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Reversal of Resistance to Adriamycin by 8-Chloro-Cyclic AMP in Adriamycin-Resistant HL-60 Leukemia Cells Is Associated with Reduction of Type I Cyclic AMP-Dependent Protein Kinase and Cyclic AMP Response Element-Binding Protein DNA-Binding Activities

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SUMMARY

8-Chloro-cyclic AMP (8-Cl-cAMP) produces growth-inhibitory and differentiating activity in the promyelocytic leukemia cell line HL-60. Adriamycin (ADR)-resistant HL-60 (HL-60/AR) cells exhibit the multidrug-resistant phenotype but do not express the *mdr1* gene product P-glycoprotein. To explore potential signaling processes that may be involved in this atypical form of drug resistance, 8-Cl-cAMP was used as a modulator of the cAMP second messenger signal transduction pathway. Treatment for 48 hr with a 10% inhibitory concentration of 8-Cl-cAMP potentiated ADR cytotoxicity 14-fold in HL-60/AR cells but not in the parental cell line. 8-Cl-cAMP was stable to hydrolysis in the medium after 48 hr and was present intracellularly predominantly as phosphorylated metabolites (70%) and the parent compound (30%). No difference occurred in ADR accumulation in HL-60/AR cells after treatment with 8-Cl-cAMP. Accompanying the 8-Cl-cAMP-mediated increase in ADR cytotoxicity in HL-60/AR cells was a reduction in the cytosolic type I cAMP-dependent protein kinase (PKA) and disappearance of the nuclear PKA holoenzyme. Coincident with these changes in drug-resistant cells was a marked reduction in the DNA-binding activity of the cAMP response element-binding protein to levels equivalent to those in sensitive cells. This effect appears to result from reduced phosphorylation of the cAMP response element-binding protein. These results suggest that the potentiation by 8-Cl-cAMP of ADR cytotoxicity in HL-60/AR cells occurs through down-regulation of nuclear type I PKA and cAMP response element-binding factors whose activities are regulated by PKA.

8-Cl-cAMP is a site-selective cAMP analog that produces antiproliferative effects in a broad spectrum of human and rodent cancer cell lines (1-3). 8-Cl-cAMP also has the capacity to induce growth inhibition and differentiation in a variety of human myeloid and lymphoid leukemia cell lines (4). The molecular mechanism of 8-Cl-cAMP is related to its ability to modulate the intracellular levels of the two isoforms of the cAMP-binding regulatory subunit of PKA, R-I and R-II, that act as positive and negative transducers of growth, respectively (5). R-II is perinuclearly localized, whereas R-I is broadly distributed in the cytoplasm, suggesting that they act as nuclear and cytoplasmic anchors, respectively, of the catalytic subunit (6, 7). 8-Cl-cAMP reduces the level of R-I and increases the level of R-II, resulting in a reduction of type I PKA activity (1, 2, 5, 31). A similar increase in the ratio of type II to type I PKA can be produced by an antisense oligodeoxynucleotide to R-I (8). R-II but not R-I may also act in *trans* to stimulate transcription through a CRE (9). Thus, the major action of 8-Cl-cAMP appears to be mediated through down-regulation of type I PKA, with a commensurate increase in type II PKA in some cell lines.

The MDR phenotype is typically characterized by overexpression of P-glycoprotein, a drug efflux pump that is the product of the *mdr1* gene (10, 11). However, not all MDR cell

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ABBREVIATIONS: 8-Cl-cAMP, 8-chloro-cAMP; PKA, cAMP-dependent protein kinase; CRE, cAMP response element; CREB, cAMP response element-binding protein; CIP, calf intestine alkaline phosphatase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; PBS, phosphate-buffered saline; MDR, multidrug resistance (or resistant); ADR, Adriamycin; HL-60/AR, Adriamycin-resistant HL-60 cells; R-I, type I regulatory subunit; R-II, type II regulatory subunit; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibitory concentration; IC_{10}, 10% inhibitory concentration.

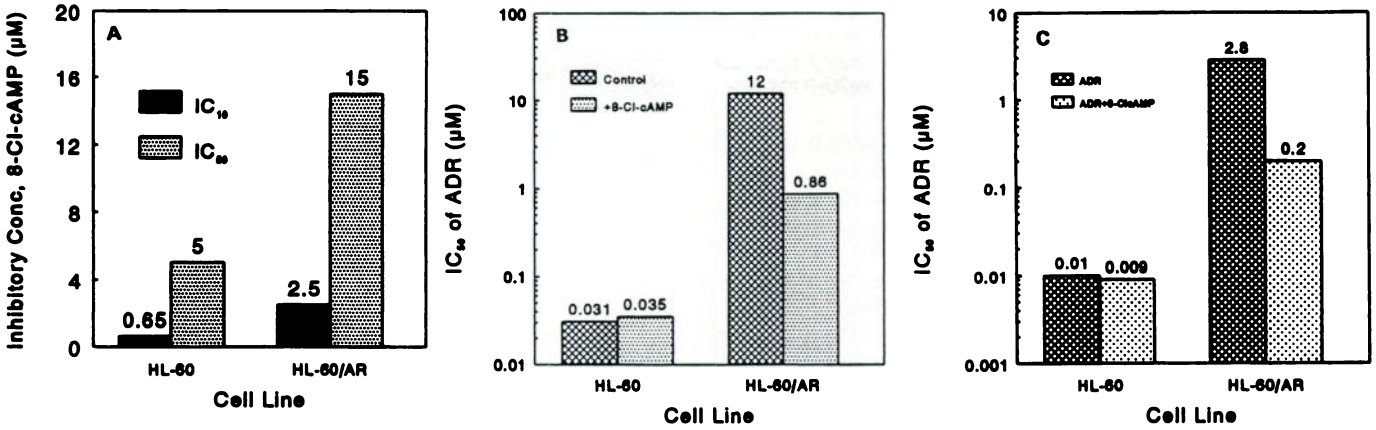

Fig. 1. Cytotoxicity of 8-Cl-cAMP and its effect on ADR cytotoxicity in HL-60 and HL-60/AR cells. A, Cells were treated for 48 hr with varying concentrations of 8-Cl-cAMP, and IC_{10} and IC_{50} values were determined on the basis of surviving viable cells that excluded trypan blue. Each value is the mean of three determinations, where the variation was 10% or less. *Numbers above each bar*, IC_{10} or IC_{50} value. B, Cells were treated for 48 hr with ADR in the presence or absence of an IC_{10} of 8-Cl-cAMP (0.65 μM and 2.5 μM for HL-60 and HL-60/AR cells, respectively), and the IC_{50} value for ADR was determined as in A. C, Cells were treated for 48 hr in the presence or absence of an IC_{10} of 8-Cl-cAMP, as in B, and cell viability was determined by a soft agar clonogenic assay. Each value is the mean of triplicate determinations from three separate experiments, where the variation was 10% or less. *Numbers above each bar*, IC_{50} of ADR in the absence or presence of 8-Cl-cAMP.

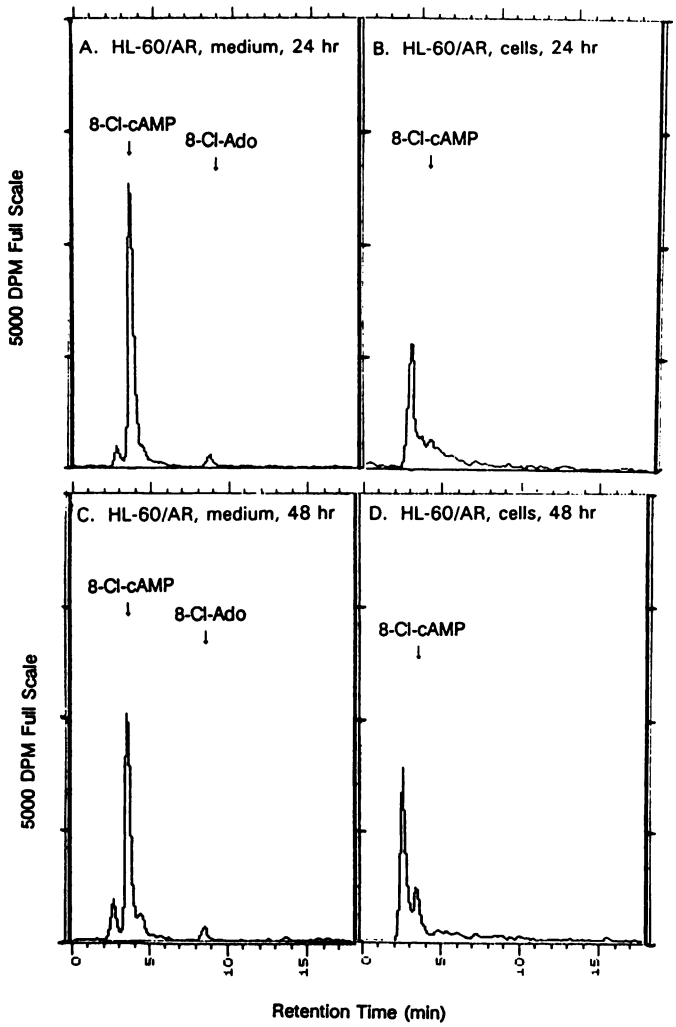

Fig. 2. HPLC analysis of [^{3}H]8-Cl-cAMP in HL-60/AR cells. Either the medium or the cells were extracted with 10% trichloroacetic acid, neutralized, and separated on a reverse phase column, as described in Experimental Procedures. *8-Cl-Ado*, 8-chloroadenosine.

lines express P-glycoprotein (12, 13) and the loci of resistance responsible for these atypical forms of MDR have not been unequivocally established. One such cell line, HL-60/AR (14), exhibits MDR without expression of P-glycoprotein (15). This cell line has been characterized as having altered sequestration of anthracyclines (16, 17), higher Ca^{2+}-dependent protease activity (18), elevated protein kinase C activity and expression of the atypical γ isoform of protein kinase C (19, 20), and increased activity and levels of transcription factor Sp1 (21). It has not been established whether any of these parameters are associated with modulation of resistance or whether they are simply epiphenomenae to the development of the resistant phenotype.

In the present study, we investigated the role of the cAMP signal transduction pathway in the MDR phenotype expressed by HL-60/AR cells. We report that 8-Cl-cAMP at minimally toxic concentrations can increase the sensitivity of this cell line to ADR and that this effect is associated with down-regulation of type I PKA as well as the DNA-binding activity of transcription factor CREB.

Experimental Procedures

Materials. 8-Cl-cAMP sodium salt was provided by K. P. Flora, National Cancer Institute, National Institutes of Health, and [^{14}C] ADR was provided by the Natural Products Branch, National Cancer Institute, National Institutes of Health. [^{32}P]8-Azido-cAMP was obtained from ICN Biochemicals. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Du Pont-New England Nuclear. Poly(dl-dC), phenylmethylsulfonyl fluoride, pepstatin, antipain, chymostatin, leupeptin, aprotinin, and soybean trypsin inhibitor were obtained from Sigma Chemical Co.

Cell culture. The human promyelocytic leukemia cell line HL-60 was obtained from the American Type Culture Collection. HL-60/AR cells (14) were obtained from Dr. James E. Gervasoni Jr., Columbia University. HL-60 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Biofluids, Inc.), 40 mM HEPES, pH 7.4, and 50 μg/ml gentamicin, and HL-60/AR cells were maintained in the same medium containing 1 μM ADR. HL-60/AR cells were diluted 10-fold in ADR-free medium before initiation of the experiments.

Cell viability. HL-60 and HL-60/AR cells (10^5/ml) were treated for 48 hr with either 8-Cl-cAMP or ADR, and cell viability was assessed by exclusion of trypan blue. IC_{10} and IC_{50} values were extrapolated from dose-response curves. Cell viability was also determined by a soft agar clonogenic assay as described previously (22).

Cellular drug accumulation. HL-60 and HL-60/AR cells were coincubated with 8-Cl-cAMP and 1 μM [^{14}C]ADR for varying time intervals. After incubation, the cells were washed three times with 2 ml of ice-cold PBS and then resuspended in 2 ml of ice-cold 50% ethanol/0.3 N HCl to extract ADR (22). The suspension was centrifuged at 1500 × *g* for 10 min, and the radioactivity in the supernatant fluid was determined in an LKB Rack Beta liquid scintillation counter.

HPLC analysis of nucleotides. For analysis of intracellular levels of 8-Cl-cAMP, HL-60 and HL-60/AR cells were grown in complete RPMI 1640 medium as described above. Control incubations contained 8-Cl-cAMP in cell-free medium. Each incubation contained either 0.65 μM (HL-60 cells) or 2.5 μM (HL-60/AR cells) 8-Cl-cAMP and 1 μCi of [^{3}H]8-Cl-cAMP. Cells were incubated for 24–48 hr, harvested by centrifugation, washed with cold PBS, and extracted with 250 μl of cold trichloroacetic acid. Extracts were neutralized by shaking with 2 volumes of 0.5 M trioctylamine in trifluorotrichloroethane and the aqueous phase was separated on a Beckman Ultrasphere 4-μm ODS column (4.6 mm × 25 cm), using a Hewlett Packard 1090 HPLC connected inline to a Radiomatic Flo-One Beta A-140 radioactivity detector. Elution was carried out at 1 ml/min in 10 mM sodium acetate, pH 5.0/10% (v/v) acetonitrile.

Preparation of cell extracts for PKA assays. Cell pellets (5 × 10^8 cells) were washed three times with ice-cold PBS and suspended in 2 ml of buffer A (20 mM potassium phosphate, pH 6.8, 2 mM EDTA, 1 mM 2-mercaptoethanol) containing 0.1 mM pepstatin, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 0.4 mg/ml aprotinin, and 0.5 mg/ml soybean trypsin inhibitor. The cell suspension was homogenized with 70 strokes of a Dounce homogenizer and centrifuged at 10,000 × *g* for 10 min at 4°. The resulting supernatants were used for analysis of cytosolic PKA activity by FPLC. In some experiments, nuclear extracts prepared as described below were used to assess PKA activity.

FPLC. Cytosolic extracts (5 mg of protein) were injected into an HR5/5 Mono Q column (Pharmacia) attached to a Pharmacia FPLC system. Elution was carried out at 4° at a flow rate of 1.0 ml/min, with a linear gradient of 0–400 mM NaCl in buffer A, and 1.0-ml fractions were collected.

PKA activity. PKA activity was measured in a reaction mixture (final volume of 50 μl) containing 30 μM Kemptide (LRRASLG; Peninsula Laboratories), 200 μM [γ-^{32}P]ATP (100–200 cpm/pmol), 10 mM $MgCl_2$, 1 mM theophylline, and 5 μM cAMP (23). After incubation for 10 min at 30°, the reaction mixture was spotted onto phosphocellulose filters (Whatman P81) and washed three times in 75 mM phosphoric acid (10 ml/sample). Filters were air dried and radioactivity was determined in a liquid scintillation counter.

Preparation of nuclear extracts. Cells (10^8 to 10^9) were washed twice in cold PBS, suspended in 10 mM Tris·HCl, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 0.2 mg/ml leupeptin, 0.4 mg/ml soybean trypsin inhibitor, 10 μM pepstatin, and incubated on ice for 10 min. The cell suspension was lysed by passage through a 24-gauge needle 30 times and was centrifuged for 5 min at 1000 × *g*. The pellet was used for the preparation of nuclear extracts by the procedure described by Borellini *et al.* (21). The pellet, representing nuclei, was suspended in 20 mM Tris·HCl, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.5 mM DTT, and KCl (4 M) was added to a final concentration of 0.3 M. The suspension was rocked for 30 min at 4° and centrifuged for 15 min at 13,000 × *g*. The supernatant was dialyzed overnight against two changes of a 200-fold excess of 20 mM Tris·HCl, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, divided into aliquots, and stored at −70°. Protein concentrations were determined with the Pierce protein reagent, with bovine serum albumin as the standard.

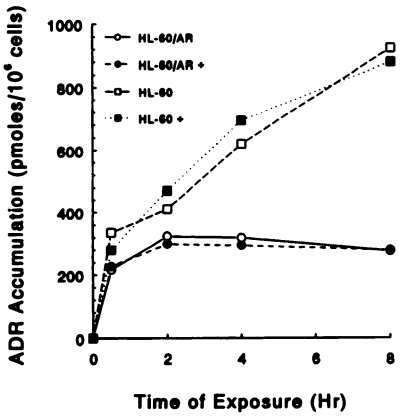

Fig. 3. Cellular uptake of [^{14}C]ADR in HL-60 and HL-60/AR cells. Cells were incubated for varying time intervals with 1 μM [^{14}C]ADR in the presence (+) or absence of an IC_{10} of 8-Cl-cAMP. The accumulation of [^{14}C]ADR was measured as described in Experimental Procedures. Each value is the mean of triplicate determinations.

Mobility shift assay. Mobility shift assays were performed with the Gelshift assay kit from Stratagene. Nuclear extracts (7 μg of protein) were incubated for 30 min at room temperature with 2 fmol (680 Ci/mmol) of double-stranded oligodeoxynucleotides end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The double-stranded probes contained the consensus DNA-binding element for CREB, AP-1, or AP-2. Competitor DNA was used at a 200-fold molar excess. The reaction mixtures were loaded directly onto 4% polyacrylamide gels and separated by electrophoresis at 100 V for 4 hr at room temperature. Autoradiography was performed by exposure of the dried gels to Kodak X-Omat XK-1 film.

In some experiments, nuclear extracts were either incubated with 2 units of CIP (Promega) in CIP buffer (50 mM Tris·HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine) or incubated with 50 units of the catalytic subunit of PKA (Sigma) in the presence of 40 μM ATP before the mobility shift assay was carried out. In these experiments, the overhang in the probe was filled in with Klenow DNA polymerase and [α ^{32}P]dGTP.

Photoaffinity labeling of cAMP receptor proteins. The photoactivated binding of [^{32}P]8-azido-cAMP was carried out by a slight modification of the method described previously (24). The reaction mixture (final volume of 50 μl) contained 4 × 10^{-7} M [^{32}P]8-azido-cAMP and samples (20–40 μg of nuclear extract) in buffer A. Incubations were carried out at room temperature for 45 min and the reaction mixtures were then irradiated for 30 sec at 254 nm, by placement of a Mineralite UVS-11 lamp directly onto the microtiter plate. The samples were mixed with 25 μl of 3× Laemmli sample buffer (3% SDS, 15% 2-mercaptoethanol, 30 mM Tris·HCl, pH 6.8, 30% glycerol, 1% bromphenol blue) and boiled for 3 min. The samples, containing 25–75 μg of protein, were separated by SDS-PAGE in 8% gels, followed by electrophoretic transfer to nitrocellulose in 25 mM Tris, 192 mM glycine, 20% methanol, pH 7.4. The nitrocellulose membrane was air-dried and exposed to Kodak XAR-2 film.

Immunoblotting. Nuclear extracts (50 μg of protein) were separated by SDS-PAGE and blotted onto nitrocellulose as described previously (21). The primary rabbit polyclonal antibody 244 to CREB (25) was kindly provided by Dr. Marc Montminy, The Salk Institute. The antigen was detected by using alkaline phosphatase-conjugated goat anti-rabbit IgG as the secondary antibody and 3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy)phenyl-1,2-dioxetane, disodium salt (AMPPD) (Tropix, Inc.) as the chemiluminescent substrate. CREB was detected by exposure of the blot to Kodak XAR-2 film.

Results

The present investigation was designed to determine whether 8-Cl-cAMP could modulate the sensitivity of HL-60/AR cells to ADR and, if so, whether this effect was associated with the

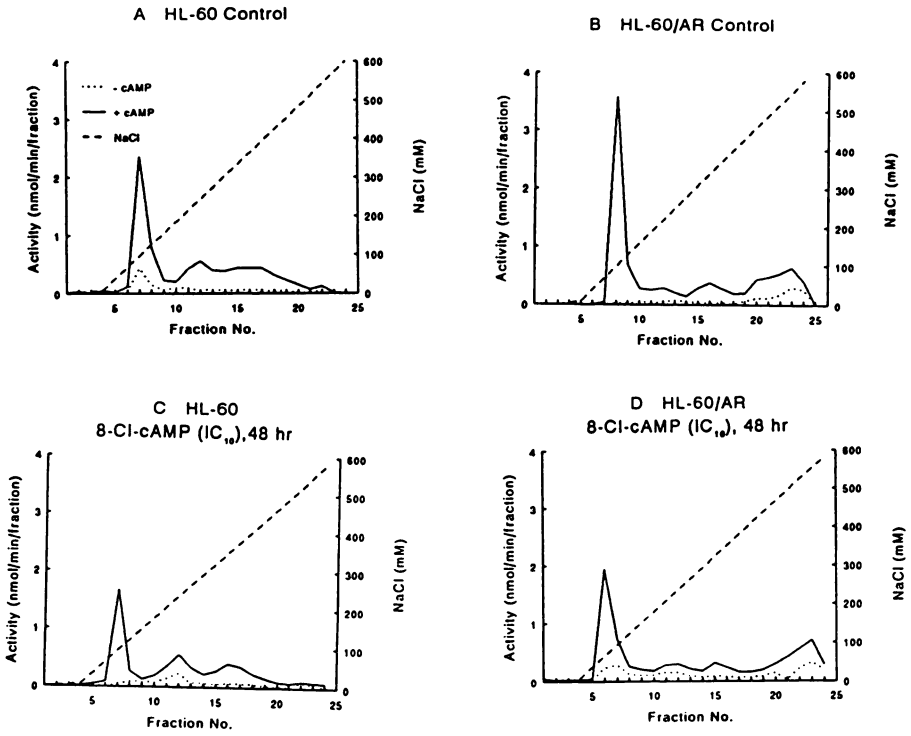

Fig. 4. Anion exchange FPLC of cell extracts from HL-60 and HL-60/AR cells after treatment with 8-Cl-cAMP. Cells were treated with an IC_{10} of 8-Cl-cAMP as described for Fig. 1, and cell extracts were prepared and separated by FPLC using a Mono Q column as described in Experimental Procedures.

down-regulation of type I PKA (1, 2, 31) and processes known to be dependent on PKA for their activity.

Cytotoxicity of 8-Cl-cAMP and its effect on ADR cytotoxicity. The cytotoxicity of 8-Cl-cAMP to HL-60 and HL-60/AR cells was initially determined by quantitation of viable cells that excluded trypan blue (Fig. 1A). The respective IC_{10} and IC_{50} values for 8-Cl-cAMP after a 48-hr exposure interval were 0.65 and 5 μM for HL-60 cells and 2.5 and 15 μM for HL-60/AR cells.

To determine whether 8-Cl-cAMP at marginally toxic concentrations could affect the cytotoxicity of ADR, cells were exposed simultaneously to an IC_{10} of 8-Cl-cAMP and varying concentrations of ADR for 48 hr and cell viability was determined by trypan blue exclusion (Fig. 1B). Treatment of HL-60 cells with 8-Cl-cAMP did not affect the cytotoxicity of ADR in HL-60 cells; however, exposure of HL-60/AR cells to 8-Cl-cAMP reduced the IC_{50} of ADR by approximately 14-fold. Similar inhibitory effects were observed at these concentrations of 8-Cl-cAMP by using a soft agar clonogenic assay to assess cell viability (Fig. 1C). The toxicity of ADR in HL-60 cells was unaffected by pretreatment for 48 hr with an IC_{10} of 8-Cl-cAMP, whereas a 14-fold potentiation of ADR toxicity was obtained in HL-60/AR cells under the same conditions.

Metabolism of 8-Cl-cAMP. Because the potentiating effect of 8-Cl-cAMP on ADR cytotoxicity required drug exposure for 48 hr, we sought to determine the amount of parent compound remaining in the medium and intracellularly (Fig. 2). Cells were incubated for 24 or 48 hr with an IC_{10} of [^{3}H]8-Cl-cAMP and the metabolite pattern was determined by HPLC. The parent drug was the major species present in the medium after incubation for 24 and 48 hr and comprised 87% and 86% of the total radioactivity, respectively (Fig. 2, A and C). Intracellular 8-Cl-cAMP represented 28% of the total radioactive metabolites at 24 or 48 hr, with the major metabolic fraction eluting in the breakthrough volume and representing other nucleotide metabolites of 8-Cl-cAMP. Little or no 8-chloroadenosine was evident either in the medium or intracellularly (Fig. 2, B and D). Identical results were obtained for HL-60 cells (results not shown).

Cellular drug accumulation. To determine whether 8-Cl-cAMP affected ADR accumulation in HL-60 or HL-60/AR cells, the cellular accumulation of 1 μM [^{14}C]ADR was measured after coincubation with an IC_{10} of 8-Cl-cAMP (Fig. 3). Drug accumulation increased over 8 hr in HL-60 cells but was unaffected by 8-Cl-cAMP. Drug accumulation reached a plateau within 30 min in HL-60/AR cells and was not altered by 8-Cl-cAMP.

Cytosolic PKA activity in HL-60 and HL-60/AR cells. The primary effect of 8-Cl-cAMP is believed to reside in its ability to down-regulate the level of type I PKA activity (1, 2, 5, 31). To assess this mechanism, cytosolic extracts were prepared from HL-60 and HL-60/AR cells after treatment with an IC_{10} of 8-Cl-cAMP and were assayed for PKA activity after anion exchange FPLC (Fig. 4). Type I PKA activity eluted at 70 mM NaCl and was the major form of PKA detected in either cell line (Fig. 4, A and B). HL-60/AR cells expressed approximately 2-fold greater type I PKA activity than did HL-60 cells. After treatment of HL-60 or HL-60/AR cells for 48 hr with an IC_{10} of 8-Cl-cAMP, PKA activity was reduced by 20% in the parental cell line and by 55% in the resistant cell line (Fig. 4, C and D). Treatment of HL-60/AR cells for 72 hr with an IC_{50} of 8-Cl-cAMP abolished PKA activity without up-regulation of type II PKA,[1] whereas in HL-60 cells the progressive down-regulation of type I PKA at an IC_{50} of 8-Cl-cAMP resulted in a compensatory increase in type II PKA (31).

Nuclear R-I levels and PKA activity. To determine whether the nuclear species of type I PKA was down-regulated in a manner similar to that of the cytosolic enzyme, nuclear

[1] Unpublished observations.

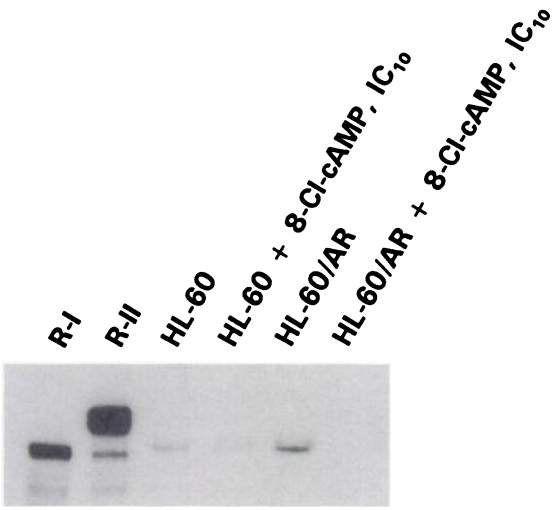

Fig. 5. Photoaffinity labeling of the cAMP regulatory subunit in nuclear extracts of HL-60 and HL-60/AR cells treated with 8-Cl-cAMP. Cells were treated for 48 hr with an IC_{10} of 8-Cl-cAMP as described for Fig. 1, and photoaffinity labeling of nuclear extracts with [^{32}P]8-Cl-cAMP was performed as described in Experimental Procedures.

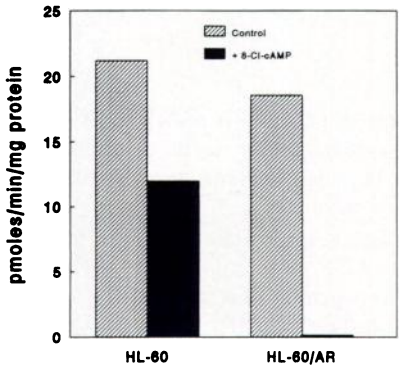

Fig. 6. Nuclear PKA activities in HL-60 and HL-60/AR cells after treatment with 8-Cl-cAMP. Nuclear extracts were prepared from HL-60 and HL-60/AR cells and assayed for PKA activities as described in Experimental Procedures.

extracts from HL-60 and HL-60/AR cells were assayed for the presence of R-I (Fig. 5). Photoaffinity labeling of nuclear proteins with [^{32}P]8-azido-cAMP identified a 48-kDa protein in both cell lines that comigrated with authentic R-I. The level of R-I was greater in resistant than in sensitive HL-60 cells, and treatment with 8-Cl-cAMP reduced the amount of R-I by 50% in HL-60 cells and virtually eliminated detectable R-I in HL-60/AR cells.

Because nuclear R-I was reduced by treatment with 8-Cl-cAMP, nuclear extracts were also assayed for cAMP-activated PKA activity (Fig. 6). Basal nuclear holoenzyme activity was similar in both cell lines; however, treatment of HL-60 cells with an IC_{10} of 8-Cl-cAMP reduced PKA activity by 45%, whereas exposure of HL-60/AR cells to an IC_{10} of 8-Cl-cAMP completely abolished PKA activity.

Mobility shift assays. Transcription factors such as CREB (26–29) and AP-1 (27) are known to be regulated through phosphorylation by PKA, and AP-2 activity is increased by cAMP-elevating agents (30). To determine whether the down-regulation of PKA was associated with changes in the DNA-binding activity of CREB, mobility shift assays were carried out, using double-stranded oligodeoxynucleotides containing

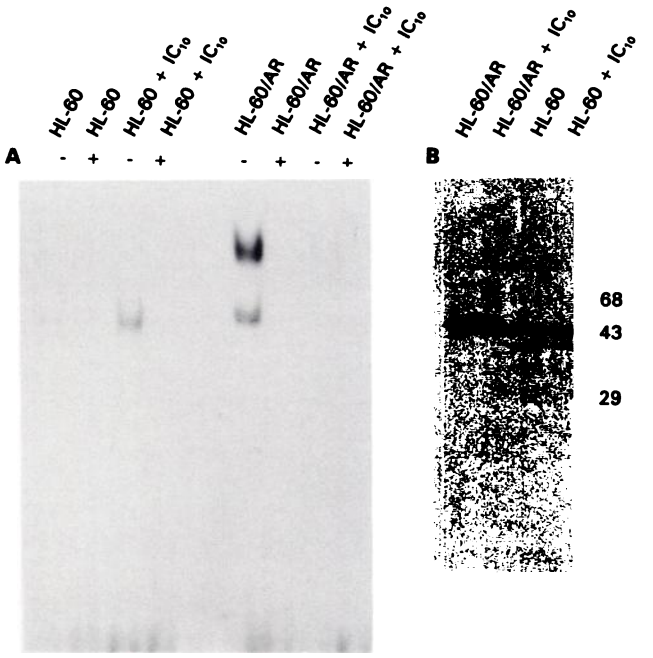

Fig. 7. Mobility shift assay for CREB DNA-binding activity, and immunoblot of CREB in nuclear extracts from HL-60 and HL-60/AR cells. A, HL-60 and HL-60/AR cells were treated for 48 hr with an IC_{10} of 8-Cl-cAMP, as described for Fig. 1, in the absence (−) or presence (+) of a 200-fold molar excess of unlabeled probe as competitor. B, Cells were treated as in A except that nuclear extracts were separated by SDS-PAGE and blotted onto nitrocellulose, and CREB was detected with rabbit polyclonal antibody 244 as described in Experimental Procedures.

the consensus sequences for CREB, in nuclear extracts from wild-type and ADR-resistant HL-60 cells (Fig. 7A). In contrast to the substantial CREB DNA-binding activity in HL-60/AR cells, HL-60 cells showed low CREB binding activity. Treatment of HL-60 cells with an IC_{10} of 8-Cl-cAMP resulted in increased CREB binding activity; however, treatment of HL-60/AR cells with an IC_{10} of 8-Cl-cAMP produced a marked reduction and disappearance, respectively, of CREB binding activity (Fig. 7A). Measurement of the amount of CREB in nuclear extracts by immunoblotting indicated that HL-60/AR cells contained approximately twice the level of CREB of HL-60 cells (Fig. 7B), a difference that could not account for the markedly higher CREB DNA-binding activity in the resistant cells.

Additional experiments were conducted to determine whether the DNA-binding activity of CREB in HL-60 and HL-60/AR cells could be modulated by the concentration of ADR or its phosphorylation state (Fig. 8). Nuclear extracts from HL-60/AR cells growing in medium containing a noncytotoxic maintenance concentration of 1 μM ADR had enhanced CREB DNA-binding activity, in comparison with cells that were not maintained in ADR for 2 weeks. Resistant cells growing in 10 μM ADR for 48 hr exhibited stronger CREB activity than cells maintained in 1 μM ADR. Although CREB activity was markedly diminished after treatment for 48 hr with an IC_{10} of 8-Cl-cAMP, its activity was increased by treatment of cells with 2.5 μM ADR in the presence of 8-Cl-cAMP (Fig. 8A).

To determine whether the 8-Cl-cAMP mediated down-regulation of CREB resulted from reduced phosphorylation due to down-regulation of PKA, nuclear extract from HL-60/AR cells was dephosphorylated with CIP and CREB DNA-binding activity was determined (Fig. 8B). Dephosphorylation reduced

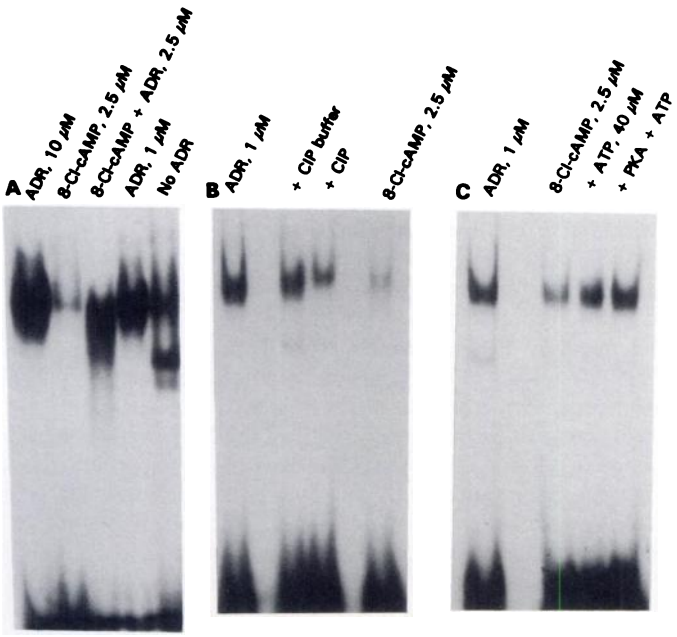

Fig. 8. Effect of ADR concentration and phosphorylation state on the DNA-binding activity of CREB in nuclear extracts from HL-60/AR cells. A, Cells were maintained in the absence or presence of 1 or 10 μM ADR and treated for 48 hr with an IC_{10} (2.5 μM) of 8-Cl-cAMP or treated with 2.5 μM ADR and 2.5 μM 8-Cl-cAMP. Mobility shift assays were performed as described in Experimental Procedures. B, Cells were maintained in 1 μM ADR and nuclear extracts were incubated in CIP buffer alone or in buffer containing CIP. CREB activity in nuclear extracts from cells treated with 2.5 μM 8-Cl-cAMP is included for comparison. C, Cells were treated with 2.5 μM 8-Cl-cAMP and nuclear extracts were incubated alone, with ATP alone, or with ATP and the catalytic subunit of PKA. CREB activity in nuclear extracts from cells maintained in 1 μM ADR is included for comparison.

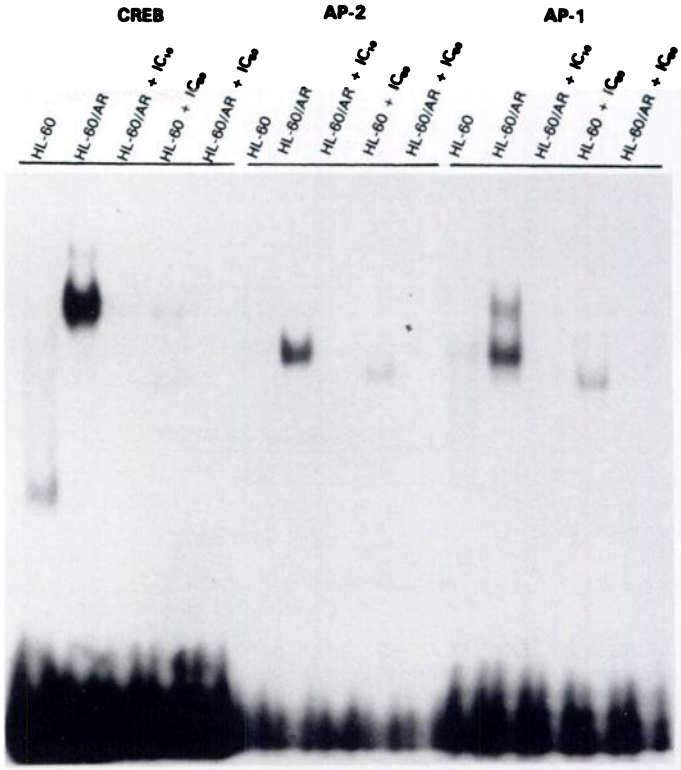

Fig. 9. DNA-binding activity of CREB, AP-2, and AP-1 in nuclear extracts of HL-60 and HL-60/AR cells. Nuclear extracts were incubated with double-stranded oligodeoxynucleotide probes containing the consensus DNA-binding elements for CREB, AP-2, or AP-1. Cells were maintained in the absence or presence of an IC_{10} or an IC_{50} of 8-Cl-cAMP for 48 hr where indicated (see Fig. 1 for drug concentrations).

CREB activity to the level present in nuclear extracts after treatment of cells with 8-Cl-cAMP. Conversely, incubation of nuclear extracts from 8-Cl-cAMP-treated cells with ATP and the catalytic subunit of PKA increased CREB activity to the level present in untreated HL-60/AR cells (Fig. 8C).

To assess whether other PKA-dependent transcription factors were affected by 8-Cl-cAMP, the DNA-binding activity of AP-1 and AP-2 in nuclear extracts from HL-60/AR cells was also measured (Fig. 9). The activities of AP-1 and AP-2 were elevated in a manner similar to that of CREB in HL-60/AR cells. Treatment of HL-60/AR cells with an IC_{10} or IC_{50} of 8-Cl-cAMP reduced or abolished, respectively, the DNA-binding activity of all three factors. In contrast, the low DNA-binding activity present in nuclear extracts from HL-60 cells was increased by treatment with an IC_{50} concentration of 8-Cl-cAMP.

Discussion

The differentiating and antiproliferative activities of 8-Cl-cAMP in leukemias and solid tumors have been attributed primarily to down-regulation of type I PKA (1–5, 8). In HL-60 cells, this mechanism is believed to involve initial activation of the stable type I holoenzyme and subsequent proteolysis of the dissociated R-I and catalytic subunits (Fig. 10) (31). The efficacy of 8-Cl-cAMP as a differentiating agent in HL-60 cells is also related to its ability to up-regulate R-II, resulting in an increase in type II PKA and maintenance of total PKA activity (4, 8, 31). The ability of 8-Cl-cAMP to sensitize HL-60/AR cells related directly to the progressive reduction of cytosolic and nuclear type I PKA activity without up-regulation of type II PKA. The inability to up-regulate type II PKA in HL-60/AR cells may also be related to the resistance of these cells to differentiating agents (19), as shown for a cAMP-unresponsive cell line (9). The absence of R-II in nuclear extracts of HL-60/AR cells before or after treatment with 8-Cl-cAMP or ADR precludes the direct involvement of this factor in resistance or in transcriptional activation or DNA binding (32, 33). Therefore, the loss of type I PKA induced by 8-Cl-cAMP in HL-60/AR cells and the absence of type II PKA appear to account for the ability of 8-Cl-cAMP to sensitize this cell line to ADR.

The disappearance of nuclear PKA activity in HL-60/AR cells after treatment with 8-Cl-cAMP suggested that processes downstream from PKA may be involved in the chemoresistance of this cell line. An analogous situation occurs in resistance mediated by overexpression of the *mdr1* gene, where cells containing a mutated R-I do not contain an active type I PKA and exhibit a down-regulation of *mdr1* expression and increased sensitivity to MDR-related drugs (34). The presence of increased DNA-binding activity of CREB, AP-1, and AP-2, levels of CREB, and levels and DNA-binding activity of Sp1 observed previously in HL-60/AR cells (21) suggests that there is a marked up-regulation of transcription factors that may be required to drive the expression of resistance-related genes. This suggests that transcription factors dependent on PKA for their activation may be involved in the up-regulation of genes related to drug resistance, regardless of the MDR phenotype (Fig. 10).

The CRE motif is TGACGTCA and binds the CREB/ATF

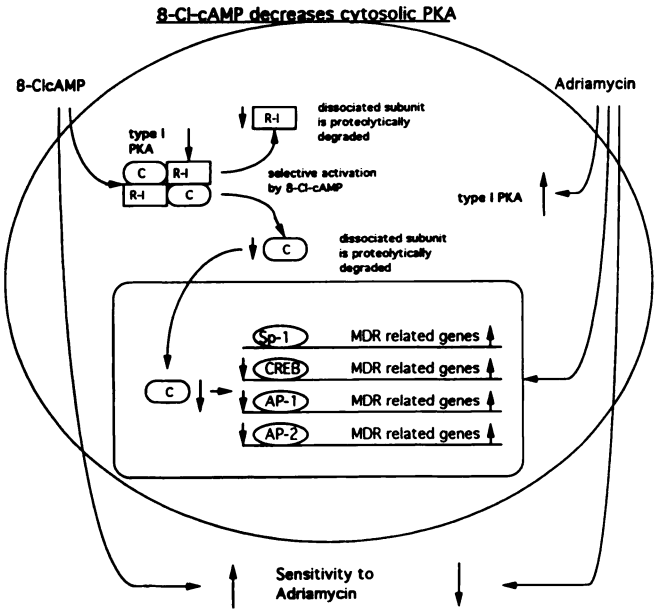

Fig. 10. Model of the effects of 8-Cl-cAMP on HL-60/AR cells that cause sensitization to ADR. 8-Cl-cAMP is taken up by the cell and exchanges for cAMP on R-I of type I PKA. 8-Cl-cAMP produces a stereoselective dissociation of the cAMP-binding (*R*) and catalytic (*C*) subunits of PKA, which are protected against proteolysis when associated as the holoenzyme. After a transient increase in phosphorylation, PKA activity progressively decreases and the reduction of cytosolic catalytic subunit results in the disappearance of nuclear catalytic subunit. Disappearance of nuclear catalytic subunit results in decreased cAMP-dependent phosphorylation of transcription factors CREB and AP-1 and possibly AP-2 and Sp1, although this has not been demonstrated directly for the latter two factors.

family of transcription factors (35), whereas the AP-1/TRE motif is TGACTCA and binds the AP-1/Jun/Fos family (36). These two families of proteins were thought to be distinct, even though the binding element differed by only one base. However, recently it was discovered that cross-family heterodimers can bind to either the CRE or the AP-1 site, with a general preference for the CRE (36). Because both CREB (25–29) and AP-1 (27) are directly activated by PKA, it is reasonable to assume that any drug that down-regulates PKA will have a similar effect on *trans*-activation. The dose-dependent reduction by 8-Cl-cAMP of nuclear PKA activity, as well as CREB, AP-1, and AP-2 binding activities in HL-60/AR cells agrees with this assertion. The differences in mobility shift pattern for CREB and AP-1 between HL-60 and HL-60/AR cells (Fig. 9) also suggest that heterodimer formation between CREB and AP-1 may account for the higher DNA-binding activity in resistant cells.

CREB binding activity in nuclear extracts from HL-60/AR cells was dependent on phosphorylation, and lower CREB binding activity after 8-Cl-cAMP treatment appeared to result, in part, from underphosphorylation. Although we cannot be sure that these effects pertain directly to CREB *per se*, they do suggest that the effect of 8-Cl-cAMP on CREB binding activity is related, to some degree, to the phosphorylation state of this factor. The binding activity of CREB was also dependent on the concentration of ADR in the medium, indicating that transactivation is also affected directly by the resistance-associated drug. Precedence for such an effect has been demonstrated for ADR, as well as other anticancer drugs associated with MDR, on the activation of transcription of the *mdr1* gene (37).

HL-60/AR cells have been shown to exhibit verapamil-sensitive drug accumulation (14) and altered sequestration of anthracyclines (16, 17). We have confirmed that there is less drug accumulation in this cell line, but the ability of 8-Cl-cAMP to sensitize HL-60/AR cells to ADR did not result from immediate changes in drug accumulation.

It has been suggested that the effects of 8-Cl-cAMP are mediated via its hydrolysis product, 8-chloroadenosine, when fetal calf serum that is not heat inactivated is used in the medium (38). Although that may be true under those conditions, our results demonstrate that in medium supplemented with heat-inactivated fetal calf serum, 8-Cl-cAMP is relatively stable over a 48-hr period and >85% of the drug in the medium is unmetabolized. There was no indication that 8-chloroadenosine was deaminated in the medium and, therefore, this metabolite does not serve as a substrate or is a poor substrate for adenosine deaminase. 8-Cl-cAMP accumulated intracellularly primarily as nucleotide metabolites and unmetabolized drug and no 8-chloroadenosine was evident. The formation of other nucleotide metabolites of 8-Cl-cAMP, such as 8-Cl-5′-AMP, could result from hydrolysis by cyclic nucleotide phosphodiesterases or possibly by the phosphorylation of 8-chloroadenosine, if formed, by nucleoside and nucleotide kinases. Formation of mono-, di-, and triphosphate metabolites of 8-Cl-cAMP in HT-29 colon carcinoma cells was noted previously (3). Because 8-Cl-cAMP accounted for approximately 30% of the intracellular drug after 48-hr incubation, these data also suggest that this drug is a poor substrate for cyclic nucleotide phosphodiesterases.

In summary, 8-Cl-cAMP selectively sensitizes HL-60/AR cells to ADR and results in the complete down-regulation of nuclear type I PKA. This effect occurs concurrently with a marked reduction in the DNA-binding activity of CREB, AP-1, and AP-2, whose activities were markedly enhanced in resistant cells. These data suggest a novel mechanism of reversing MDR by 8-Cl-cAMP in MDR cells that do not express P-glycoprotein.

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