Protein Kinase A, not Epac, Suppresses Hedgehog Activity and Regulates Glucocorticoid Sensitivity in Acute Lymphoblastic Leukemia Cells*

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Cyclic AMP synergizes strongly with glucocorticoids (GC) to induce apoptosis in normal or malignant lymphoid cells. We examined the individual roles that CAMP-dependent protein kinase (PKA) and Epac (exchange protein directly activated by cAMP), two intracellular CAMP receptors, play in this synergistic effect. Our studies demonstrate that PKA is responsible for the observed synergism with GC, whereas Epac exerts a weak antagonistic effect against GC-induced apoptosis. We find that endogenous PKA activity is higher in the GC-sensitive clone than in the GC-resistant clone. In the GC-sensitive clone, higher PKA activity is associated with lower Hedgehog (Hh) activity. Moreover, inhibition of Hh activity by Hh pathway-specific inhibitors leads to cell cycle arrest and apoptosis in CEM (human acute lymphoblastic leukemia, T lineage) cells, and the GC-sensitive clone is more sensitive to Hh inhibition. These results suggest that Hh activity is critical for leukemia cell growth and survival and that the level of Hh activity is in part responsible for the synergism between cAMP and GC.

Glucocorticoids (GC) induce apoptosis in lymphoid cells and have been used widely as a mainstay therapy for lymphoid malignancies (1). Although the mechanism of GC-induced apoptosis is not entirely clear yet, sufficient levels of active intracellular GC receptor (GR) are necessary, and suppression of c-Myc, the proper balance of mitogen-activated protein kinases, and ultimately, activation of effector caspases are involved (1–6). The second messenger cAMP can either promote cell growth or induce cell death depending on cell types (7). CAMP synergizes strongly with the GC-GR system in inducing lymphoid cell apoptosis (8–10). The mechanism underlying this synergy is poorly understood, although induction of Bcl-2-interacting mediator of cell death (BIM), which is a member of Bcl-2 family, has been suggested to play a role in the eventual apoptotic effect (9, 11, 12).

Hedgehog (Hh) signaling is critical in vertebrate development, patterning, and cell fate induction (13). The secreted protein Hh exerts its effects through binding to its receptor PTCH (Patched), which releases its inhibition of another transmembrane protein SMO (Smoothened). Activated SMO regulates gene transcription by activating transcription factors GLI (glioblastoma gene products). In mammalian cells, Hh has three isoforms: Shh (sonic hedgehog), Dhh (desert hedgehog), and Ihh (Indian hedgehog); of these, Shh is best studied. Constitutive activation of the Shh signaling pathway is associated with several forms of malignant disease, including basal cell carcinoma, medulloblastoma, rhabdomyosarcoma, prostate cancer, hepatocellular cancer, pancreatic cancer, and some gut cancers (13–19). Members of the Shh signaling pathway are expressed during thymocyte development. They regulate differentiation from the double-negative (CD4−CD8−) to the double-positive (CD4+CD8+) stage of T cell development (20). Shh is also associated with the proliferation of human hematopoietic stem cells, and Shh, PTCH, and Smo transcripts are present in primitive and mature CD19+, CD33+, and CD3+ cell populations (21–23). Shh has also previously been shown to induce Bcl-2 (24), an important regulator of T cell survival.

Because of the important role that the Hh pathway plays in lymphoid cell development, differentiation, and survival and because PKA has been identified as a negative regulator of Hh pathway in Drosophila as well as mammalian cells, we hypothesize that the Hh pathway contributes to the lymphoid malignant cell growth and survival and that PKA exerts its synergism with GC through regulating Hh signaling in CEM cells. In this study, we used human GC-sensitive and -resistant CEM cells as model systems to test our hypothesis. We report that Hh pathway activity is inversely correlated with cellular PKA activity as well as the GC sensitivity.

MATERIALS AND METHODS

Reagents—Cyclopamine and tomatidine were purchased from Toronto Research Chemicals (North York, Canada). Dexamethasone (Dex) was purchased from Sigma-Aldrich. Forsko-
lin was purchased from Alexis Biochemicals (San Diego, CA). Rp-cAMP and 8-pCPT-2′-O-Me-cAMP, an Epac activator, was purchased from Biolog Life Institute (Bremen, Germany). Caspase 3 substrate was purchased from ICN Pharmaceuticals (Auraro, OH). A Peptag PKA activity assay kit was purchased from Promega (Madison, WI). Alamar Blue dye was purchased from BIOSOURCE (Camarillo, CA).

**Cell Culture**—Human CEM clonal cell lines C7–14, C1–15, and C1–8 were cells grown in RPMI 1640 supplemented with 5% fetal bovine serum (Invitrogen). 1% fetal bovine serum was used if cells were treated with KAAD-cyclopamine. The human CEM cell line was isolated from a patient with acute lymphoblastic leukemia. Subclone C7–14, sensitive to GC-evoked apoptosis, was derived from the original sensitive C7 clone. Subclone C1–15, which was derived from the original resistant C1 clone, remains resistant to GC. Many properties of the two original clones were similar. Each was pseudodiploid, contained similar concentrations of GR (12,000–14,000 GR sites/cell) and showed a similar affinity ($K_d = \sim 20\text{ nm}$) for Dex (25).

The subclones were restested as soon as they grew out and were selected for further study on the basis of their similarity to the phenotypes of their parental clones. C7–14 was found to have 9,900 GR sites/cell, and C1–15 had 10,350. They were pseudodiploid (26). Subclone CEM C1–8 is a GC-sensitive spontaneous revertent from the GC-resistant C1 clone.

**PKA Activity Assay**—A radioisotopic method (27) was used to measure PKA activity in the cell lysate. The kinase reaction mixture (50 μl) contained 50 mM Mops (pH 7.0), 10 mM MgCl$_2$, 0.25 mg/ml bovine serum albumin, 0.1 mM kemptide substrate, and 0.1 mM ATP at 100 cpm/μmol, 20 μg of total cell lysate protein, and 10 μM cAMP. The reaction was pre-equilibrated at room temperature for 10 min and initiated by adding the kemptide substrate. Following a 10-min incubation, aliquots (45 μl) were withdrawn, spotted onto discs of Whatman P81 paper, and immediately immersed in 75 mM phosphoric acid (10–20 ml/sample) to terminate the reaction. After washing three times in phosphoric acid and once with ethanol, the discs were dried under a heating lamp. Radioactivity was measured by liquid scintillation spectrometry. Background counts measured from reactions that did not contain kemptide were subtracted from the means of at least two independent experiments as defined in the figure legends.

**Cell Cycle Analysis**—Cell cycle stage was determined using flow cytometry. Briefly, treated CEM cells were fixed with 70% ethanol at −20°C for 1 h. The cells were resuspended in 1 ml of phosphate-buffered saline containing propidium iodide (5 μg/ml) and RNase A (0.1 mg/ml) and were incubated at 37°C for 30 min. The cells (10$^4$ cells/analysis) were examined by flow cytometry (Coulter, Hialeah, FL), and the cell cycle distribution was determined by DNA content. Cells distributed in sub-G$_1$ were defined as apoptotic according to the criteria described by others (29).

**Cell Viability Assay**—Cell growth was determined by Alamar Blue assay. The cells were seeded at a density of 10,000 cells (100 μl)/well in 96-well plates. The plates were incubated with drugs for specified amount of time. Alamar Blue (10 μl) was added to each well and incubated for 4 h at 37°C. Alamar Blue is a fluorescent substrate reduced by mitochondrial enzyme activity in viable cells. Fluorescence intensity was determined using a Molecular Devices plate reader (SpectraMax M2) with an excitation wavelength at 530 nm and an emission wavelength at 590 nm. The fluorescence readings were then normalized to readings from vehicle-treated cells (at day 0 for time course experiments). The error bars indicate standard deviations from the means of at least two independent experiments as defined in the figure legends.

**RESULTS**

**PKA, not Epac, Is Responsible for the Synergistic Killing Effects Mediated by cAMP and GC**—The effects of cAMP are mediated by two intracellular cAMP receptors, PKA and Epac (30). Because Epac and PKA are ubiquitously expressed and distinctively localized in cells (31–33), it was possible that the synergistic effect of cAMP is mediated by PKA, Epac, or both. We examined the contribution of PKA or Epac to the cAMP-mediated synergistic cell killing effect in CEM cells using pharmacological activators and inhibitors. As shown in Fig. 1, the increase in intracellular cAMP induced by forskolin, demonstrated previously in these cells (10), led to synergistic killing of both GC-sensitive and -resistant cell lines when they were cotreated with Dex. On the other hand, selective activation of Epac using 8-pCPT-2′-O-Me-cAMP, an Epac-specific activator that did not activate PKA under the concentration used for our study, showed no synergetic effect with Dex in either C1–15 or C7–14 cells (Fig. 1). On the contrary, 8-pCPT-2′-O-Me-cAMP partially antagonized the killing effect of Dex in GC-sensitive clone C7–14 cells (Fig. 1B). Inhibition of PKA by Rp-cAMP, a specific PKA inhibitor, significantly reversed the synergy between Dex and forskolin in C1–15 cells (Fig. 1A). Taken together; these
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PKA Activity Is Lower in GC-resistant Leukemia Cell Line—Because PKA is implicated for cAMP-mediated synergistic killing effect in CEM cells, we examined the regulation of PKA in GC-sensitive and -resistant CEM cells. As shown in Fig. 2, PKA activity in the GC-resistant clone C1–15 cells, measured by the classic radioactivity kinase assay with a saturating concentration of cAMP (10 μM), was significantly lower than that in the GC-sensitive clone C7–14 cells. The difference between C1–15 and C7–14 clones, although only about 20%, is statistically significant and reproducible. In addition, when PKA activities in GC-sensitive and -resistant CEM cells were measured under a more physiological concentration of cAMP (1 μM) using an independent commercial PepTag PKA activity assay from Promega (Madison WI), the difference of PKA activity levels in GC-sensitive and -resistant CEM cells was more dramatic (Fig. 2, inset). These results indicate that the level of PKA activities is correlated to GC sensitivity in these CEM clones.

Increased Hh Pathway Activity in GC-resistant Leukemia Clone—In different cellular settings, PKA can have growth promoting or growth inhibiting effects. The precise mechanisms for this differential effect are unknown. Recent findings that PKA inhibits Hh pathway activity provide a clue for its anti-growth properties under certain cellular scenarios (34). To test whether PKA exerts its synergistic killing effect in CEM cells through the inhibition of Hh pathway, we first determined whether the Hh pathway was activated in our CEM clones. Hh pathway activity was determined by monitoring the expression of Hh target genes GLI1 and PTCH, by real time PCR. Although both GC-sensitive and -resistant cell lines showed activation of the Hh pathway, the GC-resistant clone C1–15 cells displayed significantly and consistently higher levels of GLI1 and PTCH mRNAs compared with the GC-sensitive clone C7–14 cells (Fig. 3, A and B). When treated with forskolin, both cell lines showed a significant decrease of GLI1 and PTCH mRNA expression. Although Dex treatment alone had no effects on GLI1 and PTCH expression in either clone, more pronounced inhibition of Hh target gene expression was seen in both clones when cotreated with both Dex and forskolin compared with single forskolin treatment (Fig. 3, A and B). In addition, suppressing basal PKA activity in C7–14 cells by myristoylated PKI, a membrane-permeable PKA-specific inhibitor, led to a reciprocal increase of GLI1 mRNA levels as measured by real time PCR (Fig. 3C). These data suggested that PKA negatively regulates Hh activation in acute lymphoblastic leukemia CEM cells and that inhibition of the Hh pathway by PKA might be involved in GC-induced cell death and may account for the synergistic effect of cAMP and Dex. To further determine whether Hh signal repression by cAMP/PKA is GC/GR-dependent or -independent, we cotreated CEM cells with forskolin and dexamethasone mesylate, a GR antagonist (35). As shown in Fig. 3 (A and B), dexamethasone mesylate did not block the inhibitory effects of forskolin. These observations suggest that Hh signal repression by cAMP/PKA is GC/GR-independent.
Inhibition of Hh Pathway Induces Cell Apoptosis in Both GC-sensitive and -resistant Cell Lines—If PKA-induced Hh inhibition is responsible for cAMP-mediated synergistic killing in CEM cells, one would expect that a Hh pathway-specific inhibitor would induce cell death in these cell lines. To test this hypothesis, both C1–15 and C7–14 cells were treated with various concentrations of KAAD-cyclopamine, a specific Hh pathway inhibitor that binds to SMO and inhibits the activation of the downstream transcriptional factor GLI (36). KAAD-cyclopamine at 10 μM almost completely inhibited cell growth in both cell lines (Fig. 4A), but a structurally related inactive analog, tomatidine, had no effect. When lower KAAD-cyclopamine concentration (2.5 μM) was used, the GC-sensitive clone
C7–14 cells with lesser PKA activity showed an increased sensitivity to KAAD-cyclopamine inhibition as compared with the GC-resistant clone C1–15 cells (Fig. 4B). Similar results were obtained when another small molecular inhibitor, Smo antagonist 1 (SANT-1), was used (Fig. 5). SANT-1 is a Hh pathway-specific inhibitor that is structurally and mechanistically distinct from cyclopamine (37). We also observed that KAAD-cyclopamine, but not its analog tomatidine, induced cell death (Fig. 4A). To confirm that KAAD-cyclopamine indeed induced apoptosis in these cells, we measured caspase 3 activities after treatment with the inhibitor. As shown in Fig. 6A, KAAD-cyclopamine treatment led to a significantly increased caspase 3 activity in C1–15 cells. Furthermore, cell cycle analysis based on flow cytometry showed that KAAD-cyclopamine treatment caused a significant increase in cell populations at sub-G1 and G0/G1 phases (Fig. 6B). These data are consistent with the need for Hh pathway activation for cell growth and survival of CEM lymphoid cells.

Elevated PKA Activity, Reduced Hh Activity, and Increased Cyclopamine Sensitivity in Spontaneous Revertant, GC-sensitive CEM C1–8 Cells—CEM C1–8 is a clone of the spontaneous revertant, GC-sensitive cells derived from the GC-resistant CEM C1 cells. The mechanism of GC sensitivity reversion of C1–8 cells is unknown. If our theory that increased PKA activity and subsequently decreased Hh activity are responsible for GC sensitivity in CEM cells is correct, one can predict that the C1–8 GC-sensitive revertant cells will have higher PKA activity, lower basal Hh path-
way activity and greater sensitivity to cyclopamine-induced growth arrest and cell death when compared with the sister clone C1–15 GC-resistant cells. To examine our hypothesis, PKA activities in C1–8 and C1–15 cells were measured. C1–8 cells, like C7–14, showed a small but significant increased level of PKA activity as compared with the C1–15 cells (Fig. 7A). Again, this difference of PKA activity levels was confirmed using an independent PKA activity assay (Fig. 7A, inset). The increased PKA activity in C1–8 cells was accompanied by a decreased Hh pathway activity, as reflected by the lower Hh target gene expression measured by real time PCR (Fig. 7B). As expected, C1–8 cells were also more sensitive to KAAD-cyclopamine treatment when compared with C1–15 cells (Fig. 7C). Taken together, these results demonstrate that higher PKA activity inhibits Hh pathway activity, and this contributes to the restored GC sensitivity of C1–8 cells.

**DISCUSSION**

Increased intracellular cAMP can either inhibit or promote apoptosis, depending upon the specific cellular context (7). The determinants for cAMP-mediated cell cycle arrest and cell death are poorly understood. Previous studies on CEM cell lines have documented that cAMP elevating agents such as forskolin synergistically promote Dex-induced cell death in both GC-sensitive and -resistant CEM clones (10), thus implicating the involvement of cAMP signaling in the GC-induced apoptotic process. In the current studies, we demonstrate that between the two intracellular cAMP receptors, Epac and PKA, PKA is responsible for the synergistic killing effects observed in CEM cells. This is consistent with a previous report that cAMP exerts its growth arrest effect through PKA instead of Epac pathway in Jurkat T cells (38). Activation of Epac, on the other hand, weakly antagonizes the apoptotic effect of Dex, consistent with our earlier observation that Epac and PKA can mediate the opposing cellular effects of cAMP (39, 40).

In agreement with the important role that PKA plays in cAMP-mediated synergism with GC, the cellular activity of PKA is significantly higher in GC-sensitive CEM cells. This increased PKA activity is further associated with a decreased Hh activity in GC-sensitive C7–14 cells. We also find that forskolin suppresses Hh pathway activity in both GC-resistant and sensitive CEM cells. Moreover, whereas Dex alone does not significantly suppress Hh activity, the inhibitory effect of forskolin on Hh activity is synergistically potentiated by Dex. Therefore, our studies for the first time suggest that inhibition of Hh pathway activity by PKA may represent one of the mechanisms that cAMP and GC converge to induce cell apoptosis in CEM cells.

Hh signaling regulates cell proliferation, differentiation, and survival in some adult tissues in addition to its important roles during development (41). Dysregulation of the Hh pathway has been implicated in many human cancers (13, 42). Our findings that the Hh pathway is activated in acute lymphoblastic leukemia cells broadens the growing list of Hh-associated cancers. Although the mechanism underlying Hh-mediated tumorigenesis is poorly understood, several studies indicate a role for Hh as a regulator of the cell cycle machinery (23, 43–46). These studies are in agreement with our observations that inhibition of Hh pathway by a SMO inhibitor KAAD-cyclopamine leads to cell cycle arrest in G1/S, whereas a structurally related analog, tomatidine, shows no effect on the cell cycle progression. Hh pathway blockade by pharmacological agents or small interference RNA induces cell growth arrest and apoptosis in several

**FIGURE 7.** GC sensitive revertant CEM C1–8 cells have relative higher PKA activity and lower Hh activity as well as greater sensitivity to Hh inhibition compared with C1–15 cells. A, PKA activity in C1–15 and C1–8 cells measured by radioactivity assay. Inset, PKA activity in both C1–15 and C1–8 cells measured by PepTag assay. B, GLI1 mRNA expression levels in C1–15 and C1–8 cells measured by real time PCR after treatment with Dex and forskolin (FSK) for 16 h. C, C1–8 cells growth measured by Alamar Blue assay after treatment with KAAD-cyclopamine. The data are represented as the means ± S.D., n = 2, p values are indicated by asterisks (relative to vehicle or C1–15 cells): single asterisk, p < 0.05.
solid tumors and inhibits tumor growth in mouse xenograft models (17, 47, 48). Again, these studies are consistent with our findings that Hh-specific inhibitors induce apoptosis in CEM cells.

PKA is a negative regulator of Hh signaling. In the mammalian system, Hh-activated transcription factors, GLI2 and GLI3, are sequentially phosphorylated by PKA, glycogen synthase kinase 3, and casein kinase I, before being targeted for proteolytic processing to generate a repressor form or completely degraded by the 26 S proteosome complex (49). In addition to affecting GLI protein stability, phosphorylation of GLI1 by PKA also prevents GLI1 translocating from cytoplasm to nucleus in COS7 cells (34). Consistent with these observations, our study indicates that PKA down-regulates Hh pathway activity, and PKA/Hh activities correlate with GC sensitivity in acute lymphoblastic leukemia cell lines. Our findings that increased PKA activity suppresses basal Hh activity in GC-sensitive cells provide a molecular explanation for the synergistic killing effects between GC and cAMP. Most importantly, this relationship between PKA/Hh activities and GC sensitivity is confirmed in the GC-sensitive revertant C1–8 cell line, which also contains a higher level of PKA activity, lower Hh pathway activity, and increased sensitivity to Hh inhibition.

Synergy between cAMP/PKA and GCs in promoting apoptosis perhaps involves cooperative effects on gene transcription by GRs and the PKA pathway (10). Transcriptional activation of BIM expression has been implicated as a common target for PKA and GCs (9). It has been shown that GC treatment of sensitive clones and GC/forskolin treatment of a resistant clone indicate that PKA down-regulates Hh pathway activity, and GC sensitivity in acute lymphoblastic leukemia cells. This finding is very important from the clinical viewpoint, because it suggests that Hh pathway inhibitors may be used to effectively treat GC-resistant leukemia.

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