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EVIDENCE THAT PROTEIN KINASE C-α ACTIVATION IS A CRITICAL EVENT IN PHORBOL ESTER-INDUCED MULTIPLE DRUG RESISTANCE IN HUMAN COLON CANCER CELLS

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Abstract—We previously designed and characterized an *in vitro* model of the intrinsic drug resistance of human colon cancer. The human colonic epithelium is chronically exposed to endogenous protein kinase C (PKC) stimulatory factors, and our model demonstrated that activation of PKC induces resistance to multiple anticancer drugs in the metastatic human colon cancer cell line KM12L4a. PKC is an isozyme family with ten members, eight of which are phorbol ester-responsive. In this report, we show that thymeleatoxin (Tx), a daphnane tumor promoter that selectively activates the phorbol ester-responsive isozymes cPKC-α, -β_1, -β_2, and -γ, was just as effective in inducing drug resistance in KM12L4a cells as phorbol dibutyrate, a potent activator of all phorbol ester-responsive PKC isozymes. The induction of resistance by Tx was associated with a reduction in cytotoxic drug accumulation in KM12L4a cells. We demonstrated by immunoblot analysis and hydroxylapatite chromatography that KM12L4a cells express active cPKC-α but not cPKC-β_1, -β_2, or γ. Our results provide strong evidence that phorbol-ester activation of cPKC-α is sufficient for the induction of resistance observed in KM12L4a cells. The possibility that endogenous PKC activators may induce intrinsic drug resistance in clinical colon cancer by an analogous mechanism is strongly suggested by our detection of active cPKC-α in surgical specimens of human colon carcinomas.

Key words: protein kinase C; multidrug resistance; human colon cancer; intrinsic drug resistance; thymeleatoxin; protein phosphorylation

MDR‖ is a well-characterized drug resistance phenotype of cancer cells that is associated with a marked reduction in the intracellular accumulation of diverse anticancer drugs [1]. The defect in drug accumulation is accounted for by overexpression of the drug efflux pump P-glycoprotein and its message *mdr1* [1]. Recent studies indicate that PKC contributes to MDR. Elevated PKC activity is a common feature of MDR tumor cells [2–4], and phorbol-ester PKC activators enhance MDR [3, 5, 6]. PKC phosphorylates P-glycoprotein [4], and major phosphorylated residues have been identified [7], providing evidence that the contribution of PKC to MDR may involve direct phosphorylation of P-glycoprotein.

The PKC isozyme family is composed of at least ten members that are classified in three groups, common PKC (cPKC) (α, β_1, β_2, γ), novel PKC (nPKC) (δ, ε, η, θ), and atypical PKC (aPKC) (ζ, λ) [8]. cPKC isozymes are Ca^{2+}-dependent and phorbol ester-responsive, nPKC isozymes are Ca^{2+}-independent and phorbol ester-responsive, and aPKC isozymes are Ca^{2+}-independent and phorbol ester-unresponsive [8]. Comparison of the PKC isozyme compositions of various paired drug-sensitive and cytotoxic drug-selected MDR tumor cell lines has revealed increased expression of cPKC-α [5, 9–11], cPKC-β [11], and cPKC-γ [12] in certain MDR lines. An independent line of evidence implicating cPKC-α in MDR is provided by observations that cPKC-α overexpression provides *mdr1*-transfected human breast cancer cells with an MDR phenotype, but cPKC-γ overexpression does not [6, 13].

Intrinsic drug resistance is a major impediment to the successful treatment of colon cancer [1]. Phorbol-ester PKC activators induce drug resistance phenotypes that resemble MDR in tumor cells that have never been exposed to cytotoxic drugs [3, 5, 14, 15], providing evidence that PKC activation may contribute to intrinsic drug resistance [15]. Although the PKC isozymes involved in phorbol ester-induced drug resistance have not been identified, a potential role for cPKC-β in intrinsic drug resistance is indicated by the induction of MDR in rat fibroblasts by cPKC-β_1 overexpression [16]. Previously, we showed that phorbol esters induce resistance to multiple cytotoxic drugs affected by MDR in human colon cancer KM12L4a cells. The

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‖ Abbreviations: ADR, Adriamycin®; DEAE, diethylaminoethyl; MDR, multidrug resistance; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; PS, phosphatidylserine; and Tx, thymeleatoxin.

IC_{50} values of the drugs were increased significantly by 2 to 3-fold as a consequence of phorbol-ester exposure, and the resistance phenotype correlated with a reduction in intracellular drug accumulation and activation of PKC [15]. In this report, we show that the isozyme-selective PKC activator Tx [17] can induce a drug resistance phenotype in KM12L4a cells, and we provide strong evidence that activation of cPKC-α mediates the induction of resistance by phorbol esters in the cells.

MATERIALS AND METHODS

Materials. The human colon cancer cell line KM12L4a was maintained as previously described [15]. Tissue culture reagents and polyclonal isozyme-specific antibodies and antigenic peptides for cPKC-α, cPKC-β ($\beta_1$and β_2), cPKC-γ, nPKC-δ, nPKC-ε, and aPKC-ζ were purchased from GIBCO BRL (Gaithersburg, MD). The antibodies specifically recognize the targeted isozymes in extracts of rat brain and cultured human cells [13, 18, 19]. The anti-cPKC-α monoclonal antibody M6 [20] was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Protease inhibitors, DEAE Sepharose, bovine serum albumin, Triton X-100, histone III-S, PS, PDBu, MTT, and ATP were purchased from the Sigma Chemical Co. (St. Louis, MO). Tx was purchased from LC Laboratories (Woburn, MA), and ADR from Adria (Columbus, OH). Protein concentration assay solution, Western analysis reagents, and DNA-grade hydroxylapatite resin were from Bio-Rad Laboratories (Richmond, CA). [γ-^{32}P]ATP, [^{14}C]Adriamycin, [^{3}H]vinblastine, affinity-purified peroxidase-linked sheep anti-mouse Ig and donkey anti-rabbit Ig, and an enhanced chemiluminescence (ECL) western blotting detection system were from the Amersham Corp. (Arlington Heights, IL). Purified PKC was prepared from rat brains as previously described [21].

Immunoblot analysis. Approximately 10^7 KM12L4a cells (50–60% confluent) were washed with cold PBS and then lysed with 16 mL of ice-cold 1% Triton X-100 in Buffer A (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 20 μg/mL soybean trypsin inhibitor, 15 mM 2-mercaptoethanol) [15]. All subsequent procedures were done at 4°. The lysate was stirred for 1 hr, centrifuged for 15 min at 13,800 *g* to remove debris, and applied to a 0.5-mL DEAE column equilibrated in Buffer A [15]. The column was washed with 5 mL Buffer A, and PKC isozymes were eluted with 1.5 mL Buffer A containing 0.4 M NaCl. The DEAE-purified KM12L4a lysate was subjected to immunoblot analysis using previously described methods [18, 22]. Purified PKC served as a positive control. All blots employed peroxidase-linked secondary antibodies. Immunoreactive bands were detected by enhanced chemiluminescence and quantitated with a computerized densitometer. For immunoblot analysis of surgical specimens, specimens of human colon carcinoma and adjacent normal-appearing mucosa were obtained at the time of surgery in the operating rooms of the M.D. Anderson Cancer Center. Portions of the tissues were used to establish the diagnosis. After dissecting necrotic tissue, submucosa, and muscularis from the specimens, the tissues were frozen in liquid nitrogen and stored at −70° for <2 months. For immunoblot analysis, tissues were homogenized in Buffer A (1 g/10 mL), and 1% Triton X-100 was added to the homogenates, which were then stirred at 4° for 1 hr. All subsequent procedures were as described above for KM12L4a cells.

cPKC assay. To assay the total Ca^{2+}- and PS-dependent histone kinase activity (cPKC activity) of KM12L4a cells, sample protein was obtained by DEAE chromatography of the cell lysate as described above. cPKC activity was measured as previously described [2, 21] using 5 μg sample protein; the activity was quantitated by subtracting the histone kinase activity observed in the presence of Ca^{2+} from the activity observed in the presence of Ca^{2+} and PS [2, 21] and was expressed as an average ±SD of triplicate determinations. For chromatographic identification of cPKC-α, -β, and -γ, hydroxylapatite analysis was done as previously described [5, 21], using the DEAE-extracted KM12L4a lysate as the sample.

Cell survival assay. KM12L4a cells harvested in their exponential growth phase by a 2-min treatment with 0.25% trypsin/0.02% EDTA (w/v) (viability >95%) were seeded into 96-well microculture plates (2000 cells/well). After a 24-hr preincubation period at 37° in a humidified atmosphere containing 5% CO_2, cells were incubated for 96 hr in medium containing cytotoxic drugs and phorbol esters, as described in Results. Cell survival was quantitated using the tetrazolium dye MTT as previously described [15, 16].

Intracellular drug accumulation. Intracellular accumulation of radiolabeled drugs was quantitated as previously described [5, 15]. KM12L4a cells were seeded into 24-well plates (Costar) at 5×10^5 cells/well. Following attachment of the cells, medium was removed and wells were washed with CMEM containing 10 mM HEPES (pH 7.3) at 37°. Stock solutions of 100 μM [^{14}C]ADR (sp. act. = 52 mCi/mmol) and 23 μM [^{3}H]vinblastine (sp. act. = 11 Ci/mmol) were prepared in 0.9% saline and diluted in CMEM containing 10 mM HEPES (pH 7.3). Cells were incubated with radiolabeled cytotoxic drug for various time periods (5 min–4 hr) at 37°, rapidly washed three times with cold PBS (4°), and lysed by adding 0.5 mL of 1 N NaOH for 10 min at 60°. The radioactivity in each well was counted in vials containing 15 mL scintillation fluid.

RESULTS

cPKC isozyme composition of KM12L4a cells. To identify the PKC isozyme(s) that could be activated by phorbol esters in the induction of drug resistance in human colon cancer KM12L4a cells [15], we first determined the cPKC isozyme content of the cells. cPKC activity was present in the KM12L4a cell lysate at a specific activity of 71 ± 9 pmol ^{32}P/min/mg, and 75% of the activity was cytosolic. Hydroxylapatite chromatography of the total cell lysate revealed a major peak of cPKC-α activity but no detectable cPKC-β or cPKC-γ activity (Fig. 1).

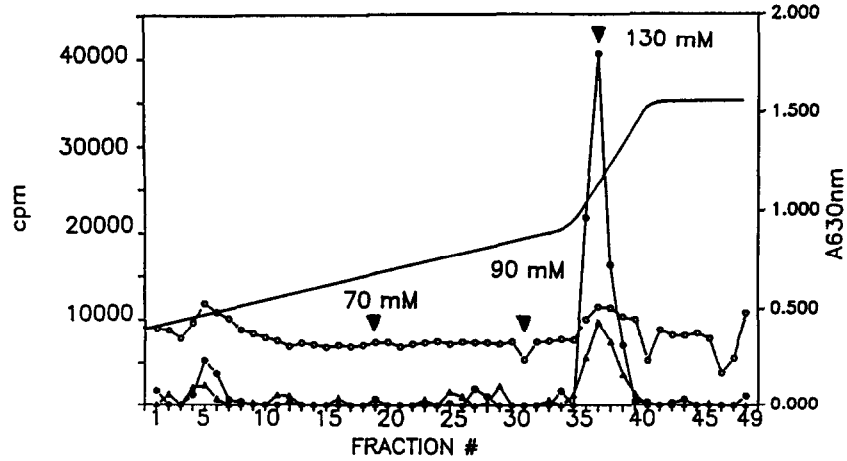

Fig. 1. Resolution of KM12L4a cPKC isozymes by hydroxylapatite chromatography. The elution profile obtained from hydroxylapatite chromatography of DEAE-extracted KM12L4a cPKC activity is shown. A 50-mL linear gradient (40–115 mM potassium phosphate, pH 8.3) corresponding to fractions 1–33 was followed by step-elution with 20 mL of 180 mM potassium phosphate, pH 8.3. The gradient is denoted by a solid line, and elution positions of cPKC isozymes are indicated by arrows (cPKC-α, 130 mM; cPKC-β, 90 mM; cPKC-γ, 70 mM). cPKC activity = Ca^{2+}- and PS-dependent histone III-S kinase activity. Shown are the histone III-S kinase activities (cpm) observed in the presence of 1 mM Ca^{2+} and 30 μg/mL PS (●) and in the presence of Ca^{2+} alone (▲) (10,000 cpm = 300 pmol ^{32}P/min), and the relative protein concentration (○). Fraction volume was 1.5 mL.

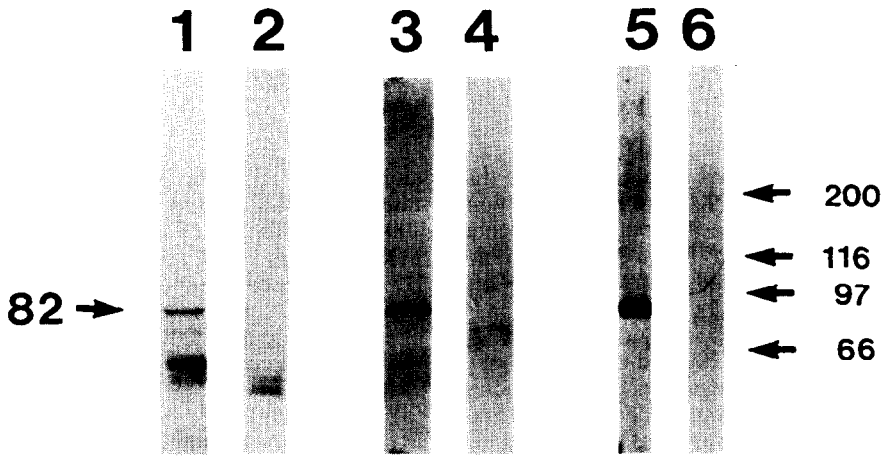

Fig. 2. cPKC-α expression in KM12L4a cells. The DEAE-extracted KM12L4a lysate was subjected to immunoblot analysis (50 μg protein/lane) with the cPKC-α antibody M6 (2 μg/mL) (lanes 1 and 2). Hydroxylapatite-purified KM12L4a cPKC activity (3 μg protein/lane) (lanes 3 and 4) and purified rat brain PKC (40 ng protein/lane) (lanes 5 and 6) were analyzed in parallel. M6 was omitted in lanes 2, 4 and 6. On the left, the position of cPKC-α (82-kDa) is indicated by an arrow. Positions of molecular weight markers (200-, 116-, 97-, 66-kDa) are indicated by arrows at the right. Bands were detected by enhanced chemiluminescence.

Next, we measured the cPKC isozyme content of the KM12L4a cells by immunoblot analysis of the DEAE-extracted cell lysate. Using a monoclonal antibody that specifically reacts with human cPKC-α and its catalytic fragment (M6) [20], we observed an immunospecific band with a molecular weight corresponding to cPKC-α (82-kDa) (Fig. 2, lanes 1 and 2). A 64-kDa immunospecific band that may correspond to the catalytic fragment of cPKC-α was also observed (Fig. 2, lanes 1 and 2). Immunoblot analysis of the hydroxylapatite-purified cPKC activity peak shown in Fig. 1 resulted in a single major immunospecific band at 82 kDa (Fig. 2, lanes 3 and 4). An 82-kDa band was also detected in the KM12L4a lysate by immunoblot analysis using rabbit polyclonal anti-cPKC-α (0.5 μg/mL), and the band was blocked by antigenic peptide (0.25 μg/mL) (data not shown). No immunoreactive bands were detected

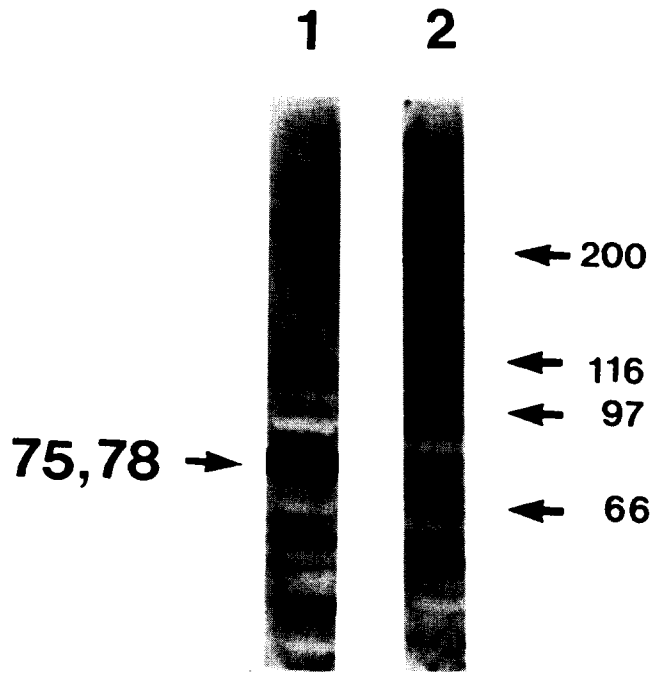

Fig. 3. aPKC-ζ expression in KM12L4a cells. The DEAE-extracted KM12L4a lysate was subjected to immunoblot analysis (50 μg protein/lane) with polyclonal anti-aPKC-ζ (0.5 μg/mL) in the absence (lane 1) or presence (lane 2) of antigenic peptide (0.25 μg/mL). Bands were detected by enhanced chemiluminescence. The arrow at the left indicates the position of the 75-, 78-kDa doublet. At the right, arrows indicate the positions of molecular weight standards (200-, 116-, 97-, 66-kDa).

in the lysate with rabbit polyclonal antibodies that recognize cPKC-β and cPKC-γ, under conditions where the antibodies detected their targeted isozymes in crude and purified rat brain PKC (data not shown). Thus, KM12L4a cells express active cPKC-α but not cPKC-β or cPKC-γ.

We also analyzed nPKC-δ, nPKC-ε and aPKC-ζ expression in the DEAE-extracted KM12L4a lysate with rabbit polyclonal isozyme-specific antibodies. A prominent 75-, 78-kDa doublet detected with anti-aPKC-ζ (Fig. 3, lane 1) was blocked by antigenic peptide (Fig. 3, lane 2). The electrophoretic mobility of the doublet was consistent with values reported for aPKC-ζ [19, 22, 23]. DEAE chromatography of the KM12L4a lysate resolved the 75-, 78-kDa doublet from cPKC-α. cPKC-α eluted at 100 mM NaCl, and the 75-, 78-kDa doublet had an elution position of 220 mM NaCl (data not shown), which is consistent with the reported elution position of aPKC-ζ [23]. The immunologic, electrophoretic and chromatographic properties of the 75-, 78-kDa doublet indicate that it is aPKC-ζ. No immunoreactive bands were detected in the KM12L4a cells with anti-nPKC-δ or anti-nPKC-ε (data not shown).

Induction of ADR resistance by Tx in KM12L4a cells. Tx is a second-stage tumor promoter that activates PKC with a unique isozyme selectivity [17]. While commonly used phorbol-ester PKC activators such as PDBu have comparable effects on all cPKC and nPKC isozymes, Tx potently stimulates cPKC isozymes (α, β, γ), but is without effect on nPKC-δ and nPKC-ε [17]. The K_a values for activation of responsive PKC isozymes are 8 nM PDBu [24] and 100–200 nM Tx [17]. We previously reported that PDBu induces ADR resistance in KM12L4a cells [15]. In this report, we show that continuous exposure to 200 nM Tx induced resistance to ADR just as effectively as 15 nM PDBu. The IC_{50} of ADR against the KM12L4a cells (22 ± 2 ng/mL) was increased to 75 ± 9 ng/mL (P = 0.004) (N = 3) by 200 nM Tx and to 61 ± 8 ng/mL (P = 0.007) (N = 3) by 15 nM PDBu. With each phorbol ester, induction of ADR resistance was statistically significant. Figure 4 shows representative results for the induction of ADR resistance by 200 nM Tx. Under these conditions, the effects of 15 nM PDBu and 200 nM Tx on PKC isozyme expression and cell growth were negligible (data not shown). The lack of down-regulation of PKC isozymes is not surprising, because down-regulation of PKC generally requires prolonged exposure to phorbol esters at saturating concentrations, and the phorbol ester concentrations employed here are near the K_a values.

Next, we measured the effects of Tx on the accumulation of [^{14}C]ADR and [^{3}H]vinblastine in the KM12L4a cells. A 30-min preincubation of KM12L4a cells with 500 nM Tx followed by simultaneous exposure of the cells to 0.1 μM [^{14}C]-ADR and 500 nM Tx reduced the intracellular accumulation of [^{14}C]ADR to 55 ± 5, 60 ± 6, and 61 ± 4% of control values at 30 min, 1 hr and 2 hr, respectively; the corresponding values obtained with

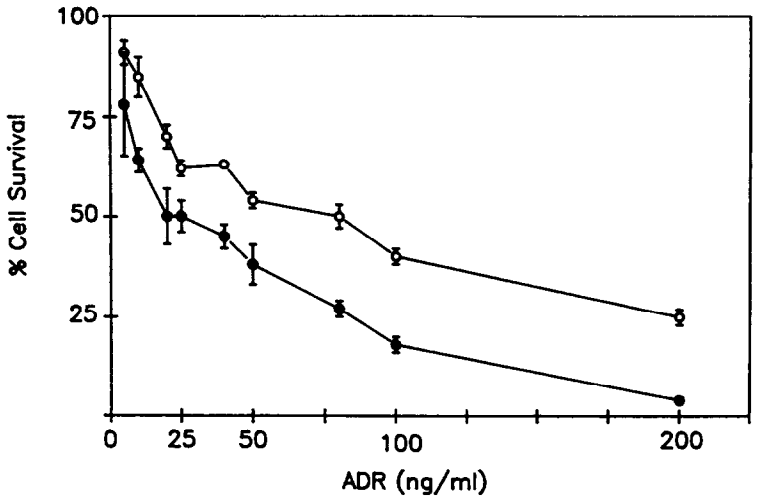

Fig. 4. Induction of ADR resistance by Tx in KM12L4a cells. KM12L4a cells were subjected to continuous exposure to ADR (0–200 ng/mL) in the presence (○) or absence (●) of 200 nM Tx for 96 hr. Percent cell survival was calculated as the percent reduction in viable cell number elicited by ADR under the conditions shown. Data points represent means ± SD of eight determinations.

100 nM PDBu were 72 ± 6, 75 ± 9, and 71 ± 6%. Similarly, 500 nM Tx reduced [^{3}H]vinblastine accumulation in KM12L4a cells to 76 ± 2, 82 ± 3, and 80 ± 1% of control values at 30 min, 1 hr and 2 hr, respectively, and the corresponding values for 100 nM PDBu were 93 ± 4, 81 ± 2, and 86 ± 4%. Under the conditions employed for the drug accumulation studies described here, PDBu and Tx did not affect KM12L4a cell growth or PKC isozyme expression in the cells (data not shown). Taken together, our results provide strong evidence that cPKC-α is unique among PKC isozymes in that it is both expressed in the KM12L4a cells and potently stimulated by Tx (see Discussion). Our results show that phorbol ester-induced drug resistance is associated with cPKC-α activation in KM12L4a cells.

Because the phorbol ester-induced drug resistance of KM12L4a cells bears similarities to MDR [15], it appears likely that the PKC substrate P-glycoprotein [7] plays an important role in this resistance phenotype. Consistent with this possibility, we have determined by FACS analysis of KM12L4a cells using P-glycoprotein-specific monoclonal antibodies that P-glycoprotein is expressed in the cells at a level similar to those observed in several murine and human colon cancer cell lines (data not shown). The expression of P-glycoprotein was unaffected by exposure to PDBu and Tx across a wide range of concentrations, indicating that, although these PKC activators may regulate the activity of the drug efflux pump, they do not regulate its expression in this system (data not shown).

Expression of cPKC-α in human colon tumor specimens. To assess the potential relevance of cPKC-α-mediated drug resistance to clinical colon cancer, we measured cPKC-α expression in surgical specimens of human colon tumors (T) and adjacent normal tissues (N) by immunoblot analysis in the presence (+) and absence (−) of M6 (Fig. 5). An immunospecific band with the apparent molecular weight of cPKC-α (82-kDa) was observed in 5/5 human colon adenocarcinomas and in the corresponding adjacent normal-appearing colonic mucosa (Fig. 5). cPKC-α expression was also detected in the N and T specimens by immunoblot analysis with rabbit polyclonal anti-cPKC-α in conjunction with antigenic peptide, and hydroxylapatite chromatography of a Dukes' B tumor specimen revealed the presence of cPKC-α activity in the tissue (data not shown).

DISCUSSION

An understanding of the mechanism of the intrinsic drug resistance of human colon cancer to pleiotropic cytotoxic anticancer drugs could facilitate the development of therapies that reverse the resistance and allow chemotherapeutic management of disseminated disease. P-glycoprotein has been implicated in intrinsic drug resistance by observations of elevated *mdr1* expression in human colon cancer surgical specimens [25]. Moreover, a direct correlation between P-glycoprotein expression and malignant potential has been demonstrated in invasive human colon cancer cells [26]. Because PKC-catalyzed phosphorylation of P-glycoprotein appears to control the drug efflux activity of the pump in mammalian cells [4, 7, 27], these observations indirectly implicate PKC in the intrinsic drug resistance of human colon cancer.

Direct evidence that regulation of PKC activity modulates the chemosensitivity of human colon cancer cells to cytotoxic anticancer drugs has been provided by studies with selective PKC activators [15]. Human colon carcinomas are commonly exposed to environmental factors that can enter cells and activate PKC, e.g. bile acids and dietary fat metabolites [5, 15, 28]. We recently designed and

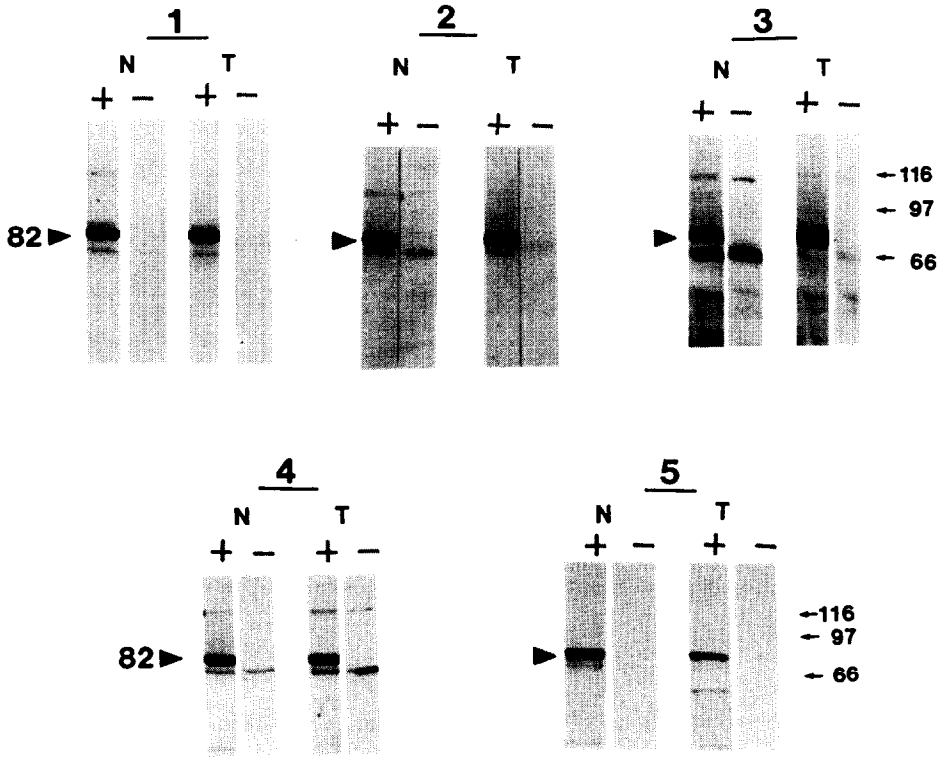

Fig. 5. cPKC-α expression in surgical specimens of human colon cancer. Sample protein (25 μg/lane) for immunoblot analysis was obtained from surgical specimens of human colon carcinomas and adjacent, normal-appearing colonic mucosa. Immunoblot analysis of cPKC-α expression with the antibody M6 (2 μg/mL) is shown for normal (N) and tumor (T) paired specimens from five colon cancer patients (1–5). Blots were done in the presence (+) and absence (−) of M6. At the left, an arrow indicates the position of cPKC-α (82-kDa). Molecular weight standards are indicated by arrows at the right. The tumors employed were Dukes' B (1, 3, 4 and 5) and Dukes' C (2) adenocarcinomas. Bands were detected by enhanced chemiluminescence.

characterized an *in vitro* model that provides evidence that stimulation of PKC by environmental factors contributes to intrinsic drug resistance in human colon cancer [15]. In the model, activation of PKC by phorbol-ester tumor promoters induces resistance to ADR, vincristine and vinblastine in cultured human colon cancer KM12L4a cells without affecting their sensitivity to 5-fluorouracil [15]. A reduction in intracellular drug accumulation is associated with the phorbol ester-induced drug resistance, and it is also achieved with a PKC-stimulatory diacylglycerol [15].

The phorbol ester Tx selectively activates cPKC-α, -β_1, -β_2, and -γ [17]. We show that the KM12L4a cells express active cPKC-α but not cPKC-β_1, -β_2, or -γ. Because Tx does not stimulate nPKC-δ or nPKC-ε activity [17], phorbol-ester activation of these isozymes clearly cannot account for the induction of drug resistance in KM12L4a cells; likewise, an essential role for phorbol-ester activation of nPKC-θ and -η in the induction of resistance is very unlikely, because the regulatory domains of these isozymes, including their phorbol-ester binding regions, are very closely related to the corresponding regions in nPKC-δ and nPKC-ε [29, 30]. The remaining PKC isozymes, aPKC-ζ and aPKC-λ, are phorbol ester independent [8, 23]. Taken together, our results provide strong evidence that phorbol-ester activation of cPKC-α mediates phorbol ester-induced drug resistance in KM12L4a cells.

The MDR phenotype of an ADR-selected human breast cancer MCF-7 cell line can be partially reversed by expression of anti-sense cPKC-α, indicating a requirement for cPKC-α activation in acquired drug resistance [31]. KM12L4a cells express aPKC-ζ, and it is possible that phorbol-ester stimulation of cPKC-α may lead to the activation of a PKC-ζ or other PKC isozymes in the KM12L4a cells. In future studies, antisense technology will be used to determine if cPKC-α and/or other PKC isozymes are required for the induction of drug resistance triggered by phorbol esters in KM12L4a cells. Involvement of cPKC-β_1, cPKC-β_2, cPKC-γ, nPKC-δ and nPKC-ε can be ruled out because they are not expressed. Finally, the detection of active cPKC-α in human colon carcinoma specimens in this report indicates the potential significance of cPKC-α-mediated drug resistance in clinical colon cancer.

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