Interaction of P-Glycoprotein with Protein Kinase C in Human Multidrug Resistant Carcinoma Cells

Jin-Ming Yang, Khew-Voon Chin, and William N. Hait

Departments of Medicine and Pharmacology, The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

ABSTRACT

Indirect evidence has suggested that P-glycoprotein (P-gp), the multidrug transporter, is phosphorylated by protein kinase C (PKC) and that phosphorylation modulates its transport function. To address the first premise more directly, i.e., that P-gp is phosphorylated by PKC, we investigated the interaction between P-gp and PKC in sensitive and multidrug-resistant MCF-7 and KB human carcinoma cell lines. We found that P-gp and PKC were coimmunoprecipitated from the multidrug-resistant cell lines MCF-7/AdrR and KB-V-1, using antibodies to either protein. The association between the two proteins was enhanced by phorbol 12-myristate 13-acetate, an analogue of diacylglycerol that induces translocation of PKC to the plasma membrane. The anti-P-gp immunoprecipitates contained PKC activity as measured by direct phosphorylation reactions. The interaction of PKC with P-gp displayed isozyme specificity: PKC-α, β, γ, ε, and θ, but not δ, ρ, ξ, and ζ, were found to coimmunoprecipitate with P-gp. These studies indicate that P-gp closely interacts with PKC and serves as a substrate, and that specific isozymes of this kinase may be involved in the phosphorylation of the multidrug transporter.

INTRODUCTION

The overexpression of P-gp, the MDR1 gene product, is closely associated with the MDR phenotype in human cancer cells. P-gp is a 150,000–180,000 plasma membrane phosphoglycoprotein that functions as an energy-dependent drug transporter with broad substrate specificity (1–3). P-gp possesses homology with bacterial transport proteins (4, 5), ATP binding (6) and hydrolysis (7) activities, drug binding (8) and efflux (9) properties, and the ability to bind compounds that reverse MDR such as verapamil and cyclosporin A (10, 11).

P-gp expressed in MDR cells undergoes covalent modification by phosphorylation. The phosphorylation of P-gp occurs in cells selected for resistance (12–14) and in cells transfected with the MDR1 gene (15, 16). The universal occurrence of this posttranslational modification suggests an important, yet unknown, role for phosphorylation in the regulation of the function of P-gp. Several enzymes have been shown to phosphorylate P-gp (7); our group and others have focused on the role of PKC (18–24). Several lines of evidence suggest that PKC is involved in MDR. For example, our laboratory and others have shown that PKC interacts with P-gp and PKC in MDR human carcinoma cells using coimmunoprecipitation techniques. We demonstrate that PKC and P-gp can be coimmunoprecipitated and that this interaction is stimulated by phorbol esters and is isoform specific.

MATERIALS AND METHODS

Cell Lines and Antibodies. The MDR human breast cancer cell line MCF-7/AdrR and the sensitive parental line MCF-7 were kindly supplied by Dr. Kenneth Cowan of the National Cancer Institute (Bethesda, MD) and were maintained in RPMI 1640 containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2/95% air. The MDR human oral carcinoma line KB-V-1, developed by Dr. Michael Gottesman’s laboratory, and the sensitive parental line KB-3–1 were grown in DMEM containing 10% fetal bovine serum under the same condition as described above. For KB-V-1, 1 μg/ml of vinblastine was added in the medium for the maintenance of the MDR phenotype. Cells were checked routinely and found to be free of contamination by Mycoplasma or fungi. All cell lines were discarded after 3 months, and new lines were obtained from frozen stocks.

Polyclonal antibody mdr (Ab-1), recognizing human P-gp, was purchased from Oncogene Science (Uniondale, NY). An anti-pan-PKC polyclonal antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).
C219 anti-P-gp monoclonal antibody was obtained from Signet (Dedham, MA). Anti-PKC monoclonal antibodies specific for PKC isoenzymes and anti-RACK1 monoclonal antibody were purchased from Transduction Laboratories (Lexington, KY). Additional materials were purchased from the following sources: protein A-Sepharose CL-4B, Pharmacia (Piscataway, NJ); prestained high range and low range SDS-PAGE molecular standards, Bio-Rad Laboratories (Hercules, CA); and ECL Western blotting analysis kit and [γ-32P]ATP (6000 Ci/mmol), Amersham (Arlington Heights, IL).

**Results and Discussion**

To determine whether PKC interacts with P-gp in MDR cells, cell lysates were immunoprecipitated with an antibody to PKC and probed with an antibody to P-gp. Fig. 1 demonstrates that in MDR cell lines MCF-7/AdrR (Fig. 1A) and KB-V-1 (Fig. 1B), P-gp was coimmunoprecipitated with the anti-PKCI antibody. PKC activity was measured in the presence or absence of PMA (Fig. 1). The band corresponding to approximately Mr 90,000 was identified as nonspecific binding of the anti-PKCI polyclonal antibody to goat anti-mouse IgG. These results suggest that PKC associates with P-gp and that this association is increased by PMA, a diacylglycerol analogue that activates the enzyme and induces its translocation to the plasma membrane. These data also strengthen the observations by Fine et al. (27) and others (25, 26, 28) that treatment of MDR cells with phorbol esters increases the phosphorylation of P-gp by PKC and decreases drug accumulation in a temporally consistent manner. In the PMA-treated MDR cells, a greater amount of P-gp was coimmunoprecipitated from MCF-7/AdrR than from KB-V-1 cells. This apparent difference could not be attributed to increased content of P-gp in MCF-7/AdrR cells, since both of the MDR cell lines used in this study expressed significant amounts of P-gp, with KB-V-1 showing somewhat higher levels than MCF-7/AdrR (data not shown). This difference may therefore reflect the 7-15-fold overexpression of P-gp seen in MCF-7/AdrR cells (18, 27) compared to the 2-fold increase in P-gp seen in KB-V-1 cells (41).

To address this interaction further, we studied whether the apparent association of the two proteins within the immunoprecipitates would support the phosphorylation of P-gp by PKC. The anti-P-gp immunoprecipitates were utilized as a source of both substrate and enzyme in kinase reactions. Fig. 2 demonstrates that when PKC was coimmunoprecipitated with P-gp, the enzyme maintained its ability to phosphorylate P-gp and that the phosphorylation of P-gp was increased by the treatment of cells with PMA. Other substrates for PKC, such as the myristoylated alanine-rich C kinase substrate (M.A.C.K.S.) and glycogen synthase kinase 3, have also been reported to bind PKC (42). Therefore, the data presented here support the notion that P-gp is a substrate for PKC.

We next determined whether immunoprecipitation with antibody to P-gp would coimmunoprecipitate PKC and whether the association...
INTERACTION BETWEEN P-GLYCOPROTEIN AND PROTEIN KINASE C

between the two proteins was isozyme specific. Cell lysates were incubated with mdr (Ab-1), and the immunoprecipitates were probed with isozyme-specific anti-PKC monoclonal antibodies. Fig. 3 demonstrates the presence of α, β, γ, and ε isozymes in the anti-P-gp immunoprecipitates of MCF-7/AdrR cells, but not in MCF-7 cells. Furthermore, the amount of PKC isozymes in the anti-P-gp immunoprecipitates was increased following treatment of MCF-7/AdrR cells with 200 nM PMA (Fig. 3). PKC-θ was detected only in the MCF-7/AdrR cells treated with PMA (Fig. 3). In contrast, PKC-δ, -μ, and -ζ were not detected in the anti-P-gp immunoprecipitates (Fig. 3). Similar results were obtained with the KB-V-1 and KB-3-1 cells (results not shown). PKC-λ was detected in both sensitive and resistant MCF-7 cells treated with PMA (Fig. 3). Since the sensitive MCF-7 cell line does not express detectable P-gp, the PKC-λ detected in the anti-P-gp immunoprecipitates of MCF-7 cells may represent nonspecific binding. It was reported that phorbol ester treatment of MDR cells mainly influenced the subcellular distribution of PKC-α, and other isoforms were not significantly affected (41). Therefore, the increased association of P-gp with PKC-α caused by PMA may be due to the translocation of the isozyme to the membrane, whereas for PKC-β, -γ, -ε, and -θ, the increased binding to P-gp by PMA may result from the activation of these isozymes within the cell membrane.

Table 1 shows that all PKC isoforms were expressed in MCF-7/AdrR cells, and that the α, γ, ε, ζ, and λ isoforms were expressed at the highest levels. Since there was no direct correlation with the expression of PKC isoforms and their detection in the anti-P-gp immunoprecipitates, it is also unlikely that these represent nonspecific interactions. For example, the β isozyme was readily detectable in the immunoprecipitates, although its expression in MCF-7/AdrR cells was at the lowest level of this family. Although PKC-δ, -μ, and -ζ were present in MCF-7/AdrR cell line (Table 1), these isoforms were not detected in the anti-P-gp immunoprecipitates (Fig. 3), confirming isozyme specificity for the interaction between P-gp and PKC. Although sensitive MCF-7 cells do not contain detectable P-gp, we wanted to rule out the possibility that the failure to show an interaction using the anti-P-gp antibodies was due to an absence of PKC isoforms in the sensitive cells. Table 1 demonstrates that all isoforms were readily detectable in sensitive MCF-7 cells except for ε, θ, and μ. In previous published experiments Blobe et al. (22) failed to detect PKC-γ in MCF-7 and MCF-7/AdrR cells, whereas PKC-ε was found in sensitive, but not resistant MCF-7 cells. These differences may reflect the use of different antibodies or cell culture conditions. For example, Gehr et al. (43) detected PKC-ε in both the parental and resistant MCF-7 cells using antibodies obtained from the same source as ours.

PKCs have been divided into three classes according to their requirements for the cofactors phosphatidylserine, calcium, and diacylglycerol (or phorbol ester; Ref. 44): conventional PKCs (α, β, and γ) are phosphatidylserine and calcium dependent and diacylglycerol (or phorbol ester) responsive; novel PKCs (δ, ε, θ, and μ) are phosphatidylserine dependent and diacylglycerol (or phorbol ester)
INTERACTION BETWEEN P-GLYCOPEPTIDE AND PROTEIN KINASE C

responsive, but calcium independent; and atypical PKCs are phosphatidylinerine dependent, but calcium independent and diacylglycerol (or phorbol ester) unresponsive. Fig. 3 demonstrates that all of the conventional PKC isozymes were associated with P-gp, whereas only PKC-€ was associated with P-gp to the extent comparable to conventional PKCs among the remainder of the isozymes tested. Therefore, these isozymes may be prime candidates as P-gp kinases.

Differences in the molecular structure between the three groups of PKCs have been recently clarified (45). Conventional PKCs contain a calcium-binding domain, whereas the other two groups do not. Conventional and novel PKCs possess diacylglycerol/phorbol ester-binding regions, whereas atypical PKCs do not. Therefore, the calcium-binding domain may play an important role in the interaction between PKC and P-gp, although the diacylglycerol/phorbol ester-binding domain may also be involved. Further studies to define the critical regions involved in this interaction and the binding site(s) on both PKC and P-gp are in progress.

PKC-α has been proposed to be the major isozyme that phosphorylates P-gp (15, 22), and the interaction of P-gp with PKC-α in a baculovirus expression system was recently reported (35). Here, we demonstrate this interaction directly in human MDR carcinoma cell lines (Fig. 3). The direct interactions of P-gp with other isozymes (β, γ, ε, and θ) of PKC were also found in this study. However, the functional significance of the interactions of these individual isozymes with P-gp is not clear yet. For example, in the current study, we found that PKC-γ interacts with P-gp (Fig. 3). In contrast, Ahmad et al. (34) reported that the expression of this isozyme did not affect the phosphorylation of P-gp and the retention of doxorubicin in BC-19 cells. Whether the association of P-gp with other isozymes of PKC decreases its ability to bind PKC-α and thus may act as indirect negative regulators of phosphorylation remains an open question.

PKC is a cellular kinase that is translocated and activated in response to stimulation. RACKS are a group of PKC-anchoring proteins that are involved in the localization of PKC (46). To determine whether the interaction between PKC and P-gp requires RACKS, we measured the expression of RACKs in both sensitive and resistant cell lines using Western blot analysis. Fig. 4 shows that RACK1 was expressed at identical levels in sensitive and resistant cell lines. However, we could not detect RACK1 in either anti-P-gp or anti-PKC immunoprecipitates from MDR cells (data not shown). Several possibilities exist for this result: (a) the interaction between RACK1 and PKC may be transient and undetectable using the immunoprecipitation technique; (b) other members of RACKs, but not RACK1, are required for the interaction between PKC and P-gp; and (c) RACKs are not required for PKC-P-gp interaction. The role of RACKs in the protein-protein interaction between PKC and P-gp remains to be investigated.

The functional role of P-gp phosphorylation has yet to be elucidated. Indirect evidence suggests that phosphorylation may regulate the transport of certain cancer chemotherapeutic drugs (13, 14, 25–27). However, P-gp mutants lacking known PKC phosphorylation sites may retain basal drug-transport activity (17). We and others have shown that activation of PKC by PMA and phosphorylation of P-gp in human breast cancer cells increases the transport of radiolabeled vinblastine above the basal state (25); kinetic analysis of these data suggested that the effect of phosphorylation was to enhance membrane efflux (25). Furthermore, we have recently shown that factors that increased diacylglycerol through the activation phospholipase C, such as epidermal growth factor and heat shock, increased the phosphorylation of P-gp and that this reaction could be inhibited by PKC inhibitors (47, 48). Posada et al. (49) found that the cytotoxic agent Adriamycin increased the production of diacylglycerol and the activity of PKC in MDR cells. Therefore, phosphorylation of P-gp may not be required for basal transport activities of P-gp, but rather for increased activity following a toxic challenge.

These experiments provide direct evidence for an interaction between P-gp and PKC in human MDR cells, and suggest that this interaction is isozyme specific. The ability of diacylglycerol analogues such as PMA to increase this interaction may help explain and strengthen previous observations.

ACKNOWLEDGMENTS

We thank Dr. Xiang Xu for helpful discussions.

REFERENCES

INTERACTION BETWEEN P-GLYCOPROTEIN AND PROTEIN KINASE C


Interaction of P-Glycoprotein with Protein Kinase C in Human Multidrug Resistant Carcinoma Cells

Jin-Ming Yang, Khew-Voon Chin and William N. Hait


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/56/15/3490

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.