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Evidence That Glucocorticoid- and Cyclic AMP-Induced Apoptotic Pathways in Lymphocytes Share Distal Events

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WEHI7.2 murine lymphocytes undergo apoptotic death when exposed to glucocorticoids or elevated levels of intracellular cyclic AMP (cAMP), and these pathways are initiated by the glucocorticoid receptor (GR) and protein kinase A, respectively. We report the isolation and characterization of a novel WEHI7.2 variant cell line, WR256, which was selected in a single step for growth in the presence of dexamethasone and arose at a frequency of $\sim 10^{-10}$. The defect was not GR-related, as WR256 expressed functional GR and underwent GR-dependent events associated with apoptosis, such as hormone-dependent gene transcription and inhibition of cell proliferation. Moreover, the glucocorticoid-resistant phenotype was stable in culture and did not revert after treatment with 5-azacytidine or upon stable expression of GR cDNA. In addition, WR256 did not exhibit the diminished mitochondrial activity commonly associated with apoptosis. Interestingly, WR256 was also found to be resistant to 8-bromo-cAMP and forskolin despite having normal levels of protein kinase A activity and the ability to induce cAMP-dependent transcription. We examined the steady-state transcript levels of *bcl-2*, a gene whose protein product acts dominantly to inhibit thymocyte apoptosis, to determine whether elevated *bcl-2* expression could account for the resistant phenotype. Our data showed that *bcl-2* RNA levels were similar in the two cell lines and not altered by either dexamethasone or 8-bromo-cAMP treatment. These results suggest that WR256 exhibits a "deathless" phenotype and has a unique defect in a step of the apoptotic cascade that may be common to the glucocorticoid- and cAMP-mediated cell death pathways.

Glucocorticoid hormones are used in many chemotherapeutic regimens because of their antiproliferative and cytolytic effects on some populations of lymphocytes (15). A number of murine and human thymus-derived cell lines have been developed to study the biochemical and genetic mechanisms of glucocorticoid-induced death (23, 30, 33, 44). This type of cell death, referred to as programmed cell death or apoptosis, appears to be a complex, multistep pathway (reviewed in references 19 and 62). Apoptosis of lymphocytes is dependent on RNA and protein synthesis and is characterized by nuclear condensation and DNA fragmentation, decreased mitochondrial ATP synthesis, and a budding-off of chromatin-containing membranous vesicles called apoptotic bodies. Some lymphocyte cell lines are also killed by elevated levels of cyclic AMP (cAMP) in a manner which is morphologically similar to that observed in glucocorticoid-mediated death (11, 27, 59). Although very little is known about the molecular events responsible for cell death induced by either agent, a number of genes which are regulated during glucocorticoid- and/or cAMP-mediated lymphocytolysis have recently been identified to better define the processes involved (1, 2, 17, 46). A number of groups have also inhibited apoptosis in a variety of cells by overexpressing *bcl-2* (31, 45, 52, 54, 58), thus providing further insight into the mechanisms of apoptosis.

Glucocorticoid-induced apoptosis is mediated through the glucocorticoid receptor (GR) (35), and glucocorticoid-resistant variant cell lines have alterations almost exclusively in the level or function of the GR (see reference 4 and references therein). In comparison, the initiation of cAMP-induced cell death is dependent upon functional protein kinase A (PKA), the cAMP-dependent protein kinase (36), and the majority of cAMP-resistant variant cell lines have mutations affecting the expression or function of PKA (22). Somatic cell genetic data suggest that the glucocorticoid- and cAMP-mediated apoptotic pathways in lymphocytes may be initiated independently of each other, based on the inability to obtain doubly resistant cells by selection with a single agent (25). However, as speculated by the authors, their screening procedure with S49 cells may not have been sensitive enough to identify all possible mutations (25).

The WEHI7 murine thymoma cell line (30) is a good model for studying the mechanisms of apoptosis because it is sensitive to the glucocorticoid dexamethasone at concentrations equal to or greater than 6×10^{-9} M (4), it expresses two functional copies of the GR gene (4), and spontaneous resistance is acquired at a frequency at least 1,000-fold lower than in S49 lymphocytes (4, 34). WEHI7 is also killed in a similar manner by elevated levels of intracellular cAMP (27). This study was undertaken to isolate a glucocorticoid-resistant variant of WEHI7 which has a non-GR mutation in the apoptotic pathway. One such GR^+ variant was isolated in a single selection step in the presence of high concentrations of dexamethasone and, unexpectedly, was determined to exhibit resistance to cAMP even though it expressed functional PKA. Analysis of *bcl-2* expression in both the wild-type and variant WEHI7 cell lines revealed that steady-state *bcl-2* transcript levels were the same in both cell lines and were not regulated during apoptosis. These data suggest that glucocorticoid- and cAMP-dependent apoptosis may have common steps in some part of the apoptotic pathway.

MATERIALS AND METHODS

Materials. 8-Bromo-cAMP, forskolin, histone type IIA, 3-(4,5-dimethylthiazole-2-yl)-2,3-phenyl tetrazolium (MTT), and dexamethasone were purchased from Sigma (St. Louis,

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Mo.); [γ-^{32}P]ATP and [α-^{32}P]ATP were purchased from New England Nuclear (Boston, Mass.); [^{14}C]chloramphenicol was purchased from Amersham (Arlington Heights, Ill.). Dexamethasone and forskolin were dissolved in ethanol and stored at −20°C in the dark.

Cell culture. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% iron-supplemented defined calf bovine serum (Hyclone), 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml at 37°C in an atmosphere containing 8% CO_2 at 90% relative humidity. Cell viability was determined by trypan blue dye exclusion with a hemacytometer.

Isolation of variant colonies. WEHI7.2 cells (8×10^9) were plated at 6×10^7 cells per 150-mm^2 plate in semisolid medium (10) containing 10^{-6} M dexamethasone. Colonies were visible after 2 to 3 weeks and were plated in liquid culture medium (50% conditioned medium, 40% DMEM, 10% calf bovine serum) containing 10^{-6} M dexamethasone. Conditioned medium was prepared by growing WEHI7.2 cells in DMEM–10% calf bovine serum to $\sim 6 \times 10^5$ cells per ml; the cells were removed by centrifugation, and the medium was filter sterilized. Because the WR256 variant grew very slowly in the presence of hormone, the selection medium was slowly diluted with medium lacking hormone over a 10-week period. The WR256 cells proliferated slowly during this time, until the concentration of dexamethasone was less than 10 nM. At this point, the cells began to divide, with a characteristic cell cycle time of 18 h, and thereafter were maintained in DMEM containing 10% calf bovine serum.

Stable transfections and isolation of clones. Logarithmically growing WEHI7.2 cells were harvested, washed twice with HBS buffer (5 mM KCl, 137 mM NaCl, 6 mM glucose, 21 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.05], 0.7 mM Na_2HPO_4), and resuspended in HBS buffer at 5×10^6 cells per ml. Then, 100 μg of supercoiled GR expression plasmid, pRmGRneo (16), was added to 1 ml of the cell suspension, and electroporation was carried out at 225 mV and 800 μF with a Cell Porator (Bethesda Research Laboratories). Cells were allowed to recover in 20 ml of liquid culture medium for 24 h, and then G418 (Geneticin; GIBCO) was added to a final active concentration of 1 mg/ml, and the cells were incubated for an additional 48 h. The cells were then plated in semisolid medium (10) containing 1 mg of active G418 per ml in order to isolate individual clones. Drug-resistant colonies were visible 10 to 14 days later, removed from the plate, and grown in liquid culture medium. Colonies were tested for the expression of GR cDNA by whole-cell binding of dexamethasone (16) and by RNA analysis.

Transient-transfection assay. Transfections into all lymphocyte cell lines were performed by electroporation as described above, with 50 μg of the reporter plasmid pMMCAT or pCREtkCAT. pMMCAT encodes the chloramphenicol acetyltransferase (CAT) gene under the control of the hormone-inducible mouse mammary tumor virus (MMTV) long terminal repeat (51); pCREtkCAT encodes the CAT gene driven by the thymidine kinase promoter with two copies of the cAMP response element (CRE) from α-glycoprotein hormone (14, 53). Following electroporation, cells were placed in 12 ml of DMEM containing 10% calf bovine serum in the presence of ethanol carrier and 10^{-8} M dexamethasone (for pMMCAT) or 5 μM forskolin (for pCREtk CAT), and incubated for 12 to 16 h. Cell extracts were prepared and assayed for CAT activity as described before (48).

PKA activity. Soluble extracts of the wild-type and variant lines were prepared as described before (40), and PKA activity was determined by measuring the ability of 25 μg of cellular protein to transfer ^{32}P from [γ-^{32}P]ATP to histone type IIA (Sigma) in the presence and absence of cAMP (50). Incubations were performed at room temperature for 4 min, and cAMP-independent phosphorylation was subtracted from total phosphorylation in the presence of cAMP to determine cAMP-dependent phosphorylation.

Cell cycle analysis. Cell cycle analysis was performed basically as described by Krishan (37). Briefly, dexamethasone was added to logarithmically growing cells to a final concentration of 10^{-6} M. At the indicated times, 2×10^6 cells were harvested and resuspended in 0.5 ml of DMEM containing 10% calf bovine serum. This cell suspension was injected into 5 ml of ice-cold methanol-acetic acid (3:1) fixative and incubated at 22°C for 15 min. The cells were washed three times in DMEM containing 10% calf bovine serum, resuspended in 1 ml of DMEM containing 10% calf bovine serum, and treated with RNase (0.1 mg/ml) for 30 min at 22°C. The cells were washed, resuspended in 1 ml of cold phosphate-buffered saline, added to 1 ml of propidium iodide (100 μg/ml in DMEM), and filtered through a 95-μm-pore-size nylon mesh. The concentration of cells in the staining solution was approximately 5×10^5/ml. The DNA content in these cells was measured with a Becton Dickinson FACScan with an excitation wavelength of 488 nm, and emission was measured above 590 nm.

Northern (RNA) blot analysis. Total RNA was isolated, and 20-μg samples were subjected to Northern blot analysis as described previously (17). For the data presented in Fig. 5B, glutathione *S*-transferase (GST) transcript levels were quantitated by measuring the radioactivity in each band with a Betagen Betascope 603 blot analyzer. GST levels were normalized against histone H3.3 RNA, quantitated by densitometric scanning of the resulting autoradiogram (Bio-Rad densitometer), the exposure of which was in the linear range of the film.

MTT assay. Mitochondrial activity was assessed by measuring the conversion of MTT to MTT-formazan (55). Lymphocytes were treated with 10^{-6} M dexamethasone for the times indicated in Table 1. Then, 0.1 ml of cells was plated at 4×10^5 to 10^5 cells per ml into each well of a 96-well microculture plate and treated with 1 mg of MTT per ml at 37°C for 5 h. Cells were lysed in 5% sodium dodecyl sulfate (SDS)–5 mM HCl at 37°C for 16 h, and the amount of blue dye formed was determined by measuring the A_{570}. Measurements were performed in triplicate.

RESULTS

Isolation of dexamethasone-resistant variants. The WEHI7.2 cell line (12) used in these studies is a glucocorticoid- and cAMP-sensitive subclone of WEHI7 (30). To isolate glucocorticoid-resistant colonies, 8×10^9 cells were plated in the presence of 10^{-6} M dexamethasone in semisolid medium (10). After 17 days, three colonies were visible, and the cells were subcloned into liquid medium containing 1 μM dexamethasone. On the basis of this frequency, the spontaneous mutation rate was 3.7×10^{-10}, which was slightly higher than that reported by Huet-Minkowski et al. (34) and may reflect the use of different WEHI7 subclones. Two of the colonies we isolated were large and gave rise to the cell lines WR255 and WR317, while the third was relatively small (WR256). Unlike WR255 and WR317, the WR256 cells had a division time in dexamethasone-containing medium that was

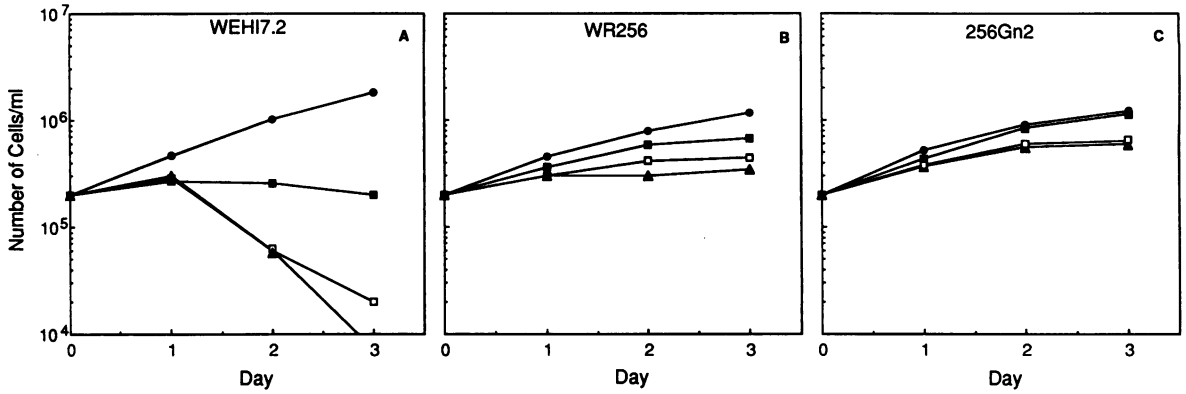

FIG. 1. Sensitivity of WEHI7.2, WR256, and 256Gn2 cells to dexamethasone. Lymphocytes were plated at 2×10^5 cells per ml in the presence of dexamethasone or ethanol vehicle. Viable cells were measured by trypan blue dye exclusion. Dexamethasone was used at 10^{-8} M (■), 10^{-7} M (□), or 10^{-6} M (▲). ●, ethanol. Data are the averages of at least three experiments.

5 to 10 times longer than that of the parental WEHI7.2 line. However, when hormone selection was removed after approximately 10 weeks, the proliferation rate of the cells increased. Consistent with this observation, ligand-binding assays (16) of the three dexamethasone-resistant variants revealed that the hormone-resistant WR255 and WR317 cell lines had reduced dexamethasone-binding activity, indicative of defects in GR, whereas WR256 was found to have the same level of binding as WEHI7.2 (data not shown).

On the basis of the ligand-binding data, the WR256 cell line was chosen for further study. The results of growth analysis of WR256 and WEHI7.2 cells in the presence of increasing concentrations of dexamethasone are shown in Fig. 1. The population doubling time of untreated WR256 cells was approximately 18 h (Fig. 1B), similar to that observed with the wild-type cell line (Fig. 1A) (3). The concentrations of hormone which led to apoptosis in WEHI7.2 did not kill the WR256 cells, although proliferation was markedly inhibited. To eliminate the possibility that the resistance to dexamethasone was due to a subtle alteration in GR which did not affect hormone binding, WR256 cells were stably transfected with a eukaryotic expression vector containing GR cDNA under the control of a Rous sarcoma virus promoter (16). Expression of functionally active GR should cause the cells to revert to a sensitive phenotype if the defect was only in the receptor (16, 28, 57). A stable transfectant, 256Gn2, was determined by Northern blot analysis to express the GR cDNA and by whole-cell binding analysis to exhibit approximately twice the level of dexamethasone binding (data not shown). 256Gn2 exhibited the same response to dexamethasone as WR256 did (Fig. 1C), suggesting that the defect in WR256 was not due to altered GR, since expression of additional GR had no effect on the dexamethasone-resistant phenotype.

The WR256 variant was subcloned in order to verify that the dexamethasone-resistant phenotype was heritable. Twelve of 12 colonies tested exhibited the same response to glucocorticoids as the parent WR256 (data not shown). Moreover, after 8 months of continuous culture in the absence of dexamethasone, the growth characteristics in the presence of hormone were unchanged. In addition, WR256 was treated with 1 to 6 μg of 5-azacytidine per ml to determine whether demethylation of the DNA could reverse the phenotype, as has been reported for other steroid-resistant variants (8, 24, 56). 5-Azacytidine treatment had no effect on the dexamethasone-resistant phenotype of WR256 (data not shown). Together, these data suggest that WR256 is a clonal line containing a stable genetic defect.

Effect of dexamethasone on cell cycle and mitochondrial activity. The unusual growth characteristics of WR256 treated with dexamethasone (Fig. 1B) suggested that they may be the result of either cell cycle arrest or a balance of cell death and proliferation induced by the hormone. Since the parent WEHI7.2 cells undergo cell cycle arrest in G_1 prior to glucocorticoid-mediated apoptosis (17), WR256 was examined to determine the distribution of cells in the cell cycle following dexamethasone treatment (Fig. 2A). Both WEHI7.2 and WR256 were arrested in G_1, and within 48 h, approximately 70% of the WEHI7.2 and WR256 cells had undergone cell cycle arrest. Harmon et al. (29) reported that in human CEM C7 cells, the cell cycle arrest prior to lymphocyte apoptotic cell death was irreversible; however, this was not the case for WR256 (Fig. 2B). Growth inhibition of WR256 cells (triangles) was completely reversible upon removal of dexamethasone from the medium after treatment for 72 h (arrow). In contrast, the few remaining WEHI7.2 cells were committed to apoptosis and did not recover after the dexamethasone was removed (circles). However, any potential for reversibility of cell cycle arrest in the wild-type line could be obscured by cell death in this assay. Since dexamethasone-induced cell cycle arrest and cell death were functionally dissociated in WR256, this indicates that lymphocyte apoptosis was not simply a consequence of growth arrest in WEHI7.2 cells. It is possible, however, that growth arrest is necessary but not sufficient for killing of WEHI7.2.

Mitochondrial failure is associated with glucocorticoid-induced lymphocyte apoptosis (43) and anti-CD3-induced cell death of T-cell hybridomas (60). Therefore, the effects of glucocorticoid treatment on the mitochondria of WEHI7.2 and WR256 lymphocytes were determined in a standard MTT conversion assay (55) (Table 1). Mitochondrial activity in WEHI7.2 lymphocytes was reduced by 24 and 83% after 24 and 36 h of incubation with dexamethasone, respectively, and this reduction in activity was concomitant with the appearance of apoptotic cells in the population (Fig. 1A). The WR256 cells showed no decrease in activity after 24 h and only a modest decrease (22%) after 36 h. This small decrease in mitochondrial activity may reflect the large proportion of noncycling cells (Fig. 2A) having lower energy requirements.

WR256 exhibits wild-type levels of GR-mediated transcription. The presence of transcriptionally functional GR in

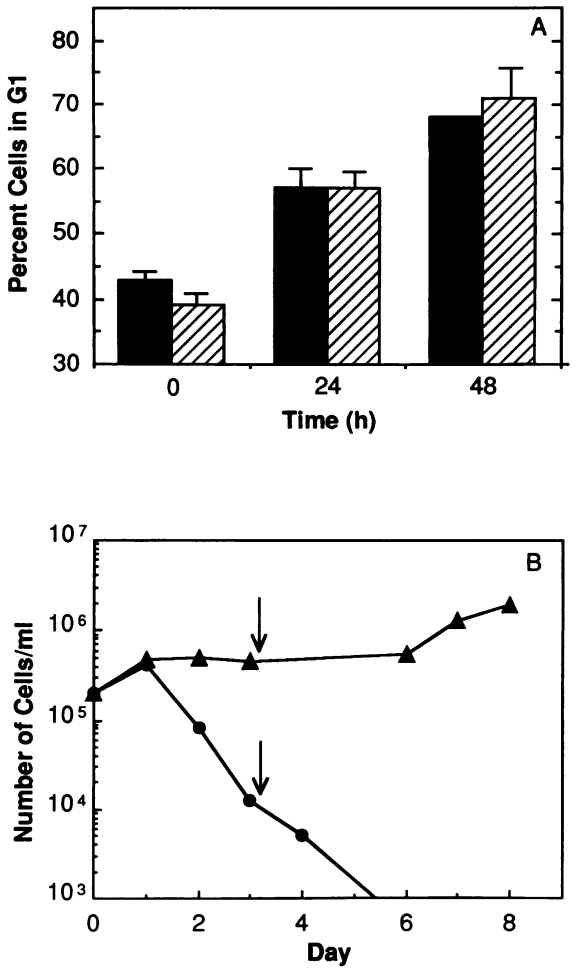

FIG. 2. Effect of dexamethasone on the cell cycle. (A) Cells were incubated with 10^{-6} M dexamethasone or ethanol vehicle for the indicated times and subjected to cell cycle analysis as described in Materials and Methods. WEHI7.2, solid bars; WR256, striped bars. Shown are the means of four determinations ± standard error. (B) WEHI7.2 (●) and WR256 (▲) were incubated for 72 h in the presence of 10^{-6} M dexamethasone. The cells were washed with phosphate-buffered saline (arrows) and incubated for an additional 5 days in the absence of dexamethasone. Shown are the averages of two experiments.

WR256 was confirmed by measuring glucocorticoid-dependent transcription of a CAT reporter gene under the control of the hormone-inducible MMTV promoter. The level of dexamethasone-inducible CAT activity was similar for the

TABLE 1. Mitochondrial activity of WEHI7.2 and WR256 cells treated with dexamethasone[a]

Dexamethasone treatment (h)	Relative activity	
	WEHI7.2	WR256
0	1	1
24	0.76 ± 0.07	1.0 ± 0.05
36	0.17 ± 0.03	0.78 ± 0.01

[a] Cells were tested for their ability to convert MTT to MTT-formazan. Results are reported relative to those for the untreated population. Values are the means of three experiments ± standard error for triplicate measurements.

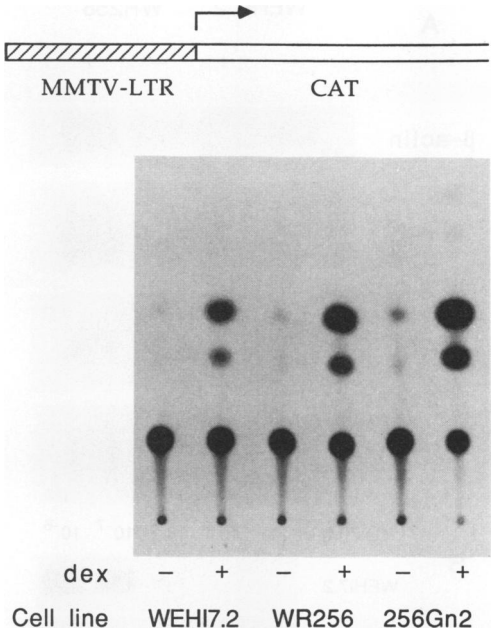

FIG. 3. Effect of dexamethasone on induction of MMTV-controlled CAT activity in WEHI7.2, WR256, and 256Gn2 lymphocytes. Cells were transfected with an MMTV-CAT reporter construct, incubated with 10^{-8} M dexamethasone (dex) or ethanol vehicle for 16 h, harvested, and assayed for CAT activity as described in Materials and Methods. Shown is a representative experiment of three. LTR, long terminal repeat.

WEHI7.2 and WR256 cell lines (Fig. 3), while 256Gn2 exhibited higher activity, consistent with the fact that 256Gn2 cells express GR cDNA. These results confirm and extend the results of the hormone-binding assays and demonstrate that WR256 and WEHI7.2 cells express similar levels of transcriptionally competent GR. Thus, the dexamethasone-resistant phenotype of WR256 was not due to reduced levels of, or a defect in, GR.

To further demonstrate that GR was functional in the WR256 cell line, we also examined the expression of cellular genes which are known to be regulated during glucocorticoid-mediated cell death. The pattern of hormone-dependent endogenous gene expression in WR256 and WEHI7.2 was examined by Northern blot analysis (Fig. 4A), and histone H3.3 (61) was used to normalize the results because this transcript is relatively unaffected by the hormone in these cells (17). In this study, a modest increase in β-actin transcript levels was observed in both WEHI7.2 and WR256, consistent with what had been reported for glucocorticoid-treated primary rat thymocytes (2). Expression of the calmodulin (CaM) gene was also examined because it is induced during apoptosis of WEHI7.2 (17), WEHI-7TG (1), and primary murine lymphocytes (1). In fact, the CaM protein has been shown to have an active role in glucocorticoid-mediated lymphocyte cell death (17, 41). We noted that the levels of dexamethasone-induced CaM gene expression are similar in WEHI7.2 and WR256 cells, suggesting that the apoptosis-resistant phenotype of WR256 does not interfere with early events that precede changes in CaM RNA levels.

Recently, our laboratory has observed that GST transcript levels increase dramatically during glucocorticoid-mediated apoptosis of WEHI7.2 and S49 lymphocytes (5). This increase in GST transcript levels may result from alterations in

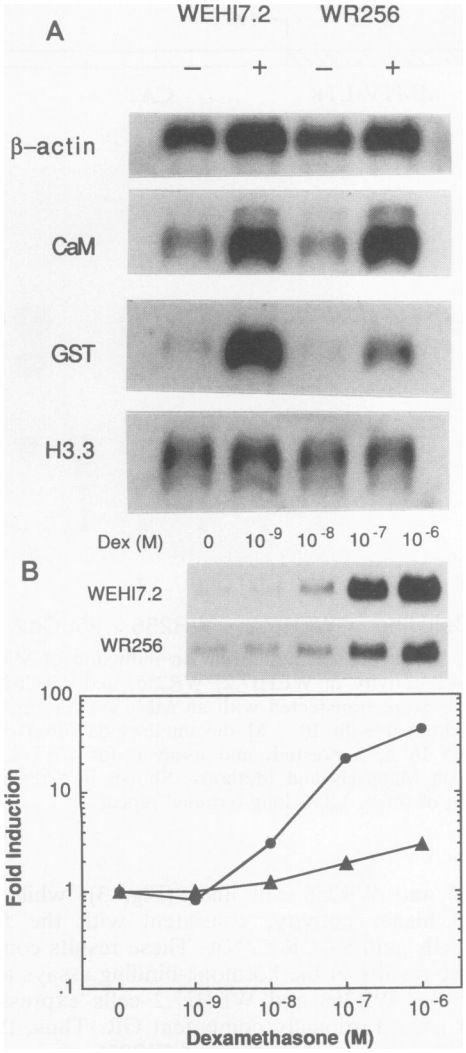

FIG. 4. Dexamethasone effects on endogenous gene expression. (A) WEHI7.2 and WR256 cells were incubated for 24 h with 10^{-6} M dexamethasone (lanes +) or ethanol vehicle (lanes −). Total RNA was isolated, and 20 μg was subjected to electrophoresis through a formaldehyde-agarose gel and transferred to a Duralon membrane (Stratagene). The resulting blot was analyzed sequentially with CaM, histone H3.3 (for normalization), β-actin, and GST probes. (B) WEHI7.2 (●) and WR256 (▲) cells were treated with the indicated concentrations of dexamethasone (Dex) or ethanol vehicle. Total RNA (20 μg) was isolated and subjected to Northern analysis. The blot was analyzed sequentially with histone H3.3 and GST probes. GST levels were quantitated by Betascope and normalized against H3.3 levels.

the redox state of the affected cell and therefore represent a stress response to cell death (6). In contrast to the observed similarities in β-actin, CaM, and H3.3 gene expression, an interesting difference in GST RNA levels was observed in the two cell lines treated with dexamethasone (Fig. 4A). To further investigate this dramatic difference in hormone-induced GST transcript levels, we examined the dependence of the response on the ligand dose (Fig. 4B). WEHI7.2 and WR256 cells were treated with increasing concentrations of dexamethasone, and total RNA was analyzed for GST expression. GST transcript levels were quantitated by Betascope analysis and normalized against H3.3 levels. Dexamethasone at 1 nM did not cause an increase in GST transcript levels; however, at dexamethasone concentrations equal to or greater than 10 nM, GST RNA levels increased in both WEHI7.2 (circles) and WR256 (triangles). At 1 μM dexamethasone, WR256 exhibited only a 3-fold increase in GST RNA levels, while WEHI7.2 had a 45-fold increase. Interestingly, although the magnitude of the response differed between the lines, both lines exhibited a half-maximal response at approximately the same hormone concentration (20 nM dexamethasone). The reduced magnitude of the response suggests that changes in the intracellular milieu leading to increased GST transcript levels may be reduced in the glucocorticoid-resistant WR256 variant. Thus, events "downstream" of CaM regulation but "upstream" of GST induction may be affected in WR256 cells.

WR256 cells are also resistant to cAMP-induced apoptosis. A priori, there was no reason to expect WR256 to be resistant to cAMP, since it was isolated in a single step for resistance to dexamethasone. Because the defect appeared to be subsequent to GR activation, we were interested in determining whether it also interfered with cAMP-induced death. To determine whether the cAMP-mediated apoptotic pathway was intact in WR256, the cells were treated with forskolin, an inducer of adenylyl cyclase activity, or with the derivative 8-bromo-cAMP, and viability was measured over a 72-h period (Fig. 5). WR256 exhibited marked resistance to both 8-bromo-cAMP (Fig. 5B, solid squares) and forskolin (open squares). 256Gn2 was also resistant to elevated levels of cAMP (data not shown). Since WR256 was unaffected by cAMP derivatives as well as forskolin, this suggests that the cAMP resistance was not due to defective adenylyl cyclase.

Many of the variant lymphocyte cell lines selected for resistance to cAMP have been shown to have mutations in the function or expression of PKA (22), the mediator of cAMP-induced cell death (36). In addition, Gruol et al. (26) have demonstrated that lymphocyte cell lines containing defective PKA activity give rise to spontaneous glucocorticoid-resistant variants at a high frequency. Therefore, WR256 was tested for the possibility that a PKA mutation resulted in resistance to cAMP and subsequently to dexamethasone. By using a standard in vitro assay specific for PKA (50), it was determined that WR256 and WEHI7.2 were indistinguishable in both the level of PKA activity and the concentration of cAMP necessary to activate the enzyme (Fig. 6).

In some cells, cAMP may exert its effects in part through a cAMP-dependent alteration in gene expression mediated by PKA (reviewed in reference 49); moreover, cAMP-induced apoptosis of a myelocytic leukemic cell line requires protein synthesis (38). Because cAMP may be exerting its adverse effects in WEHI7.2 by affecting gene expression, both WEHI7.2 and WR256 were analyzed for their ability to induce cAMP-dependent transcription. Both cell lines were transiently transfected with the vector pCREtkCAT, treated with forskolin, and analyzed for inducible CAT activity. This vector confers cAMP responsiveness to the expression of the CAT reporter gene. The results in Fig. 7 demonstrate that the minimum factors necessary for transcription of pCREtkCAT are present in both WR256 and WEHI7.2 cells. cAMP-induced CAT activity was not observed with a thymidine kinase-CAT vector lacking the CRE (data not shown). Therefore, similar to the results from the analysis of GR- and dexamethasone-mediated MMTV-CAT expression described above, the signal transduction pathways of at least some components of WEHI7.2 cAMP responsiveness are normal in WR256.

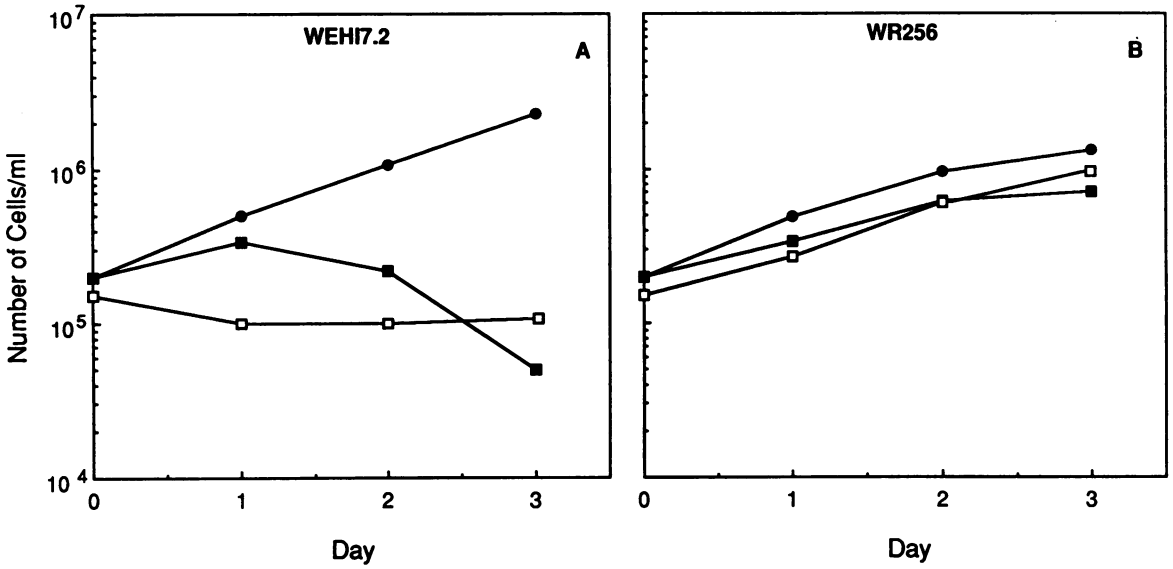

FIG. 5. Effect of cAMP on the growth of WEHI7.2 (A) and WR256 (B). Cells were untreated (●) or treated with 200 μM 8-bromo-cAMP (■) or 5 μM forskolin (□). The number of live cells was determined by trypan blue dye exclusion.

***bcl-2* transcript levels are unaltered in dexamethasone- and cAMP-treated WEHI7.2 and WR256 cells.** The *bcl-2* proto-oncogene encodes a mitochondrial inner membrane protein (31) which can protect a variety of cells against apoptosis initiated by certain stimuli (31, 45, 52, 54, 58). For example, thymocytes of transgenic mice expressing human *bcl-2* are protected from glucocorticoid-mediated apoptosis (52, 54). It was possible that the dexamethasone- and cAMP-resistant phenotype of WR256 was due to high-level constitutive expression of *bcl-2*. Thus, WEHI7.2 and WR256 were analyzed for expression of the endogenous mouse *bcl-2* gene during dexamethasone or 8-bromo-cAMP-induced apoptosis. Northern blot analysis of RNA isolated from untreated and treated cells demonstrated that expression of the primary 7-kb transcript (9) was the same in WEHI7.2 and WR256 and apparently was not regulated during apoptosis (Fig. 8). These results suggest that alterations in transcriptional regulation of the *bcl-2* gene in WR256 cells are not the molecular basis of the apoptosis-resistant phenotype. However, the possibility exists that regulation of *bcl-2* may occur posttranscriptionally. Thus, we cannot completely exclude the possibility that the *bcl-2* gene product is acting to protect WR256 from apoptosis.

DISCUSSION

Apoptosis is a form of cell death which has been observed in a number of systems, including embryonic development and clonal selection (62). Because of the complexity of the event, little is known about the molecular basis of programmed cell death. Therefore, variant cell lines which contain defects in the apoptotic cascade could be very informative in deciphering the steps involved. Although

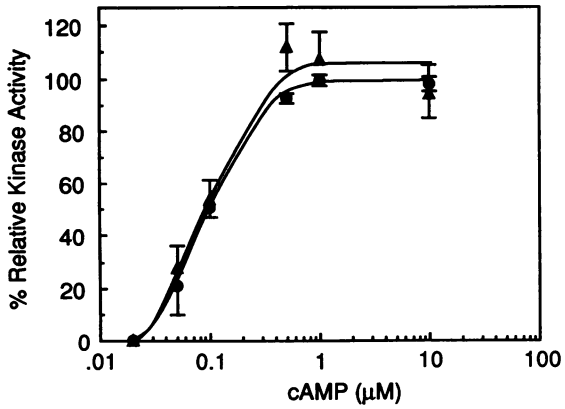

FIG. 6. Activity of PKA from extracts of WEHI7.2 (●) and WR256 (▲). Cell lysates were prepared and analyzed for cAMP-dependent protein kinase activity as described in Materials and Methods. Values are relative to maximal activity achieved with WEHI7.2 lysates. The data for each point were determined in duplicate. Shown are the means of three experiments ± standard error.

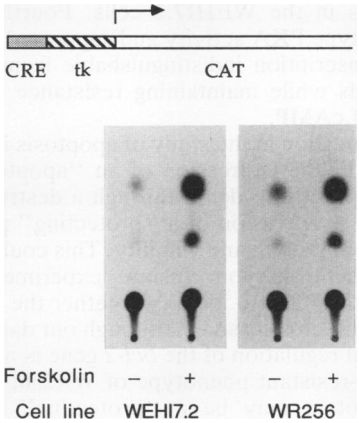

FIG. 7. Induction of CAT activity from a CRE. Cells were transfected with pCREtkCAT, incubated with 5 μM forskolin or ethanol vehicle for 12 h, harvested and assayed for CAT activity as described in Materials and Methods. Shown is a representative experiment of three. tk, thymidine kinase.

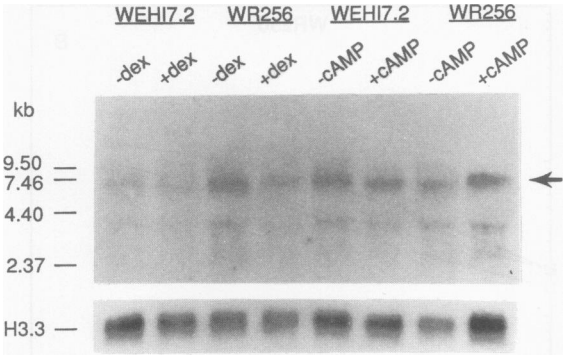

FIG. 8. Expression of the mouse *bcl-2* transcript during WEHI7 apoptosis. WEHI7.2 and WR256 cells were untreated or treated for 24 h with 1 μM dexamethasone (dex) or 200 μM 8-bromo-cAMP. RNA was isolated, and 20 μg was subjected to Northern analysis. The resulting blot was analyzed sequentially with *bcl-2* and H3.3 probes.

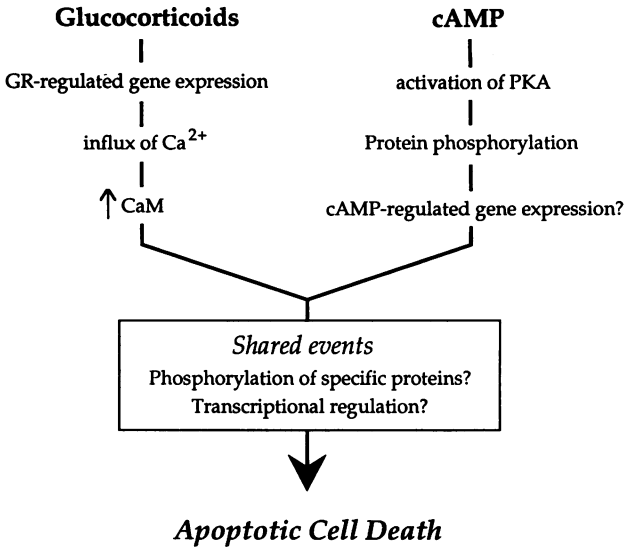

FIG. 9. Model of convergent apoptotic pathways.

hundreds of glucocorticoid-resistant variants have been isolated, almost all contain alterations in the level or activity of GR (24). Likewise, only one cAMP-resistant mutant which expresses functional PKA has been reported (39). WR256 is unique because it is the only cell line to date that is resistant to apoptosis from both glucocorticoids and cAMP while retaining fully functional GR and PKA. Although selection occurred exclusively in dexamethasone, it is possible that the WR256 apoptosis-defective phenotypes are due to two separate mutations.

By the criteria that GR and PKA in WR256 are fully functional and are expressed at wild-type levels, we propose that WR256 is a "deathless" mutant. This is supported by several lines of evidence. First, the cells exhibited dexamethasone-mediated cell cycle arrest, a characteristic of early events in glucocorticoid-induced lymphocyte apoptosis (17, 29), while the GR^- variant WEHI7.418 (13) did not (18). Thus, the GR in WR256 is capable of eliciting hormone-dependent arrest of proliferation. Second, the cells are able to support steroid-regulated, GR-dependent transcription of a reporter gene under the control of the MMTV hormone-inducible promoter. Third, induction of the CaM and β-actin genes occurs in a dexamethasone-dependent fashion at the same level as in the WEHI7.2 cells. Fourth, WR256 expressed wild-type PKA activity and supported cAMP-inducible gene transcription indistinguishable from those of the WEHI7.2 cells while maintaining resistance to lethal concentrations of cAMP.

A central question in the study of apoptosis is whether cell death is due to the expression of an "apoptotic" protein, which leads directly to death through a destructive mechanism, or to the repression of a "protecting" protein that is required for cell growth and viability. This could be tested by performing heterokaryon fusion experiments between WEHI7.2 and WR256 to identify whether the WR256 mutation is dominant or recessive. Although our data suggest that transcriptional regulation of the *bcl-2* gene is not involved in the apoptosis-resistant phenotype of WR256, expression of the Bcl-2 protein may be a "protecting" factor in the developing thymus (32, 47). Bcl-2 does not appear to be expressed in immature thymocytes sensitive to glucocorticoid-induced apoptosis, but it is highly expressed in mature lymphocytes, which are insensitive to apoptosis (32, 47). Thus, Bcl-2 may act to protect mature lymphocytes from apoptotic cell death and may have a physiological role in lymphocyte development (32, 54). Interestingly, we have recently constructed and analyzed WEHI7.2 cell lines stably expressing human *bcl-2* and found that both dexamethasone and cAMP resistance correlates with the level of human Bcl-2 protein (7).

Apoptosis appears to be morphologically similar in cells treated with a variety of agents. However, genetically there must exist differences which allow various signals to independently initiate apoptosis in specific cells. On the basis of our data and that obtained in other laboratories, we have proposed a temporal model illustrating convergent events in the initiation of glucocorticoid- and cAMP-induced apoptosis in WEHI7.2 lymphocytes (Fig. 9). It is important to note that although it is not known how cAMP- and glucocorticoid-mediated cell death are mechanistically related, it is possible that there are differences in the rate-limiting steps of each pathway. Briefly, our model includes a glucocorticoid-mediated influx of Ca^{2+} (41), which is followed closely by CaM gene induction, an early event occurring within 2 h of treatment with dexamethasone (17). Extending our model to include cAMP, apoptosis of WEHI7.2 initiated by the cyclic nucleotide is dependent upon functional PKA but appears to be independent of β-actin, CaM, and GST induction (18). Because it has been suggested that glucocorticoid-induced cell death is Ca^{2+} stimulated while death caused by cAMP is not (41, 42), it is possible that GST may be induced as a response to a Ca^{2+}-stimulated event(s) which is absent in cAMP-induced death (5). This is supported by the observation that GST transcript levels are increased in response to calcium ionophore treatment (5).

As shown in Fig. 9, the shared events of these two pathways could include additional phosphorylations of specific proteins as well as coregulation of "downstream" genes. For example, in dexamethasone-induced cell death, CaM and Ca^{2+} may be involved in activating the Ca^{2+}-CaM-dependent kinase, which then affects the phosphorylation state of the cell. In cAMP-induced apoptosis, PKA could affect cellular phosphorylation in a similar manner, since PKA and Ca^{2+}-CaM-dependent kinase II can often phosphorylate identical substrates (20). The putative "cross talk" between kinases could be one of several common steps

in the two pathways. Thus, WR256 may have a mutation that affects a kinase substrate required for apoptosis or, alternatively, that affects a subsequent step in the cascade. Transcriptional regulation could represent another common event in the pathways, since both glucocorticoids and cAMP can affect transcription. It is possible that both GR- and CRE-binding proteins may regulate the expression of additional genes involved in the apoptotic cascade. To examine further the hypothesis of convergent pathways, we are currently characterizing a panel of apoptosis-defective variant cell lines which were isolated from a large pool of chemically mutagenized WEHI7.2 cells (21).

Although the exact defect(s) in WR256 has yet to be determined, the finding that dexamethasone- and cAMP-mediated cell death appear to have common features already gives us clues as to what types of mechanisms may be involved. Moreover, it is likely that the novel WR256 variant cell line will be an invaluable tool in the elucidation of early molecular events in apoptosis. Perhaps by understanding the nature of the defective phenotype in WR256, we may be able to use molecular genetic approaches to investigate critical events in the apoptotic pathway.

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