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Journal

Proceedings of the National Academy of Sciences of the United States of America, 112(41)

ISSN

0027-8424

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Publication Date

2015-10-01

DOI

10.1073/pnas.1516057112

Peer reviewed

Mechanisms of cyclic AMP/protein kinase A- and glucocorticoid-mediated apoptosis using S49 lymphoma cells as a model system

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Contributed by Susan S. Taylor, August 20, 2015 (sent for review June 2, 2015; reviewed by Antonio Feliciello and Roland Seifert)

Cyclic AMP/protein kinase A (cAMP/PKA) and glucocorticoids promote the death of many cell types, including cells of hematopoietic origin. In wild-type (WT) S49 T-lymphoma cells, signaling by cAMP and glucocorticoids converges on the induction of the proapoptotic B-cell lymphoma-family protein Bim to produce mitochondria-dependent apoptosis. Kin$^-$, a clonal variant of WT S49 cells, lacks PKA catalytic (PKA-Cα) activity and is resistant to cAMP-mediated apoptosis. Using sorbitol density gradient fractionation, we show here that in kin$^-$ S49 cells PKA-Cα is not only depleted but the residual PKA-Cα mislocalizes to heavier cell fractions and is not phosphorylated at two conserved residues (Ser^{338} or Thr^{197}). In WT S49 cells, PKA-regulatory subunit I (RI) and Bim coimmunoprecipitate upon treatment with cAMP analogs and forskolin (which increases endogenous cAMP concentrations). By contrast, in kin$^-$ cells, expression of PKA-RIα and Bim is prominently decreased, and increases in cAMP do not increase Bim expression. Even so, kin$^-$ cells undergo apoptosis in response to treatment with the glucocorticoid dexamethasone (Dex). In WT cells, glucocorticoid-mediated apoptosis involves an increase in Bim, but in kin$^-$ cells, Dex-promoted cell death appears to occur by a caspase 3-independent apoptosis-inducing factor pathway. Thus, although cAMP/PKA-Cα and PKA-R1α/Bim mediate apoptotic cell death in WT S49 cells, kin$^-$ cells resist this response because of lower levels of PKA-Cα and PKA-RIα subunits as well as Bim. The findings for Dex-promoted apoptosis imply that these lymphoma cells have adapted to selective pressure that promotes cell death by altering canonical signaling pathways.

cAMP | apoptosis | PKA | lymphoma | glucocorticoids

Glucocorticoids and cAMP regulate many key biological processes, including metabolism, gene transcription, cell proliferation, and apoptosis (1–5). The regulation of apoptosis occurs in a cell type-specific manner, such that cAMP and glucocorticoids can be either pro- or antiapoptotic (6, 7). A major effector of cAMP signaling is cAMP-dependent protein kinase A (PKA), a Ser/Thr protein kinase that consists of an R_2C_2 holoenzyme with a regulatory subunit (PKA-R) dimer and two catalytic (PKA-Cα) subunits (8). The three major genes for C subunits—PRKACA, PRKACB, and PRKACG—encode the Cα, Cβ, and Cγ proteins, respectively. There are four genes for R subunits—PRKARIA, PRKARIB, PRKARIIA, and PRKARIIB—which encode RIα, RIβ, RIIα, and RIIβ, respectively. The PKA holoenzyme is inactive under basal conditions but increases in cAMP unleash PKA-Cα activity, which then catalyzes substrate phosphorylation (8). Abnormal regulation of cAMP/PKA signaling occurs in a variety of disorders, including Carney complex disease as well as tumors and human cancer cell lines (2, 9, 10). Targeting of cAMP/PKA signaling may have potential for numerous disease settings, but understanding is limited regarding the molecular mechanisms for its antiapoptotic and proapoptotic effects (6, 7).

Many characteristics of the cAMP/PKA signaling system have been identified in the CD4$^+$ CD8$^+$ S49 T-lymphoma cell line (6, 11–13), which has served as a model system to understand cAMP-dependent gene expression networks and actions of PKA (6, 14). Treatment of wild-type (WT) S49 cells with cAMP analogs, agents that increase endogenous cAMP concentrations, or with glucocorticoids (e.g., Dexamethasone, Dex) will produce G_1-phase cell-cycle arrest and then apoptosis (11). Moreover, cAMP/PKA- and Dex-promoted apoptosis of WT S49 cells both occur by enhanced expression of Bim (a proapoptotic B-cell lymphoma-family protein) and activation of the intrinsic mitochondria-dependent apoptotic pathway (15). Based on the proapoptotic response to cAMP, clones of cells resistant to cAMP-promoted cell killing were isolated from WT S49 cells. One such clonal variant, kin$^-$, lacks PKA activity (16). In kin$^-$ cells, expression of the mRNA transcript of the PKA-Cα subunit is normal, but there is no soluble PKA-Cα protein (17–19). We have previously shown that *cis*-autophosphorylation of Ser^{338} ribosome-associated PKA-Cα occurs cotranslationally, but posttranslational phosphorylation of the activation loop Thr^{197} is mediated in *trans*; absence of phosphorylation of Ser^{338}leads to the accumulation of insoluble, inactive PKA-Cα (20). Ser^{338} is thus critical for processing and maturation of PKA-Cα and is a prerequisite for phosphorylation of Thr^{197} (20). However, the precise mechanism by which kin$^-$ cells are able to escape cAMP/PKA-mediated apoptosis is not known, and understanding is limited regarding the mechanisms that may link cAMP- and glucocorticoid-promoted cell killing (21, 22). The current studies using WT and kin$^-$ S49 cells address both these issues and provide insights regarding the mechanisms of cAMP/PKA- and

Significance

Cyclic AMP, the first identified second messenger, regulates a wide array of cellular functions including apoptosis by activating protein kinase A (PKA) and, in turn, the phosphorylation of target proteins. The current study uses a variety of biochemical and functional analyses to assess wild-type S49 lymphoma cells and kin$^-$, a clonal variant that lacks PKA. The results identify key alterations in the ability of kin$^-$ cells to process PKA and also define previously unidentified alterations in cAMP- and glucocorticoid-promoted killing of kin$^-$ S49 cells. The findings provide evidence for PKA-dependent pathway switching in cell death responses and have implications for therapeutic development in diseases with aberrant apoptosis.

Author contributions: M.M.K., P.A.I., and S.S.T. designed research; M.M.K., J.R.K., Y.M., A.W., and M.D. performed research; M.M.K., P.A.I., and S.S.T. analyzed data; and M.M.K., J.R.K., P.A.I., and S.S.T. wrote the paper.

Reviewers: A.F., University of Naples; and R.S., Medical School of Hannover.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1516057112/-/DCSupplemental.

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glucocorticoid-promoted cell death by showing differences in the pathways to cell death by these two types of stimuli in these cells. The findings have implications for a number of settings, including ones in which one may seek to enhance or blunt apoptotic cell death by cAMP and PKA.

Results

PKA-Cα Is Not Processed via Phosphorylation and Is Mislocalized in Kin⁻ S49 Cells. WT and kin⁻ S49 cells were lysed and postnuclear lysates were fractionated into cytoplasmic, membrane, and insoluble fractions and assessed by immunoblot analyses for PKA-Cα and its phosphorylation at activation loop residue Thr^{197}. We used Cos7 cells, a nonlymphoma cell line, as a control. As shown in Fig. 1*A*, PKA-Cα is soluble and phosphorylated in WT and Cos7 cells but insoluble and not phosphorylated in kin⁻ cells, consistent with earlier results (19, 20). We also detected two PKA-C isoforms based on the phosphoantibody blots, both of whose isoforms are absent in kin⁻ (Fig. 1 *A*, *Bottom*). The insolubility of PKA-Cα in kin⁻ cells suggests that these cells have a defect in its processing. We thus tested whether another kinase, protein kinase C (PKC), which also requires multiple phosphorylation steps for maturation and activity (23, 24), is correctly processed in kin⁻ cells. We found that PKC is processed and phosphorylated similarly in WT and kin⁻ cells, implying that kin⁻ S49 cells have a specific defect in their processing of PKA-Cα (Fig. 1*B*) (25). Because phosphoinositide-dependent protein kinase 1 (PDK1) can interact and phosphorylate PKA-Cα at Thr^{197} (26), we tested whether kin⁻ cells lack PDK1 as a mechanism to explain the loss in Thr^{197} phosphorylation and its impact on folding of the PKA-Cα subunit. We assessed the expression of PDK1 and of HSP70, a chaperone crucial to the proper phosphorylation and maturation of PKC (27), and found that both PDK1 and HSP70 are expressed at similar levels in WT and kin⁻ cells (Fig. 1*C*). An important cellular function of PKA-Cα is to phosphorylate the transcription factor CREB on Ser^{133}; this phosphorylation regulates gene expression of multiple transcripts but can also be mediated by other kinases, such as ERK, p70S6K1, and RSK1 (28, 29). Using phospho-specific antibodies to assess the activity of other kinases that phosphorylate CREB, we found (Fig. 1*C*) a similar extent of phosphorylation of those kinases in WT and kin⁻ cells. Thus, the defect in CREB-mediated phosphorylation and transcription in kin⁻ cells is specific to the PKA pathway (30).

To further understand the insolubility of PKA-Cα in kin⁻ cells, we used sorbitol density gradient ultracentrifugation to resolve postnuclear lysates of WT and kin⁻ cells into 20 fractions and assessed by immunoblot analysis the subcellular localization of PKA-Cα. We found that in WT cells, PKA-Cα is detected in Golgi and soluble fractions, but in kin⁻ cells, the low levels of inactive PKA-Cα localize to mitochondria/ER fractions (Fig. 2*B*), such that kin⁻ cells have very little PKA-Cα in soluble fractions (Fig. S1). Because levels of PKA-Cα in kin⁻ cells were very low, detection of the signal required that the blot be overexposed. PKA-Cα is phosphorylated at both Ser^{338} and Thr^{197} in WT cells, but neither site is phosphorylated in kin⁻ cells (Fig. 2*A*), suggesting that both its cotranslational and posttranslational processing steps are compromised in kin⁻ cells, results consistent with earlier reports (17, 20). Imaging of PKA-Cα by immunofluorescence microscopy of WT and kin⁻ cells provided complementary results: PKA-Cα localizes to perinuclear (likely Golgi) regions in WT cells (Fig. 2*C*), whereas in kin⁻ cells PKA-Cα is poorly expressed and has a diffuse appearance throughout the cells (Fig. 2*D*).

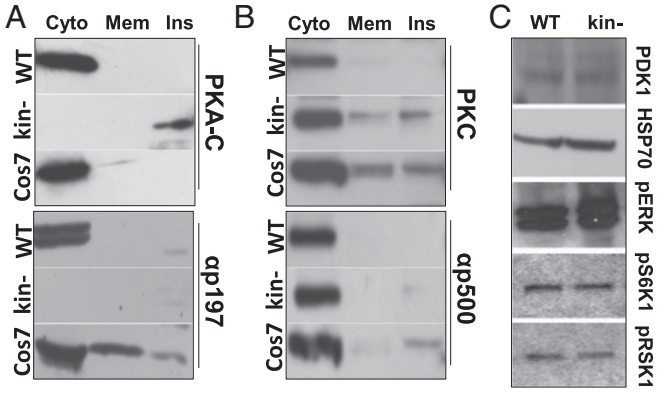

Fig. 1. Kin⁻ S49 cells have a defect in PKA-Cα phosphorylation. (*A*) Lysates from WT and kin⁻ S49 and COS7 cells were analyzed for PKA-Cα (*Above*) and phosphorylation on Thr^{197} (*Below*). (*B*) Lysates from WT and kin⁻ S49 and COS7 cells were analyzed for PKCβII (*Above*) and phosphorylation on Thr^{500} (*Below*). (*C*) Western blot analysis of PDK1 and HSP70 and phosphorylation of ERK, S6K1, and RSK1, assessed by phospho-specific antibodies, in WT and kin⁻ S49 cells.

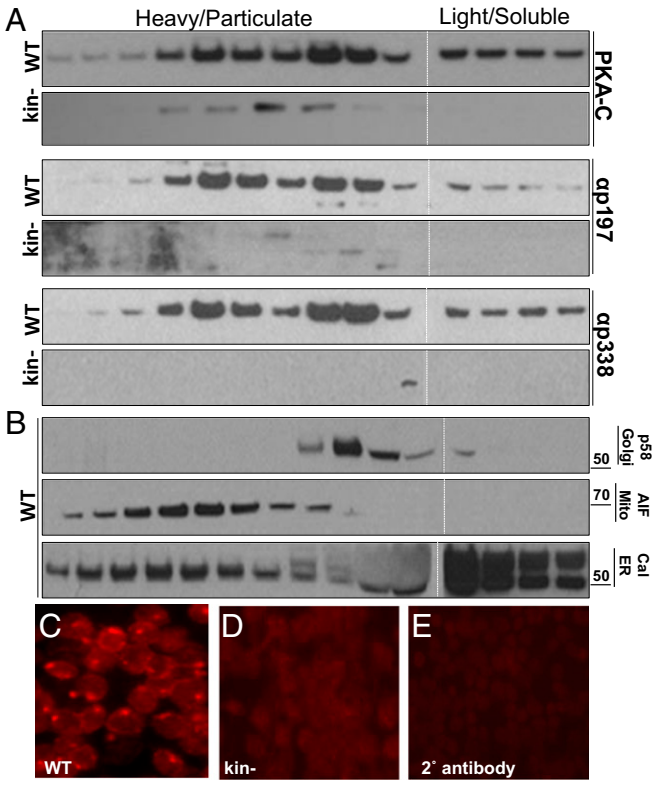

Fig. 2. PKA-Cα in kin⁻ S49 cells is mislocalized. (*A*) WT and kin⁻ S49 cell lysates were fractionated by sorbitol density fractionation and analyzed for PKA and phosphorylation of PKA-Cα on Thr^{197} and Ser^{338} (the kin⁻ blot was overexposed to visualize the image). (*B*) Fractionation controls for subcellular compartments were p58 as a Golgi marker, AIF for mitochondria, and Calreticulin for ER. (*C*) PKA-Cα staining in WT cells shows perinuclear staining. (*D*) PKA-Cα staining in kin⁻ cells shows diffuse staining throughout the cell and no prominent perinuclear localization. (*E*) Secondary rabbit antibody background staining.

PKA-RIα Protein Expression Is Prominently Decreased in Kin⁻ Cells Despite Transcription of Full-Length mRNA. Because the soluble catalytic subunit of PKA is lacking in the cytosol of S49 kin⁻ cells (Fig. 1*A*), we examined the presence of its holoenzyme partners, regulatory (R) subunits of PKA, in whole cell lysates of S49 cells. As shown in Fig. 3*A*, mRNA levels of PKA-RIα are comparable in WT and kin⁻ cells, but PKA-RIα protein was not detected by immunoblot analysis of kin⁻ cells (Fig. 3*B* and Fig. S1). cAMP-regulated transcription of PKA-RIα occurs via CREB (31),

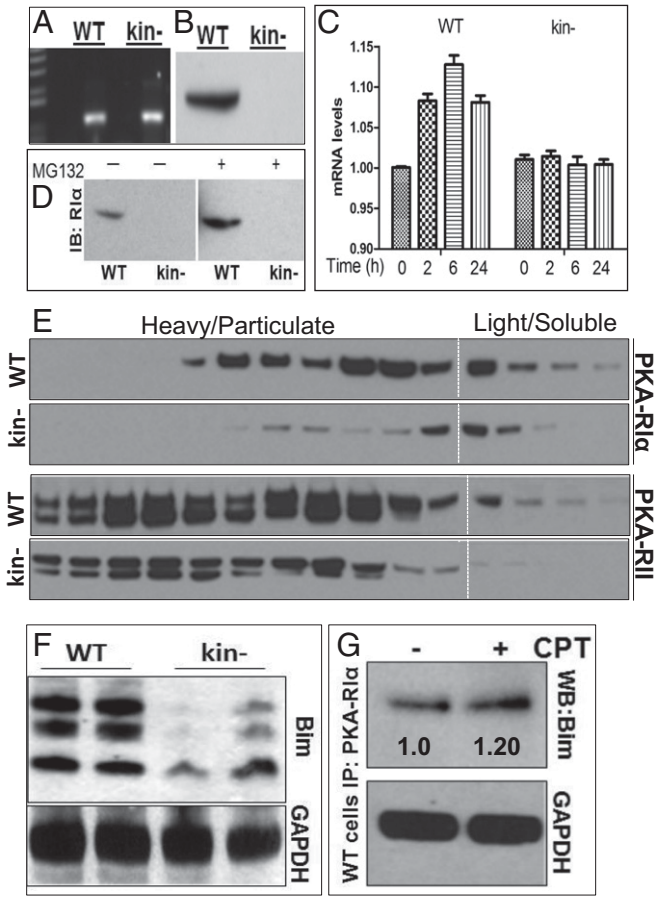

Fig. 3. PKA-RIα and Bim isoforms are down-regulated in kin⁻ S49 cells. (*A*) PKA-RIα mRNA in WT and kin⁻ cells. (*B*) Immunoblot of PKA-RIα (present in WT, not in kin⁻). (*C*) PKA-RIα mRNA levels respond to cAMP treatment in WT but not kin⁻ cells. (*D*) MG132 treatment increases the level of PKA-RIα protein in WT but not kin⁻ cells. (*E*) Lysates from WT and kin⁻ S49 cells were fractionated and analyzed for PKA-RIα and PKARII subunits. (*F*) Immunoblot analysis of Bim in WT and kin⁻ cells. (*G*) Immunoprecipitation of Bim by PKA-RIα in CPT-cAMP–treated WT cells. The data shown are representative of those obtained in four separate experiments.

whose phosphorylation is regulated by cAMP and PKA-Cα. We tested whether mRNA levels of PKA-RIα increase in response to increases in cellular cAMP in WT and kin⁻ cells. PKA-RIα mRNA increased in WT, but not kin⁻, S49 cells incubated with the cAMP analog 8-(4chlorophenylthio) cyclic AMP (CPT)-cAMP (Fig. 3*C*). By contrast, mRNA levels of the other PKA C and R subunits did not change upon CPT-cAMP treatment of WT or kin⁻ cells, thus indicating the unique response of PKA-RIα to cAMP/PKA (Fig. S2). These results suggest that PKA-RIα is not expressed in kin⁻ cells because of the loss in PKA-Cα activity, but do not rule out the possibility that kin⁻ cells have increased degradation of PKA-RIα. PKA-RIα can be degraded by the ubiquitin RING ligase praja2, the activity of which is increased in response to increased cellular cAMP levels, thereby stimulating proteolysis of R subunits (32). To assess if increased proteasome-mediated degradation might mediate the loss in PKA-R1α in kin⁻ cells, we treated WT and kin⁻ cells with the proteasomal inhibitor MG132. Treatment with MG132 increased the levels of PKA-RIα in WT, but not kin⁻, cells (Fig. 3*D*), implying that increased degradation is not the mechanism for the lower expression of PKA-RIα. In addition, we used sorbitol density gradient centrifugation to assess expression and subcellular localization of the R subunits in WT and kin⁻ cells. The expression of PKA-RII protein is similar in WT and kin⁻ cells, but as noted above, much less RIα is expressed in the kin⁻ cells (Fig. 3*E*).

Proapoptotic Bim Expression Is Decreased in Kin⁻ Cells, and Bim Immunoprecipitates with PKA-RIα in WT Cells. Bim is a mediator of cAMP-promoted apoptosis in WT S49 cells, at least in part by induction of the Bim transcript by cAMP (15, 33, 34). We found that the three isoforms of BIM have much lower protein expression in kin⁻ than WT cells (Fig. 3*E*). When overexpressed, Bim (extralong) and PKA-RIα interact, an interaction that is required for the subsequent phosphorylation and stabilization of Bim by PKA-Cα (35). Consistent with those results in overexpressing cells, immunoprecipitation of endogenous PKA-RIα in WT and kin⁻ S49 cells revealed that PKA-RIα interacts with Bim in WT cells incubated with CPT-cAMP (15) (Fig. 3*G*). The latter findings suggest that in addition to induction of the Bim transcript by cAMP/PKA, PKA-RIα/Bim interaction and subsequent phosphorylation of Bim by PKA-Cα stabilize Bim and facilitate its proapoptotic actions. Immunoprecipitation could not be performed with kin⁻ cells due to their low expression and lack of interaction with Bim and the PKA-RIα proteins. The lack of induction of Bim and Bim/PKA-RIα interaction thus contributes to the inability of cAMP/PKA to promote apoptosis in kin⁻ S49 cells.

Kin⁻ Cells Are Susceptible to Dex-Promoted Apoptosis via an AIF-Mediated Pathway. In addition to cAMP/PKA-promoted apoptosis, WT S49 cells undergo glucocorticoid (Dex)-promoted mitochondria-dependent apoptosis; this apoptotic response also involves increased Bim expression (17). Because kin⁻ cells are resistant to cAMP-promoted apoptosis, we assessed their susceptibility to killing by Dex. Unlike their resistance to cAMP-promoted apoptosis, kin⁻ cells incubated with Dex (1 μM) for 48 h undergo apoptosis, as detected by annexin V staining (Fig. 4*A*). By contrast, with WT cells incubated with Dex, which show cleavage of the apoptotic effector caspase 3, caspase 3 is not

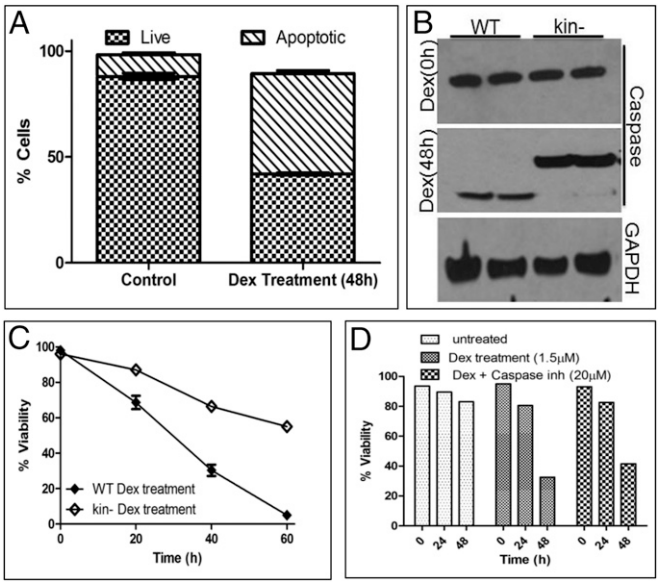

Fig. 4. Dex promotes apoptosis of kin⁻ S49 cells. (*A*) Kin⁻ cells treated with Dex and analyzed by Annexin V staining using flow cytometry. (*B*) Immunoblot analysis of caspase 3 cleavage in Dex-treated WT and kin⁻ cells. (*C*) Time course of Dex treatment shows a slower rate of induction of apoptosis in kin⁻ compared with WT S49 cells. (*D*) Kin⁻ cells were treated with Dex (1.5 μM) without and with a pan-caspase inhibitor (z-vad-fmk, 20 μM), and viability was assessed over 48 h by trypan blue staining.

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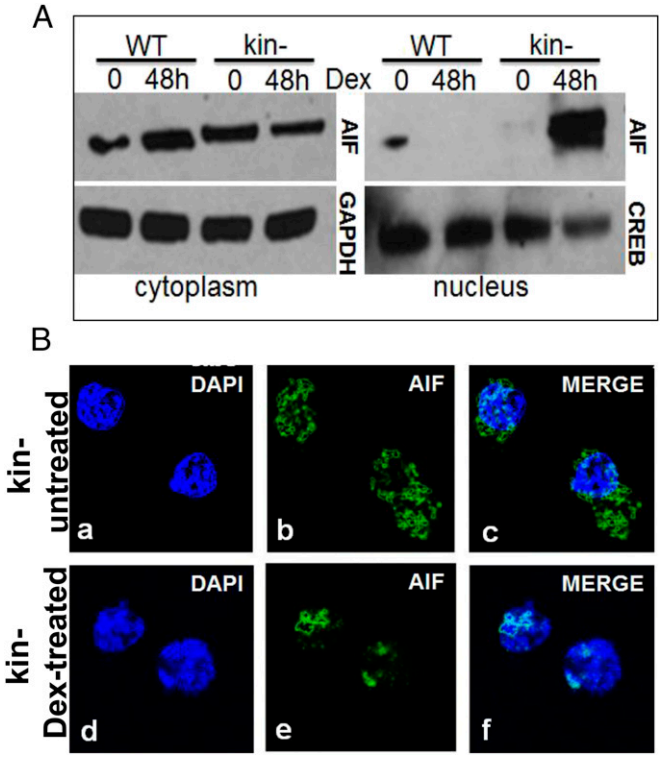

Fig. 5. AIF translocates to the nucleus in kin$^-$ S49 cells incubated with Dex. (*A*) Detection of AIF in cytoplasmic and nuclear fractions of WT and kin$^-$ cells in response to treatment with Dex (1 μM). (*B, a*) DAPI staining of untreated kin$^-$ cells. (*b*) AIF imaging of untreated kin$^-$ cells. (*c*) Merge of images from *a* and *b*. (*d*) DAPI staining of Dex-treated kin$^-$ cells. (*e*) AIF imaging of Dex-treated kin$^-$ cells. (*f*) Merge of images from *d* and *e*.

cleaved in Dex-treated kin$^-$ cells (Fig. 4*B*), implying that Dex-promoted apoptosis of kin$^-$ cells is independent of caspase 3. Moreover, kin$^-$ cells undergo Dex-promoted apoptosis at a slower rate than do WT cells (Fig. 4*C*). Incubation of kin$^-$ cells with a pan-caspase inhibitor did not prevent Dex-induced apoptosis, providing further evidence that in kin$^-$ cells, the apoptotic response to Dex is independent of caspase activation (Fig. 4*D*).

The apoptosis-inducing factor (AIF) pathway can induce apoptosis in a caspase-independent manner (36, 37). The proapoptotic mechanism of AIF, a mitochondrial protein, involves translocation to the nucleus. Immunoblot analysis of WT and kin$^-$ cells revealed that AIF translocates from the cytoplasm to the nucleus in response to Dex treatment of kin$^-$ but not WT S49 cells (Fig. 5*A*), implying that Dex activates the AIF pathway in kin$^-$, but not WT, cells. Consistent with those findings, confocal microscopy revealed that Dex treatment promotes the translocation of AIF from the cytoplasm to the nucleus of kin$^-$ cells (Fig. 5*B*).

Discussion

cAMP/PKA signaling regulates many cellular processes, including cell proliferation, growth arrest, and apoptosis. Using WT and kin$^-$ S49 lymphoma cells, a model system for cAMP/PKA action, we identified a mechanism by which cells can resist cAMP/PKA-promoted apoptosis. WT S49 cells express PKA-Cα, PKA-RIα, and Bim. In response to increases in cellular cAMP concentrations, Bim is stabilized by PKA phosphorylation, is increased in expression, and promotes apoptosis. By contrast, kin$^-$ S49 cells lack PKA-Cα and PKA-RIα and have lower levels of Bim, which collectively enable those cells to resist cAMP-induced apoptosis (Fig. 6).

Evading apoptosis is a major hallmark of cancer (38). The kin$^-$ S49 cells were isolated from WT cells treated with 1 mM cAMP (39). The resistant cells were then placed in cAMP-containing media and incubated for 3 mo until kin$^-$ clones were obtained. Under selective pressure to resist cAMP-mediated apoptosis, the kin$^-$ cells decreased their expression of PKA-Cα. This occurred in association with blunting of proper maturation, folding, and localization of PKA-Cα (Fig. 2). The decreased PKA-RIα expression of kin$^-$ cells likely results from the decrease in PKA-Cα. The absence of PKA-RIα in kin$^-$ cells can lead to destabilization/degradation of Bim, which along with the decreased induction of Bim by the absence of functional PKA-Cα creates the resistance of the kin$^-$ cells to cAMP-induced apoptosis (14) (Fig. 6).

The precise mechanism for the decreased expression of PKA-Cα and PKA-R1α in kin$^-$ cells remains to be determined. Kin$^-$ cells express PKA-Cα mRNA but are defective in processing the protein (Figs. 1 and 2) (17). The lack of soluble PKA-Cα protein derives from a loss in Ser^{338} phosphorylation, which is essential for maturation of PKA-Cα and phosphorylation of Thr^{197} (20). Cellular expression of PKA-RIα depends in part on the ubiquitin ligase Praja (32). APC(Cdc20) ligase ubiquitinates Bim and promotes its degradation in a cell cycle-dependent manner (40). It will thus be of interest to determine if cAMP/PKA signaling regulates the expression or activity of Praja and APC(Cdc20) ligase in S49 cells and other cell types.

Bim not only mediates cAMP/PKA-promoted apoptosis of S49 cells but also is involved in glucocorticoid-promoted apoptosis of S49, other lymphoma, and immature lymphoid cells (15, 35). The data here imply that another mechanism, the mitochondrial protein AIF, is involved in glucocorticoid-promoted apoptosis in kin$^-$ cells. The AIF inhibitor, *N*-phenylmaleimide, at very low concentrations (0.5 μM) was toxic to S49 cells, limiting our ability to assess its effect on cell death. Furthermore, AIF nuclear entry is regulated by interaction with PKA kinase interacting protein (AKIP), but we were not able to detect AKIP in kin$^-$ cells (41). Based on the response of kin$^-$ cells, we speculate that PKA-Cα may be a negative regulator of AIF-promoted apoptosis, an action that might contribute to antiapoptotic responses to cAMP (7).

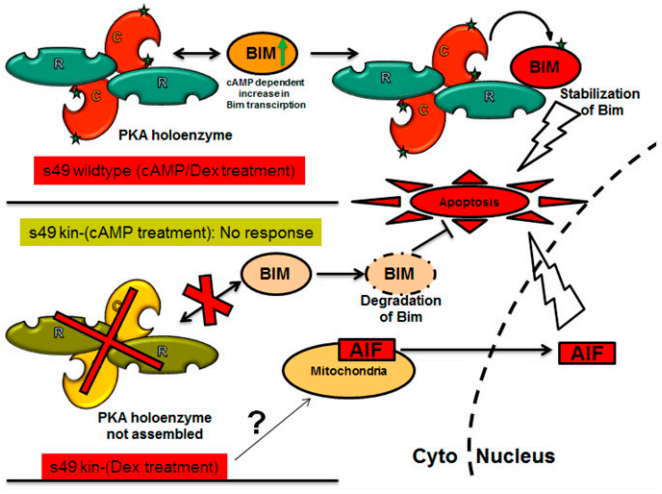

Fig. 6. Model for resistance of kin$^-$ S49 cells to cAMP/PKA-promoted apoptosis and Dex-mediated apoptosis. WT cells respond to cAMP/Dex by enhancing PKA-Cα–mediated transcription, phosphorylation, and the stabilization of proapoptotic Bim, which leads to apoptosis. Kin$^-$ cells have mislocalized and inactive PKA-Cα subunits and decreased levels of PKA-RIα and Bim and are thus resistant to cAMP/PKA-promoted apoptosis. Kin$^-$ cells are killed by Dex treatment, which activates AIF and presumably its promotion of apoptosis.

Our findings have implications for cellular regulation, in particular apoptotic cell death and therapeutic approaches that promote mitochondria-dependent apoptosis (42). Hormones and drugs that increase cAMP are proapoptotic for many cell types; this action may have therapeutic utility, such as in treating certain cancers (43). In other settings (e.g., heart failure), cAMP/PKA-promoted apoptosis (i.e., of cardiac myocytes) may lead to disease progression; thus, ways to prevent such cell death need to be considered (44–46). The current data highlight the potential importance of approaches that alter the promotion of BIM-mediated apoptosis by cAMP/PKA. The results regarding glucocorticoid-mediated cell killing of S49 lymphoma cells imply that kin$^-$ S49 cells, and perhaps other types of cancer cells, adapt to selective pressure and avoid cell death by altering canonical signaling pathways. Efforts to enhance killing of such cells thus may need to use approaches that attack such alternative mechanisms.

Our results have implications for cancer progression as well as diseases with aberrant apoptosis. A hallmark of cancer cells is their ability to avoid apoptosis (38) by rewiring the apoptotic program and enhancing cell survival—features demonstrated by kin$^-$ S49 cells. Such switching has been observed in T cells, cardiomyocytes, and leukemia (47–49). The survival of kin$^-$ S49 cells involves the elimination of cAMP/PKA signal transduction, a central signaling module in eukaryotic biology. cAMP/PKA signaling in immature T cells is apoptotic but has other functional roles, including in metabolism and stress responses. Because kin$^-$ cells are devoid of cAMP/PKA signaling, they respond differently to oxidative and metabolic stress (50, 51).

Our data imply that S49 cells have two cell death pathway programs: Bim/caspase-driven and caspase-independent AIF-driven pathways. Cells subjected to cell death-promoting stimuli that trigger the primary pathway may switch to a secondary pathway and have enhanced resistance to cell death. As such, the results shown here have implications for cancer therapeutics. One is the need to define how particular cancer cells are programmed to undergo cell death so that therapies can target such programs. Second, because cancer cells also have enhanced proliferation, chemotherapy might combine proapoptotic and antiproliferative agents to target both mechanisms and thereby help inhibit the development of resistance.

Materials and Methods

Reagents. Buffer reagents, forskolin, and CPT-cAMP were from Sigma; PKA-Cα subunit antibody was purchased from BD Biosciences; phospho-specific antibody for p197 (PKA-Cα) was generated in the S.S.T. laboratory; and antibodies for p338 (PKA-Cα), GAPDH, PKA-R1α, and Bim were from Cell Signaling. AIF, p58 golgi, and ER calreticulin antibodies were from Abcam. The phospho antibodies for S6K1, ERK, and RSK as well as antibodies for PDK1, PKC, and HSP70 were generously provided by Alexandra Newton, University of California, San Diego, La Jolla, California.

Cell Culture. WT and kin$^-$ S49 cells were grown in suspension in DMEM media with 10% heat-inactivated horse serum and maintained at 10% CO_2. Cells were harvested in radioimmunoprecipitation assay (RIPA) (Invitrogen) and assessed by SDS/PAGE (4–12% gradient gels; Invitrogen) followed by immunoblot analysis.

Cell Fractionation. WT, kin$^-$, and Cos7 cells were lysed in buffer (20 mM Hepes pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, and protease inhibitor mixture III from Calbiochem) followed by centrifugation at 100,000 × *g* for 20 min at 4 °C in a Beckman Optima TLX Benchtop Centrifuge. The supernatant was collected as cytosol (cyto). The pellet was resolubilized in lysis buffer with 1% triton and spun again at 100,000 × *g* for 20 min at 4 °C to obtain the membrane (mem) fraction (supernatant). The subsequent pellet was resolubilized in SDS Electrophoresis sample buffer and considered as an insoluble (Ins) fraction (Fig. 1).

Sorbitol Density Gradient Centrifugation. WT and kin$^-$ S49 cell lysates were spun for 10 min at 1,000 × *g* to remove nuclear debris. Postnuclear lysates were loaded on 10 mL 30–80% sorbitol gradients and spun at 34,000 × *g* for 48 h; 20 fractions were collected and analyzed by immunoblotting.

S49 Cells Immunocytochemistry. S49 cells were plated onto coverslips coated with polylysine and fixed in 4% paraformaldehyde. Coverslips were incubated in a blocking buffer consisting of 1% normal donkey serum, 1% fish gelatin, 1% BSA, and 0.2% Triton X-100. The coverslips were incubated with anti–PKA-Cα rabbit polyclonal antibody (generated in the S.S.T. laboratory; CAT856) at 1:200 dilution for 1 h. After rinsing in PBS, cells were incubated in secondary antibody, rhodamine anti-rabbit, for 45 min. Fluorescent images were acquired with a 63× oil objective on a Zeiss AxioImager M1 upright light microscope with a Hamamatsu Orca ER camera.

PKA-RIα mRNA Isolation, Western Blot, and MG132 Treatment Analysis. RNA was isolated from WT or kin$^-$ cells with RNeasy (Qiagen). cDNA was transcribed with SuperScript III First Strand Synthesis System (Invitrogen), and PCR was conducted using primers for the mouse PKA-RIα subunit. PCR primers were as follows: 5′, ATGGCGCTC TGGCAGTATGGCAAC, and 3′, GACCGACAGGGACACGAACGTG. The PCR product was cloned into the cloning vector pCR4-TOPO (Invitrogen) for sequencing. For immunoblot analysis, RIPA extracts of 10^7 S49 WT and kin$^-$ cells were resolved on SDS/PAGE and probed with PKA-RIα antibody from BD Biosciences. WT and kin$^-$ cells were incubated with vehicle or 5 μM MG132 (Calbiochem) for 4 h before cell lysis in RIPA buffer. Total cell extracts were run on SDS/PAGE gels. RIα was detected as described above. The cAMP effect on expression of various PKA subunits was assessed as described in ref. 14.

Immunoprecipitation. WT S49 cells were seeded at 5×10^5 cells per mL, incubated with CPT-cAMP (100 μM) or forskolin (10 μM) for 24 h, pelleted by centrifugation, washed with ice-cold PBS, and lysed in RIPA buffer. Protein was quantified by BCA assay, and 2 μg cell lysate was precleared by incubation with protein A/G agarose, incubated overnight with 1 μg/μL antibody, and precipitated by incubation with protein A/G agarose for 4 h. The beads were washed three times with RIPA, and the protein complex was removed from the beads by adding 2× SDS loading buffer (Invitrogen) and heating to 95 °C for 5 min.

Apoptosis Assay. Apoptosis was monitored by Annexin V staining as per the manufacturer's instruction (Trevigen). The Annexin V-positive cells were quantified by FACS using a Guava flow cytometer.

Confocal Microscopy. Kin$^-$ cells were grown in 3.5 cm^2 Mat Tek poly-d-lysine–coated dishes and treated with Dex for 48 h. The cells were washed with PBS and fixed with 4% paraformaldehyde. The cells were blocked with 1% donkey serum and 0.5% BSA in PBS for 1 h followed by overnight incubation with AIF antibody at 4 °C and secondary antibody (goat anti-rabbit 488). The cells were mounted, stained with DAPI, and imaged using a Fluo View 1000 confocal laser scanning microscope with a 60× objective. We acquired 10–15 slices using a sequential scanning method and processed them by ImageJ.

ACKNOWLEDGMENTS. This works was supported by NIH Grant DK54441 (to S.S.T.) and grants from the Lymphoma and Leukemia Society (to P.A.I.).

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