel of the same size which did not contain protein material was cut out and used to determine background radioactivity. The data are presented as the relative increase in P-180 phosphorylation as a function of TFP concentration. The phosphorylation level of P-180 in membranes from cells incubated in the absence of TFP is set at 1.

Experiments were also carried out in which phosphoproteins were analyzed in plasma membranes isolated from prelabeled cells incubated in the absence and presence of TFP. The results of these studies show that TFP induced a major increase in P-180 phosphorylation (Fig. 3, A and B). Of interest is the finding that in several different experiments of this type P-180 was the only plasma membrane protein which increased in [^{32}P]phosphate content. We also observed that TFP induced an increased phosphorylation of P-180 in the endoplasmic reticulum (data not shown).

P-180 phosphorylation in the presence of various concentrations of TFP. Detailed studies were carried out to examine P-180 phosphorylation at TFP concentrations that are minimally required for inducing the onset of drug uptake in resistant cells. Using [^{3}H] daunomycin, a highly sensitive probe for measuring cellular drug accumulation, we found that resistant cells began to show a significant uptake of daunomycin at a TFP concentration of 10 μM (Fig. 1A). When prelabeled resistant cells were treated with 10 μM TFP for 20 min, we also observed a significant increase in the phosphorylation levels of P-180 (Fig. 4). Under these conditions, the [^{32}P]phosphate content of this protein increased about 1.8-fold. As the TFP concentration was increased, there was a corresponding increase in the phosphorylation of P-180 (Fig. 4). In two additional experiments of this type, essentially identical results were obtained. Analysis of the values obtained from the three different experiments revealed a highly consistent pattern with only minor deviation of the values for each point. The values obtained at a given TFP concentration were all within 15% of each other. These results therefore show that the concentration range of TFP required to enhance P-180 phosphorylation is similar to that required to induce an increase in the cellular accumulation of drug.

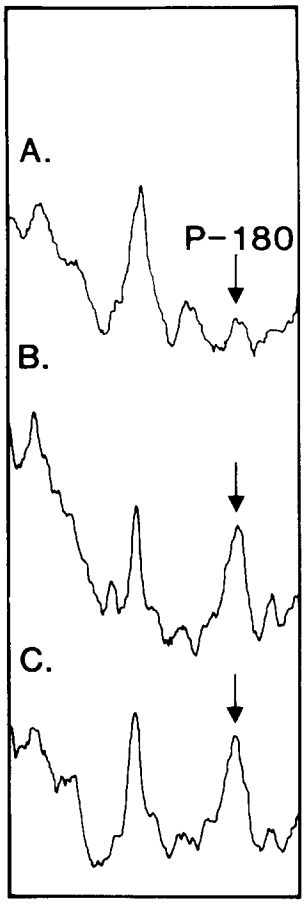

Fig. 5. Effects of TFP and verapamil on P-180 phosphorylation. Resistant cells were prelabeled with $^{32}P_i$ and then incubated in the absence (A) or presence of TFP (B) or verapamil (C) for 20 min. Both agents were used at a final concentration of 25 μM. At the end of the incubation period, cell membranes containing endoplasmic reticulum and plasma membranes were prepared, and the proteins were analyzed after polyacrylamide gel electrophoresis. The results are presented as densitometer scans of the autoradiogram.

P-180 phosphorylation in cells treated with verapamil. Tsuruo *et al.* [17–19] have shown previously that, in addition to calmodulin inhibitors, a number of Ca^{2+} channel blockers are also active in bringing about a reversal of adriamycin resistance. One of the most active agents in this system is the phenylalkylamine verapamil [24]. We therefore carried out studies to determine the effect of this agent on P-180 phosphorylation in drug resistant cells. Cells prelabeled with $^{32}P_i$ were treated with either TFP or verapamil (25 μM) for 20 min, and the proteins in isolated membranes were analyzed after polyacrylamide gel electrophoresis. As shown in Fig. 5, both TFP and verapamil were capable of inducing

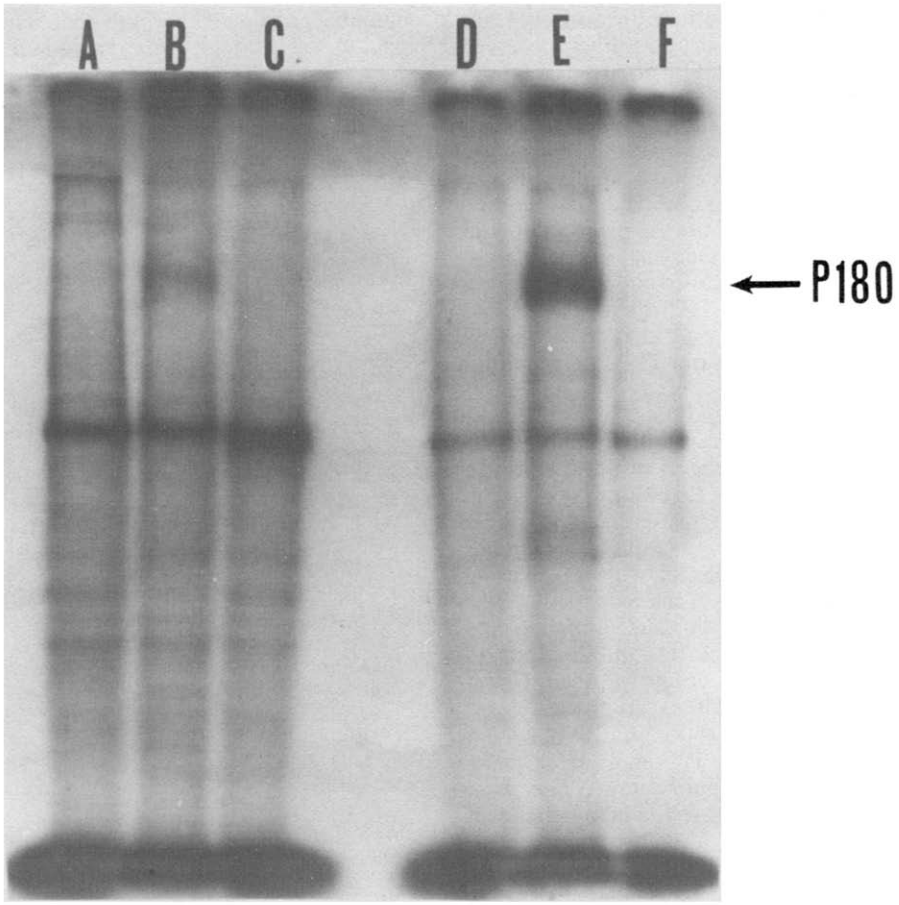

Fig. 6. Effect of TFP on P-180 phosphorylation in drug sensitive, resistant and revertant cells. Cells were prelabeled with $^{32}P_i$ and then incubated for 20 min with 30 μM TFP under conditions described in Materials and Methods. Endoplasmic reticulum (lanes A, B, and C) and plasma membranes (lanes D, E and F) were prepared, and the proteins were analyzed after electrophoresis in a 7% polyacrylamide gel. Lanes A and D, B and E, and C and F show the results obtained with membranes isolated from sensitive, resistant, and revertant cells respectively. The revertant cells used in these experiments were those that had undergone an essentially complete conversion to drug sensitivity.

a major increase in the phosphorylation of P-180. Both agents were about equally active and, under the conditions of the experiment, induced about a 3-fold increase in the P-180 [^{32}P]phosphate content. Verapamil was also found to induce drug uptake in adriamycin resistant cells at a level comparable to that observed for NEM.

TFP-induced phosphorylation of P-180 in cells that have reverted to drug sensitivity. Recently we observed that certain isolates of drug resistant Chinese hamster lung cells undergo a spontaneous reversion to adriamycin sensitivity after growth in culture for about 8 months [15]. Analysis of these cells reveals that they have undergone an essentially complete reversion to drug sensitivity [15]. Studies were therefore carried out to determine if the reversion to drug sensitivity resulted in a parallel loss in the TFP-induced phosphorylation of P-180. In these studies sensitive, resistant and revertant cells were prelabeled with $^{32}P_i$ and then treated with 30 μM TFP for 20 min. Endoplasmic reticulum and plasma membranes were isolated and the proteins were analyzed after polyacrylamide gel electrophoresis. Analysis of endoplasmic reticulum and plasma membranes from drug resistant cells revealed the presence of P-180 in a superphosphorylated form (Fig. 6, B and E). In similar fractions from drug sensitive cells, P-180 was not detectable (Fig. 6, A and D). Analysis of the membrane fractions from drug revertant cells clearly showed that there was essentially a complete loss of phosphorylated P-180. We have also observed that [^{14}C]glucosamine-labeled P-180 [1] is absent in cells which have reverted to drug sensitivity. It thus may be indicated that in revertant cells there is a loss of the P-180 polypeptide.

DISCUSSION

The results of the present study demonstrate that treatment of adriamycin resistant cells with the calmodulin inhibitor trifluoperazine [21] resulted in a significant increase in the cellular accumulation of drug and a parallel superphosphorylation of a plasma membrane glycoprotein (P-180). Previous studies have shown that there is a strong correlation between the presence of this protein and the drug resistant

phenotype [7, 9, 14, 15]. Certain lines of evidence suggest that the reversion to drug sensitivity observed in resistant cells treated with TFP occurred as a result of an enhanced phosphorylation of P-180. This is based on the finding that similar concentrations of TFP were required for increasing the phosphorylation of this protein and for inducing drug uptake in resistant cells. It was also observed that the Ca^{2+} channel blocker verapamil was capable of modulating drug uptake and inducing protein phosphorylation changes identical to those observed in resistant cells treated with TFP. Finally, we found that, as resistant cells reverted to drug sensitivity, there was a parallel loss in the TFP-induced phosphorylation of P-180.

Based on these findings and those which we have described previously [15, 16], it is indicated that the cellular accumulation of drug in resistant cells can be modulated by the level of P-180 phosphorylation. It is suggested that in the resistant cell P-180 is phosphorylated at a small number of sites and in this form is active and functions in a drug exclusion mechanism. In the presence of calmodulin inhibitors such as TFP or Ca^{2+} channel blockers, P-180 becomes phosphorylated at additional sites and this leads to the biological inactivation of the protein. Under these conditions, high levels of drug are able to accumulate in the resistant cell.

The mechanism by which TFP and similar acting agents modulate P-180 phosphorylation is unknown. One possibility is that a Ca^{2+}–calmodulin-dependent protein kinase system [25, 26] is involved in maintaining P-180 in its active form. Alternatively, since TFP is an inhibitor of the phospholipid-sensitive Ca^{2+}-dependent protein kinase C [27, 28], this enzyme may have a role in this process. Under conditions in which the involved enzyme system is inhibited, a second protein kinase would be activated and this enzyme would be involved in the superphosphorylation of P-180. The nature of this enzyme is unknown but previous studies have shown that P-180 in isolated plasma membranes is highly phosphorylated by a Mg^{2+}-dependent protein kinase [16]. Other types of mechanisms may account for the action of TFP in inducing the reversal of adriamycin resistance. It seems possible that agents like TFP may induce specific alterations in the plasma membrane and this, in turn, would result in P-180 becoming more accessible to phosphorylation reactions.

The mechanisms by which P-180 acts to exclude drug in resistant cells is unknown. Since cells resistant to adriamycin are cross-resistant to a number of structurally unrelated agents [5, 12, 13], it may be possible that P-180 acts to form a channel which is capable of bringing about the efflux of a variety of compounds. The activity of this channel would then be modulated by the phosphorylation state of P-180.

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REFERENCES

1. D. Garman and M. S. Center, *Biochem. biophys. Res. Commun.* **105**, 157 (1982).
2. M. Inaba and R. K. Johnson, *Biochem. Pharmac.* **27**, 2123 (1978).
3. M. Inaba, H. Kobayashi, Y. Sakurai and R. K. Johnson, *Cancer Res.* **39**, 2200 (1979).
4. T. Skovsgaard, *Cancer Res.* **38**, 1785 (1978).
5. T. Skovsgaard, *Cancer Res.* **38**, 4722 (1978).
6. R. Ganapathi, W. Reiter and A. Krishan, *J. natn. Cancer Inst.* **68**, 1027 (1982).
7. R. L. Juliano and R. V. Ling, *Biochim. biophys. Acta* **455**, 152 (1976).
8. W. T. Beck, T. J. Mueller and L. R. Tanzer, *Cancer Res.* **39**, 2070 (1979).
9. R. H. F. Peterson, M. B. Meyers, B. A. Spengler and J. L. Biedler, *Cancer Res.* **43**, 222 (1983).
10. R. H. F. Peterson and J. L. Biedler, *J. supramolec. Struct.* **9**, 289 (1978).
11. N. Kartner, M. Shales, J. R. Riordan and V. Ling, *Cancer Res.* **43**, 4413 (1983).
12. J. L. Biedler and H. Riehm, *Cancer Res.* **30**, 1174 (1970).
13. V. Ling, *Can. J. Genet. Cytol.* **17**, 503 (1975).
14. N. Kartner, J. R. Riordan and V. Ling, *Science* **221**, 1285 (1983).
15. M. S. Center, *Biochem. biophys. Res. Commun.* **115**, 159 (1983).
16. D. Garman, L. Albers and M. S. Center, *Biochem. Pharmac.* **32**, 3633 (1983).
17. T. Tsuruo, H. Lida, S. Tsukagoshi and Y. Sakurai, *Cancer Res.* **42**, 4730 (1982).
18. T. Tsuruo, H. Lida, S. Tsukagoshi and Y. Sakurai, *Cancer Res.* **43**, 2267 (1983).
19. T. Tsuruo, H. Lida, M. Nojiri, S. Tsukagoshi and Y. Sakurai, *Cancer Res.* **43**, 2905 (1983).
20. R. Ganapathi and D. Grabowski, *Cancer Res.* **43**, 3696 (1983).
21. R. M. Levin and B. Weiss, *Molec. Pharmac.* **12**, 581 (1976).
22. G. L. Peterson, *Analyt. Biochem.* **83**, 346 (1977).
23. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
24. A. Fleckenstein, *A. Rev. Pharmac. Toxic.* **17**, 149 (1977).
25. H. Schulman and P. Greengard, *Proc. natn. Acad. Sci. U.S.A.* **75**, 5432 (1978).
26. C. O. Brostrom, Y. C. Huang, B. M. Breckenridge and D. H. Wolff, *Proc. natn. Acad. Sci. U.S.A.* **72**, 64 (1975).
27. T. Mori, Y. Takai, R. Minakuchi, B. Yu and Y. Nishizuka, *J. biol. Chem.* **255**, 8378 (1980).
28. B. C. Wise and J. F. Kuo, *Biochem. Pharmac.* **32**, 1259 (1983).