Regulation of P-Glycoprotein Expression in Cyclic AMP-dependent Protein Kinase Mutants

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Abstract

Multidrug resistance (MDR) in cancer poses a major obstacle to the success of chemotherapy. We previously reported that cyclic AMP (cAMP)-resistant mutants of the Chinese hamster ovary and the mouse adrenal cortical carcinoma cells harboring defective regulatory (Rlα) subunits of the cAMP-dependent protein kinase (PKA) are more sensitive than wild-type cells to chemotherapeutic agents that are substrates for P-glycoprotein. In addition, a transfectant overexpressing a mutant Rlα cDNA showed similar increased sensitivity to these drugs. The altered drug sensitivity in the Rlα mutants results from reduced expression of the mdr gene, suggesting that PKA may regulate its expression. In this study, we evaluated the sensitivity of several Chinese hamster ovary catalytic (C) subunit mutants to various anticancer drugs. Like the Rlα subunit mutant, the C subunit mutants also exhibit decreased kinase activity and unresponsiveness to growth inhibition by cAMP. However, in contrast to the Rlα subunit mutant, the C subunit mutants are not multidrug sensitive and maintain P-glycoprotein expression levels comparable to those of wild-type cells. Furthermore, the C subunit mutants display the same resistance patterns as wild-type cells to P-glycoprotein substrates, including Adriamycin, Taxol, and colchicine. No significant difference was observed in their sensitivity to non-MDR drugs, such as 5-fluorodeoxyuridine, between wild-type, Rlα, and C subunit mutant cells. These results suggest that the increased multidrug sensitivity in the PKA mutant cells results from alteration of the Rlα subunit and not the kinase activity, thus implying novel functions for the Rlα subunit. Therefore, genetic alteration of the Rlα subunit of PKA may modulate drug resistance in cancer.

Introduction

Emergence of MDR1 is a serious problem in cancer chemotherapy because tumors become refractory to treatment with a variety of natural product anticancer agents that are both chemically and physically unrelated. This increased resistance to multiple cytotoxic drugs is due to overexpression of the mdr gene, which encodes a plasma membrane ATP-dependent drug efflux pump termed P-glycoprotein. The resultant resistance limits the choice of cytotoxic agents that are available for chemotherapy and leads to treatment failure (1–3).

Expression of the MDR1 gene occurs commonly in human cancers, and its regulation has been actively investigated in recent years (4–6). It has been shown that the CHO and the mouse adrenal cortical carcinoma Y1 cells harboring a defective Rlα subunit of the cAMP-dependent PKA are sensitive to multiple chemotherapeutic drugs that are substrates for P-glycoprotein (7). In addition, a transfectant overexpressing a dominant mutant Rlα cDNA shows similar increased sensitivity to these drugs (8). We have shown subsequently that the increase in drug sensitivity is due to decreased expression of the mdr gene in these mutants (9). Therefore, an intact PKA system is apparently required for maintaining the basal levels of mdr RNA in these cells. The role of PKA in P-glycoprotein expression was further supported by the discovery that a selective inhibitor of PKA, H-87, inhibited MDR1 gene expression and promoter activity in the mouse P388 cells (10). These studies suggest that alteration of PKA activity may modulate cellular sensitivity to chemotherapeutic agents and establish that the cAMP signal transduction pathway may be involved in regulating drug resistance.

To provide further evidence that changes in drug sensitivity are due to alteration of PKA activity, we examined in this study the sensitivity of CHO cells with mutations in the C subunit of PKA to various chemotherapeutic agents that are substrates for P-glycoprotein. Despite their cAMP-resistance and decreased kinase activity, the C subunit mutants maintained the same drug resistance patterns as the wild-type cells, whereas the Rlα subunit mutants displayed increased sensitivity to these cytotoxic agents. Furthermore, P-glycoprotein levels in the C subunit mutants were comparable to wild-type levels but were reduced in the R subunit mutants. These results suggest that decreased mdr gene expression in the R subunit mutants may be a direct result of the mutational alteration of the Rlα subunit.

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3 The abbreviations used are: MDR, multidrug resistance; CHO, Chinese hamster ovary; R, regulatory; C, catalytic; PKA, cAMP-dependent protein kinase; 8-Br-cAMP, 8-bromo-cAMP; RI, regulatory subunit of type I PKA; Rlα, alpha subtype of RI; RII, regulatory subunit of type II PKA.
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Results
Analysis of genetic mutants in the cAMP signal transduction pathway has increased our understanding of the role of cAMP and PKA in the modulation of cellular processes. The pioneering approach of Tomkins and colleagues (11, 12) in developing the mutant genetic systems with the mouse S49 lymphoma cells through a combination of mutagenesis and exploitation of their growth characteristics in cAMP permitted the isolation and selection of mutant cells that do not respond to cAMP. On the basis of this approach, the CHO cells used in the present study have impaired PKA activity resulting from mutations in either the R or the C subunit gene of PKA (13, 14). The 10248 mutant cells contain a dominant mutation (glycine to glutamic acid substitution at amino acid 200) in the cAMP-binding domain of the RIα subunit that decreases its affinity for cAMP and prohibits holoenzyme dissociation (15, 16). Consistent with previously reported results (15, 16), the RIα mutants have a 50% reduction in PKA activity in comparison to the wild-type 10001 cells (Fig. 1).

It has been shown that mutations affecting the R or C subunit of PKA caused an increase in resistance to the growth inhibitory effects of cAMP in CHO cells (13, 20). Consequently, activation of the mutant PKA in these cells requires much higher concentrations of the second messenger. Cell cycle analysis of the PKA mutants by flow cytometry confirmed this cAMP-resistant phenotype observed previously. Treatment with 1 mM 8-Br-cAMP did not significantly alter the cell cycle patterns of the RIα and C subunit mutants after 24 or 48 h (Table 1). However, a G1 growth arrest was observed with the wild-type cells 24 h after exposure to 1 mM 8-Br-cAMP. A further increase in G1 block was observed in 10001 cells after 48 h of treatment (79% of the cell population in G1 after treatment versus 58% of the untreated control). These results confirmed that the PKA mutants are resistant to the growth inhibitory effects of cAMP.

We reported previously that the RIα subunit mutants have decreased mdr gene expression, and this reduced P-glycoprotein expression was thought to be associated with altered kinase activity (8, 9). Therefore, it was expected that the C subunit mutants suffering from a similar loss in PKA activity would also have altered P-glycoprotein expression. Surprisingly, Western blot analysis showed that the C subunit mutants have levels of P-glycoprotein expression comparable to those of the wild-type 10001 cells (Fig. 2). Consistent with previous results (8, 9), a 50% reduction in P-glycoprotein expression was observed in the 10248 RIα mutant. Densito-

\[ \text{Fig. 1. PKA activity in wild-type and mutant CHO cells. PKA activity was measured relative to 100% activation of the wild-type at 100 \, \mu M} \]

\[ \text{cAMP. All values were corrected for activity in the absence of cAMP. Columns, average of three separate experiments repeated in duplicate. 10001, wild-type; 10248, RIα subunit mutant; 10215, 10260, and 10265, C subunit mutants; bars, SE.} \]

\[ \text{Table 1: Effect of cAMP on cell cycle distribution of wild-type and PKA mutant cells}^a \]

\[ \begin{align*}
\text{Cell type} & & & \text{24 h} & & & \text{48 h} \\
& & \text{Without cAMP} & \text{With cAMP} & \text{Without cAMP} & \text{With cAMP} \\
10001 & 22 & 50 & 58 & 79 \\
10248 & 24 & 23 & 56 & 51 \\
10215 & 47 & 48 & 76 & 73 \\
10260 & 48 & 49 & 66 & 66 \\
10265 & 48 & 53 & 72 & 74 \\
\end{align*} \]

\[ ^a \text{Data presented are based on at least three independent experiments performed in duplicate. Numbers denote the percentage of cells in G_0-G_1.} \]

\[ \text{Fig. 2. P-glycoprotein expression in KB and CHO cells. Cell extracts (100 \, \mu g} \text{ of protein) from KBV-1 cells that overexpress P-glycoprotein, KB3-1 cells that are MDR1-null mutants, and CHO cells were run on a 6\%} \text{ denaturing polyacrylamide gel. Protein was transferred to a polyvinylidene} \]

\[ \text{difluoride membrane and probed with 1 \, \mu g \text{/ml C219 monoclonal antibody for 12 h. The immunoreactive bands were detected by use of an ECL kit using a secondary goat antimes} \]

\[ \text{antibody conjugated to horseradish peroxidase. A representative of four separate experiments is shown.} \]
metric scanning confirmed the altered levels of P-glycoprotein in the 10248 mutant cells (data not shown). Because mdr gene expression was not altered in the C subunit mutants, therefore, our results suggest that the altered expression may be associated with the defective RIA subunit and not decreased kinase activity.

We then further examined the changes in drug sensitivity in these PKA mutants to various drugs that are substrates for the multidrug transporter. As shown in Fig. 3, C subunit mutants 10215, 10260, and 10265 exhibited the same pattern of resistance as wild-type cells to Adriamycin, Taxol, and colchicine. These results correlate with the level of P-glycoprotein expression in wild-type and C subunit mutant cells. Furthermore, consistent with earlier studies (7–9), the R subunit mutants displayed increased sensitivity to these drugs. There was no difference in the sensitivities of wild-type or R or C subunit mutant cells to the non-MDR drug 5-fluorodeoxyuridine (Fig. 3D). These data support the idea that mutation in the RIA subunit of PKA contributes to the altered drug sensitivity and suggest a novel function for the RIA subunit.

Discussion
Expression of P-glycoprotein has been shown to play a significant role in the clinical resistance of a large variety of human malignancies (21-23). Therefore, studies of the regulation of the mdr gene may yield novel insight into circumventing MDR in cancer. Previously, we showed that RIA subunit mutants of CHO cells, as well as wild-type cells, transfected with a dominant mutant RIA cDNA have reduced mdr gene expression and display increased sensitivity to chemotherapeutic agents that are substrates for P-glycoprotein (7, 9). These data suggest that alteration in P-glycoprotein levels may be regulated by PKA. Although nuclear run-on assay results suggest that expression of mdr genes may be posttranscriptionally regulated, the precise regulatory input of PKA remains undefined (9). To substantiate the role of the kinase in the regulation of mdr gene expression, we further evaluated, in the present study, the P-glycoprotein levels and the cellular sensitivity of several C subunit PKA mutants to anticancer agents. Despite their decreased PKA activity (Fig. 1) and resistance to growth inhibition by cAMP (like the R subunit mutants; Table 1), surprisingly, mutations in the C subunit in 10215, 10260, and 10265 cells did not alter their P-glycoprotein expression levels (Fig. 2). The C subunit mutants also had MDR patterns comparable to wild-type cells (Fig. 3, A-C). Moreover, wild-type and RIA and C subunit mutants maintained the same sensitivity to non-MDR drugs, including 5-fluorodeoxyuridine (Fig. 3D). Changes in P-glycoprotein levels and altered drug sensitivity could not have been due to changes in either PKA type I or PKA type II activity because the mutants 10215 (missing type II but retaining a reduced amount of type I activity; Ref. 15), 10265 (missing type I kinase but having reduced type II activity; Ref. 15), and 10260 (very little type I or type II activity; Ref. 19) all exhibited patterns of resistance to Adriamycin, Taxol and colchicine similar to those of wild-type cells. Therefore, there appears to be no direct correlation between alterations in type I and type II PKA activity and P-glycoprotein levels.
These results support our hypothesis that decreased mdr gene expression in 10248 cells may be a result of the mutation in the Rα subunit gene and suggest that the Rα subunit but not the C subunit kinase may modulate cellular multidrug sensitivity.

Other recent studies lend further support for a function of Rα in modulating drug sensitivity. It has been shown that overexpression of the Rα subunit in CHO-Ki cells via retrovirus-mediated gene transfer increases their sensitivity to topoisomerase II poisons (24). Furthermore, the CHO-Ki-derivative ADR-5 mutant, which overexpresses the endogenous Rα, is also hypersensitive to topoisomerase II inhibitors and displays cross-sensitivity to the site-selective cAMP analogue 8-Cl-cAMP (24). Although neither the kinase activity nor P-glycoprotein levels were examined in these studies, they demonstrate a relationship between Rα expression and cellular sensitivity to drugs that target topoisomerase II. In addition, it has been shown that treatment of the multidrug-resistant human MCF-7 breast cancer and the A2780 ovarian carcinoma cells with 8-Cl-cAMP leads to decreased MDR1 gene expression and increased vinblastine accumulation (25). 8-Cl-cAMP has the ability to down-regulate Rα expression and activity (26). The down-regulation of Rα in MCF-7 and A2780 cells correlates with the decreased P-glycoprotein expression and enhanced cellular sensitivity in these cells, suggesting a role for Rα in regulating MDR1 expression. Taken together, these findings seem to indicate that changes in drug sensitivity may be due to genetic alterations of the Rα subunit of PKA via mutation or altered gene expression.

Our laboratory has also examined the role of PKA in the development of drug resistance to cisplatin (27). We have found that CHO and mouse adrenocortical carcinoma Y1 cells with defective Rα subunits but not altered C subunits exhibit increased resistance to cisplatin. The Rα mutants are also cross-resistant to other DNA-damaging agents, and further analysis by the host cell reactivation assay revealed an enhanced DNA repair capacity in these mutants. We speculate that the altered resistance may be a function of the Rα subunit, as opposed to the activity of the kinase (27). Furthermore, a study examining the effects of 8-Cl-cAMP on parental and cisplatin-resistant PC-14 non-small cell lung cancer cell lines showed a correlation between a low RI:RII ratio and increased resistance to cisplatin, supporting the idea that Rα deregulation is specific to cisplatin resistance (28).

The precise mechanisms by which Rα modulates drug resistance are unclear at present. It is conceivable that Rα may interact with other proteins that subsequently alter cellular sensitivity to cytotoxic agents. For example, the RII subunit of PKA has been found to associate with the A-kinase anchor protein AKAP70, demonstrating that the R subunit can interact with other proteins (29). Furthermore, the phosphorylated RII subunit has been shown to interact with DNA and to possess DNA relaxing activity (30). Therefore, the R subunit may have the ability to positively or negatively regulate factors involved in cellular drug resistance via protein-protein interaction or posttranslational modification.

Certainly, our results and those of others suggest that the Rα subunit of PKA may have other cellular functions in addition to inhibition of the kinase activity and that mutation of the Rα subunit may affect drug sensitivity in these cells. Therefore, exploitation of the cAMP levels and the R subunit function may potentiate the cytotoxicity of chemotherapeutic agents and reduce drug resistance in cancer. More importantly, the drug resistant phenotypes and their mechanisms may enable us to begin to examine potentially novel regulatory functions of the Rα subunit.

Materials and Methods

Cell Culture and Drug Sensitivity Assays. CHO and KB cells were cultured in MEM and DMEM, respectively, supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg of streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cytotoxicity was evaluated using the clonogenic assay. For clonogenic survival, 200 cells were plated in 60-mm diameter Petri dishes and immediately treated with various concentrations of drugs. After a 7–10 day incubation, colonies were stained with methylene blue (0.01 g/ml in 50% methanol), and colonies containing greater than 50 cells were scored.

Cell Cycle Analysis and Flow Cytometry. Approximately 1 x 10⁶ cells were plated in 100-mm dishes and incubated with or without 1 mM 8-Br-cAMP (Sigma Chemical Co., St. Louis, MO) for 24, 36, or 48 h. After fixation in 70% ethanol, cells were stained with a 10 μg/ml propidium iodide/1 mg/ml RNase/PBS solution, and the DNA content of the cells was analyzed in duplicate by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Cell cycle data analysis was performed with the ModFit LT program (Verity Software House, Inc., Topsham, ME).

Western Blot Analysis. For Western blot analysis of P-glycoprotein expression, protein lysates (100 μg) were separated by 6% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated for 12 h with 1 μg/ml of the monoclonal antibody C219 against P-glycoprotein. The immunoreactive bands were detected by use of an ECL kit (Amersham Corp., Arlington Heights, IL) using a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Life Technologies, Inc., Grand Island, NY).

PKA Activity Assay. Kinase activity was determined by measuring the transfer of ³²P from [γ-³²P]ATP to Kemptide (Sigma), a specific substrate for PKA (8). Cell extracts were prepared by homogenization in 10 mM Tris, pH 7.4, 0.8 mM EDTA, and 1 mM DTT. Homogenates were centrifuged for 5 min at 14,000 rpm. The reaction mixture contained 27.5 mM Mes, 1.0 mM EDTA, 2.75 mM NaF, 5.5 mM magnesium acetate, 1.25 mg/ml BSA, 0.1 mg Kemptide, 100 μM ATP, 100 μM cAMP, and 5.0 μCi [³²P]ATP in a total volume of 0.1 ml. The reaction was initiated by the addition of cell extract (80 μg of protein). After 10 min of incubation at 30°C, 40-μl aliquots were immediately spotted on phosphocellulose discs (Whatman P81 filter paper), washed four times with 3% phosphoric acid, and quantitated by scintillation counting. A no-enzyme blank was subtracted from the total incorporation, and all values were corrected for activity in the absence of cAMP.

Statistics. Statistical significance was assessed where appropriate by a paired Student’s t test (significant difference at P < 0.05).

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References


