A New Determinant of Glucocorticoid Sensitivity in Lymphoid Cell Lines

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ABSTRACT The SAK cell line, derived from a spontaneous thymic lymphoma in an AKR mouse, is resistant to lysis by glucocorticoids in spite of the presence of functional glucocorticoid receptor. Receptor function was determined by hormone binding analyses, as well as characterization of hormonal effects on cell growth and on the accumulation of murine leukemia virus and metallothionein mRNAs. SAK cells were fused with a receptor-defective (and therefore resistant) variant of a well-characterized murine thymoma line, W7. The resulting hybrids are glucocorticoid sensitive, demonstrating complementation of the receptor defect in W7 cells by the functional glucocorticoid receptor of SAK. This fusion shows that SAK cells are resistant to the hormone due to the absence of another function designated "I" for lysis. SAK cells were also fused with glucocorticoid-sensitive W7 cells (containing wild-type receptor), generating glucocorticoid-sensitive hybrids, which demonstrate that the dexamethasone-resistant phenotype of the SAK cells is recessive. Resistant derivatives of this hybrid were found which still contain the full amount of receptor. Chromosome analysis revealed that, on the average, the resistant derivatives had lost two chromosomes, suggesting segregation of chromosomes carrying genetic material necessary for the "lysis" function.

The drug 5-azacytidine (a known inhibitor of DNA methylation) has been shown to cause heritable changes in gene expression. Treatment of SAK cells with 5-azacytidine generated glucocorticoid-sensitive clones at high frequency, suggesting that the gene(s) involved in the "lysis" function are intact and have been inactivated through a process such as differentiation.
more differentiated dexamethasone-resistant T-cell and provide insights into this mode of acquisition of glucocorticoid resistance in vivo.

MATERIALS AND METHODS

Cell Lines and Culture Conditions: The SAK cell line (SAKRTLS.12.1) is a cloned murine T-cell line derived from a spontaneous tumor in an AKR mouse. The SAK cells were a generous gift from Dr. Robert Hyman (Salk Institute). Both SAK and its subclone, SAK8, show the same properties with respect to glucocorticoid receptor and response. The receptor-defective W7 variant used is Moua101 derived by M. Huet-Minkowski (Institut Pasteur) by treatment of W7TG with bleomycin to produce a receptorless variant which was then selected for ouabain resistance (8). This variant has been shown by Scatchard analysis to contain <10% of the amount of specific binding seen in the parent (data not shown) and is completely resistant to lysis. The W7 line containing two copies of the wild-type receptor locus, which was used in this work, is W7TGoua1 isolated by M. Pfahl (Salk Institute; 11). All cell lines and hybrids were grown in Dulbecco's Modified Eagle's medium (DME) containing 10% fetal calf serum (FCS) in a humidified atmosphere at 37°C.

Steroid Binding Assays: The binding of [3H]dexamethasone to whole cells and the nuclear transfer of the receptor-hormone complex were measured as described (12).

Analysis of mRNA Accumulation: The accumulation of leukemia virus mRNA in SAK8 cells was measured by the method of Wahl et al. (13). Briefly, oligo(dT) cellulose columns were used to select poly(A)-containing mRNA. The mRNA was denatured with glyoxal, fractionated in a 1% agarose gel, transferred to nitrocellulose paper, and probed with Moloney leukemia virus cDNA (generously provided by Dr. Inder Verma, Salk Institute). The nitrocellulose was washed and exposed to film. The developed film was analyzed by scanning the bands which correspond to viral mRNA in an LKB densitometer (LKB Instruments, Bromma, Sweden). The area under the peaks was compared to quantitate the increase in viral mRNA.

5-Azacytidine Treatment: Exponentially growing SAK8 cells were treated for 13 h with 3 μM 5-azacytidine (Calbiochem-Behring Corp., San Diego, CA). After extensive washing, the cells were allowed to recover until exponential growth had resumed (24-48 h), then subcloned at a dilution of 0.25 cells/well in tissue culture trays. Clones were picked 9 d later and screened for dexamethasone sensitivity. The cloning efficiency of the 5-azacytidine-treated cells is 30%-50%; the cloning efficiency of untreated cells is 100%.

RESULTS

Growth of SAK8 Cells in the Presence of Dex

SAK8 cells normally grow in suspension as single cells or small clumps of three to eight cells (Fig. 1A). After 48 h of culture in medium containing 5 x 10⁻⁶ M dexamethasone, the cells do not lyse; instead, large aggregates of up to 100 cells appear (Fig. 1B). Such aggregates of cells are also seen in the presence of other glucocorticoid hormones such as cortisol (data not shown). If the formation of the aggregates is specific for glucocorticoid hormones, it should be possible to prevent the response by adding an excess of an antiglucocorticoid compound. In the presence of 5 x 10⁻⁶ M progesterone alone, a known antiglucocorticoid (17), the gross phenotypic charac-
teristics of the cells remain unchanged (Fig. 1 C). Fig. 1 D shows that a 100-fold excess of progesterone (5 × 10⁻⁶ M) does in fact block the aggregation response to dex (5 × 10⁻⁶ M).

In addition to the aggregation response, SAK8 cells grow more slowly in the presence of dex. The doubling time is increased from 12 h to ~18 h and the cultures reach a final cell density of 1 to 1.5 × 10⁶ cells/ml rather than 2.5 to 3.5 × 10⁶ cells/ml (refer to Fig. 7 A). The ability of single cells to form clones is also decreased from 100% seen in the absence of hormone to between 20% and 50% in 10⁻⁶ M dex. This inhibition in cloning efficiency is overcome by increasing the FCS concentration from 10% to 20%, conditions in which the cloning efficiency increases to between 70% and 100% of the control. These hormonal effects on cell doubling time and cloning efficiency could result from a decrease in growth factor production caused by glucocorticoid hormones. Such an effect on growth factor production has been described in mitogen-induced T-cells where clonal expansion is inhibited by dex (18).

These effects of dex on the growth of SAK8 cells indicate that, in spite of the absence of the cytolytic response, glucocorticoid hormones have specific effects mediated by functional glucocorticoid receptor. To confirm this observation, we turned to other, more biochemical approaches, to demonstrate functional hormone receptor in these cells.

Glucocorticoid Receptor in SAK Cells

Fig. 2 shows the Scatchard analysis (19) of [³H]dex-binding data from SAK cells and several hybrids generated from SAK (described in following sections). SAK cells contain approximately 31,000 (mean ± SE = 30,700 ± 2,800) glucocorticoid-binding sites per cell, with a dissociation constant of 10 nM for dex. In addition, nuclear transfer assays show that >60% of the receptor-hormone complexes are translocated into the nucleus (data not shown). The results of the Scatchard and nuclear transfer analyses are very similar to the values obtained for the WEHI-7 (W7) cell line (4) and another AKR cell line (unpublished observations), both of which are lysed by dex. The hormone binding analyses used here have detected alterations in receptor number or nuclear transfer capacity of hundreds of W7 variant clones (8, 9) which are resistant to dex due to receptor defects. Thus, abnormal binding of glucocorticoid hormone in SAK cells would be detectable by these procedures.

Increased Accumulation of Specific mRNAs in the Presence of Dex

The SAK8 cell line was derived from a spontaneous thymic lymphoma in an AKR/J mouse. AKR/J mice have stably integrated copies of murine leukemia virus (type C retrovirus) in their genome. Glucocorticoid hormones have been reported to enhance the synthesis of virus in an AKR mouse embryonic cell line (20) and other cell lines which express type C retroviruses (21, 22). Thus, it was of interest to determine whether the endogenous murine leukemia virus (MuLV) mRNA in SAK8 cells was increased by dex, especially in view of the fact that we had seen an increase in synthesis of the viral gp70 envelope glycoprotein (23).

RNA was prepared from SAK8 cells incubated in medium containing 10⁻⁷ dex for 6 or 24 h. Poly(A)-containing RNA was selected using oligo(dT) cellulose. Samples of untreated control mRNA as well as mRNA from dex-treated cells were fractionated in an agarose gel, transferred to nitrocellulose paper, and the transfer was probed with Moloney leukemia virus cDNA. The hybridized blot was exposed to film, and the densitometric scan of the film was analyzed. Table I shows that the samples from the cells treated with dex for 6 or 24 h contain over threefold more mRNA that hybridized to the leukemia virus cDNA probe than the control.

Work by Palmiter et al. (24) has shown that glucocorticoid hormones increase the levels of metallothionein mRNA in a variety of cell types. Metallothionein mRNA was quantitated by solution hybridization (25) using nucleic acids extracted from untreated SAK8 cells and those grown in the presence of 10⁻⁶ M dex for 20 h. Table I shows a 2.5-fold increase in metallothionein mRNA molecules/cell after dex treatment. This induction is similar to that obtained in W7 cells after demethylation of the metallothionein gene by treatment of W7 cells with 5-azacytidine (26). The metallothionein gene is not expressed in W7 cells before treatment with 5-azacytidine.

We have shown that both MuLV mRNA and metallotheonine mRNA levels are increased in SAK8 cells treated with dex. These results together with the effects of glucocorticoids on SAK8 growth and the dex binding assays leave little doubt that SAK8 cells contain functional glucocorticoid receptor. Therefore, SAK cells must be resistant to lysis by glucocorticoid hormones because they lack another function involved in the cytolytic response. We have used a genetic approach to address the basis for glucocorticoid resistance in these cells.

### Table I

| Concentration of dex | Time | No dex treated | Dex-treated |
|----------------------|------|----------------|-------------|
| MuLV                 | 10⁻⁷ | 6              | 1*          | 3.3         |
|                      |      |                | 24          | 1           | 3.2         |
| Metallothionein      | 10⁻⁶ | 20             | 90*         | 224         |

* PolyA⁺ RNA was prepared and MuLV mRNA analyzed as described in Materials and Methods. Metallothionein mRNA was assayed by R. Palmiter as described (personal communication; 25).
* Relative units as derived from densitometer tracings of film exposed to Northern blot (see Materials and Methods).
* Units are molecules per cell.
Genetic Complementation

To demonstrate the existence of another function involved in the cytolytic response, we fused SAK cells with a receptor-defective variant of the dex-sensitive W7 cell line. Fig. 2 shows the Scatchard analysis of the [3H] dex-binding data from this hybrid. The hybrid contains essentially the same number of sites (27,100 ± 1,800) with the same affinity for hormone as the SAK parent. This is the expected result since nearly all of the dex binding sites in the hybrid are contributed by the SAK cells. After fusion, no appearance of previously unexpressed hormone binding sites contributed by the W7 parent is seen.

Fig. 3 shows the growth of the two parental cell lines and four hybrid clones in varying concentrations of dex. Both the parental lines and hybrid clones grow in the absence of hormone (top row), but only the two dex-resistant parental lines grow in the presence of dex \(10^{-8}\) to \(10^{-4}\) M). The SAK line appears to have fewer cells in the presence of dex, due to cell aggregation and the decreased final cell density described above. The small white areas seen in the wells containing the hybrids in the presence of dex are cell debris.

Fig. 4 shows growth curves of the SAK parent, the receptor-defective W7 parent and one of the hybrid clones in \(10^{-5}\) M dex. It is evident from the growth curve that the hybrid clone is killed by dex while the two parental cell lines continue to grow. Direct microscopic observation showed that all seven hybrids derived from this fusion were lysed by dex.

Since the defect conferring resistance to the W7 derivative is known to be the absence of glucocorticoid receptors (<10% of the wild-type receptor number), the observed complementation (Figs. 3 and 4) demonstrates that the SAK line is resistant to lysis by dex because it lacks a function other than the receptor, designated "I" for lysis. The lysis function in the hybrid is contributed by the receptor-defective W7 variant, and functional receptor is contributed by the SAK parent. This complementation can be expressed in the following simplified way:

SAK \(2r^+/21^-\) × W7 \(2r^-/21^+\) → hybrid \(2r^+2r^-/21^+21^-\).

dex-resistant  dex-resistant  dex-sensitive

**Figure 3** Complementation between two dex" cell lines. Parental cell lines (SAK and W7) and four independently arisen hybrids were inoculated into a 24-well tissue culture tray at a cell density of \(10^6\) cells/ml. The molar concentration of dex is indicated for each row. The cells were allowed to grow for 4 d before the tray was photographed. The white areas are generated by the presence of cells or cellular debris.

**Figure 4** Kinetics of lysis in a hybrid cell line. Growth curves were performed in the presence of \(10^{-8}\) M dex on the SAK (X) and receptor-defective W7 (Δ) parents and one of the hybrids (○) shown in Fig. 3. Living cells were determined by trypsin blue exclusion and counted in a hemocytometer.

FIGURE 3  Complementation between two dex' cell lines. Parental cell lines (SAK and W7) and four independently arisen hybrids were inoculated into a 24-well tissue culture tray at a cell density of \(10^6\) cells/ml. The molar concentration of dex is indicated for each row. The cells were allowed to grow for 4 d before the tray was photographed. The white areas are generated by the presence of cells or cellular debris.
In this diagram, \(r^+\) indicates the presence of a functional receptor locus and \(1^+\) indicates the presence of the lysis function which may be encoded by one or several loci.

**Segregation of the Lysis Function**

Since intraspecies hybrids have been shown to segregate chromosomes (10, 27, 28), we wished to obtain hybrid derivatives which had become dex resistant through the loss of the "I" function, while retaining the full amount of receptor. We constructed another type of hybrid containing four copies of the glucocorticoid receptor, but only two copies of the functional "lysis" locus or loci, in order to favor segregation of the "I" function. We fused SAK cells with dex-sensitive W7 cells which contain 30,000 glucocorticoid receptors per cell to produce hybrids tetraploid for the receptor. All six hybrids tested had between 50,000 and 60,000 receptors per cell. One hybrid with 50,900 (+3,600) receptors per cell (Fig. 2) was used for further studies. The number of receptor sites should approach 60,000 sites/cell (the sum of the receptor contents of SAK and W7). However, previous reports have shown that in parental fusions between W7 lines, some variability in the number of receptor sites of hybrid clones is seen (10, 29). This appears to be due to random segregation of chromosomes. The karyotype that the hybrids produced by the fusion of SAK with dex-sensitive W7 cells (Fig. 6 A) shows many cells with fewer than the modal number of chromosomes. The hybrids obtained from this fusion are sensitive to dex. The fusion can be described as:

\[
\text{SAK} (2r^+/21^-) \times \text{W7} (2r^+/21^+) \rightarrow \text{hybrid} (4r^+/21^+21^-).
\]

The fact that dex-sensitive hybrids are generated from the fusion of a dex-sensitive parent with a dex-resistant parent indicates that the dex-resistant phenotype is recessive. This supports the hypothesis that the SAK cells are resistant to dex because of the absence of the lysis function and not due to the presence of a "protecting" substance.

Dex-resistant derivatives of the hybrid containing four copies of the receptor allele were selected at a frequency on the order of \(2.5 \times 10^{-5}\). Of the six dex-resistant derivatives tested, four retained the full amount (50,000 to 60,000 sites per cell) of glucocorticoid receptor; in all cases, the nuclear transfer of the receptor was essentially the same as that of the dex-sensitive hybrid parent.

Fig. 5 shows the growth of the dex-sensitive hybrid, which is killed in \(10^{-5}\) M dex, and of one dex-resistant derivative. The resistant derivative which still contains 50,900 (+4,300) receptors/cell (Fig. 2) continues to grow in the presence of the hormone. Since chromosome segregation would provide a likely explanation for the appearance of dex-resistant derivatives from the dex-sensitive phenotype, we determined the number of chromosomes in the dex-sensitive hybrid and its dex-resistant derivative.

The modal number of chromosomes in the dex-sensitive hybrid is 80 (Fig. 6 A). In the dex-resistant derivative (shown in Fig. 5) the modal number of chromosomes has shifted to 78 (Fig. 6 B). This result is consistent with the idea that the dex-resistant derivative has segregated two chromosomes which carry the genetic information for the "lysis" function involved in the response:

\[
(4r^+/21^+21^-) \rightarrow (4r^+/21^-)\]

dex sensitive dex resistant

Previous work has shown that fusion of two wild type W7 parents yielding a hybrid with four copies of the receptor locus gave rise to dex-resistant variants with a frequency of \(<2.5 \times 10^{-8}\) (28). This low frequency is expected since this hybrid would also have four copies of the lysis function.

In other experiments, dex-resistant derivatives were selected using the hybrid which only contains two receptor alleles. In this case, segregation of chromosomes carrying genetic material involved in the "lysis" function is not favored \((2r^-2r^-/21^+21^-)\) over segregation of chromosomes bearing receptor loci. Predictably, 18 out of 21 clones analyzed had \(<50\%\) of the hormone binding seen in the dex-sensitive hybrid parent. Such dex-resistant clones arose at a frequency on the order of \(5 \times 10^{-8}\), two orders of magnitude higher than the frequency of resistance derived from the hybrid tetraploid for the receptor. These results are in good agreement with previous studies using hybrids of W7 and its derivatives (10, 28).
**Activation of the Lysis Locus**

We have shown that the SAK cell line contains functional glucocorticoid receptor and is resistant to lysis by dex due to a deficiency in the "lysis" function. The origin of dex resistance in SAK could be a mutation in gene(s) encoding the lysis function or the inactivation of the lysis gene(s) through a process such as demethylation. In vivo, mature immunocompetent T-cells are resistant to lysis by glucocorticoids; it seems possible that SAK might be an example of this more mature dex-resistant T-cell type. If this were the case, one might be able to activate the expression of the "lysis" gene(s) in these cells. To distinguish between these possibilities, we have employed a technique previously shown to activate silent genes.

Recently, a large amount of work from a number of laboratories has established a correlation between hypomethylation of DNA and expression of specific genes (reviewed in reference 30). The drug 5-azacytidine inhibits DNA methylation and has been shown to cause heritable changes in gene expression in a variety of cell lines (26, 31-33). We treated SAK8 cells with 5-azacytidine, allowed them to recover, and subcloned the population (as described in Materials and Methods). Since the incorporation of 5-azacytidine into DNA is thought to be random, it is important to screen for clones expressing the specific function under investigation.

The subclones were tested for sensitivity to glucocorticoids and at least 10% of the individual clones tested were lysed by dex. The frequency of dex-sensitive clones was always high but varied with the length of the 5-azacytidine treatment and with the time after treatment at which the clones were assayed. Fig. 7 shows the growth of SAK8 (Fig. 7A) and one such dex-sensitive clone derived by treatment of SAK8 with 5-azacytidine (Fig. 7B) in the presence and absence of 10^{-7} M dex. Thus, it is possible to "activate" the lysis function in SAK8 cells by treatment with 5-azacytidine. To demonstrate that the dex-sensitive subclones did not pre-exist in the SAK8 population, SAK8 cells were subcloned and 100 independent clones were assayed for response to glucocorticoids. All 100 clones were dex-resistant. The high frequency at which dex-sensitive clones are generated after 5-azacytidine treatment must be as a result of the action of the drug on SAK8 cells.

The generation of dex-sensitive subclones of SAK8 through demethylation of DNA supports the hypothesis that the absence of the lysis function in SAK8 is not due to a mutation; it appears that the necessary genetic material is "intact" in SAK8 but inactivated through DNA methylation. These results extend our genetic studies showing that the recessive absence of the "lysis" function in SAK is complemented by functional lysis locus or loci in W7 cells.

**DISCUSSION**

To date, the hundreds of dex-resistant derivatives obtained from the murine T-cell lines S49 and W7, as well as the human CEM-C7 line, have been shown to be the result of defects in the glucocorticoid receptor (reviewed in references 5 and 7). Two general types of receptor defects have been characterized: (a) defects in receptor quantity and (b) defects in receptor quality, such as nuclear transfer defective. Somatic cell hybrids have been used to demonstrate that a receptor defect of one type is not complemented by a different type of receptor defect (8, 10). These data are in agreement with recent work showing that a single purified polypeptide is capable of binding both glucocorticoid hormones and specific DNA sequences (34). There is no evidence that more than a single protein molecule is required to bind glucocorticoid hormones and elicit a biological response.

SAK cells are resistant to glucocorticoids in spite of the fact that by both biological and biochemical criteria they contain functional receptor. Glucocorticoid hormones have specific effects on the growth of SAK cells, as well as on the accumulation of metallothionein and MuLV mRNAs. The increase in MuLV mRNA by glucocorticoids has not been reported previously.

The studies described here provide the first genetic evidence for a function, other than the glucocorticoid receptor, involved in the cytoplectic response. The glucocorticoid receptor of the SAK cell line complements the receptor-defective W7 line, resulting in a dex-sensitive hybrid. No increase over the sum of the parental hormone binding sites is seen in the hybrid, which rules out an "activation" of the defective receptor of W7. Therefore, the basis for glucocorticoid resistance in SAK cells must be in another function which may be determined by a single genetic locus or several loci.

The hybrid between the dex-resistant SAK cells and the dex-sensitive W7 cell line is dex sensitive. In this fusion the dex-resistant phenotype is recessive. If SAK cells are not resistant due to a defect in the lysis function and instead produce a substance that "protects" the cells from lysis, one would have expected the resistant phenotype to be dominant unless this substance is present in limiting amounts such that it is not produced in a sufficient quantity to "protect" a tetraploid cell. This possibility seems unlikely since it is possible to "activate" the lysis function in SAK8 by treatment with 5-azacytidine. Although one could argue that 5-azacytidine activates an inhibitor of the "protecting" substance, this is a much more complicated model introducing additional and speculative assumptions.

We have used two different hybrids, one diploid and the other tetraploid for the receptor locus, to select dex-resistant derivatives. It is possible to generate resistance through the loss of receptor as well as through the loss of nonreceptor genetic material involved in the lysis response, depending on the ploidy.
of the receptor locus. The hybrid tetraploid for the receptor locus became dex-resistant through the segregation of only two chromosomes, on the average, without loss of receptor. We are currently constructing interspecies hybrids to perform similar experiments designed to map the “lysis” gene(s) to a specific chromosome.

It is interesting to examine the means by which SAk cells may have acquired glucocorticoid resistance. We used 5-aza-cytidine to inhibit DNA methylation in SAk cells and generated dex-sensitive derivatives. Work from several laboratories has demonstrated that thymidine kinase (33), viral (35), and metallothionein genes (26) can be activated by treatment of cells with 5-azacytidine. It appears, therefore, that the SAk line is derived from a mature dex-resistant thymocyte and that, during differentiation, nonreceptor gene(s) involved in the lytic response is/are inactivated through methylation of specific DNA sites. If so, immature thymocytes may become resistant to glucocorticoids through the inactivation of nonreceptor genes involved in the lytic response; the glucocorticoid receptor would still be expressed and capable of mediating other effects of glucocorticoid hormones on the metabolic processes of these cells. The type of glucocorticoid resistance attained by methylation of DNA would have an epigenetic origin since it would involve a modification rather than a change in the base sequence of the DNA. In contrast, the variety of receptor-defective variants generated from W7 and S49 cell lines by mutagenic treatments (5) appear to be the result of rare genetic (mutational) events.

The demonstration of a function other than the glucocorticoid receptor involved in cytokinesis raises several questions about the nature of this “lysis” function. It is certainly possible that hormone-induced cell death is the result of a cascade of events, only one of which needs to be inactive to generate resistance such as that seen in SAk cells. It may be possible, in the future, to identify defects in several loci, any one of which is sufficient to confer glucocorticoid resistance. Clearly, in the case of SAk cells, the “defect” resulting in dex resistance cannot be in a vital function since one would expect such a defect to be lethal. Finally, it remains to be determined whether the “lysis” genes are induced directly by dex or indirectly by means of one or several protein intermediates. Another possibility is that the glucocorticoid hormone receptor interacts with a constitutively expressed “acceptor” protein specifically required for the lytic response, and that the two proteins together are required to induce the cytokinesis. In that case, this “acceptor” would be the “lysis” function absent in SAk cells.

It is certainly of interest to determine whether a “lysis” function is also involved in the lytic response of human T-cell lines, and whether this epigenetic mode of the acquisition of resistance is involved in the lytic response of human T-cell lines. The type of glucocorticoid resistance attained by methylation of DNA sites. If so, immature thymocytes may become resistant to glucocorticoids through the inactivation of nonreceptor genes involved in the lytic response; the glucocorticoid receptor would still be expressed and capable of mediating other effects of glucocorticoid hormones on the metabolic processes of these cells. The type of glucocorticoid resistance attained by methylation of DNA would have an epigenetic origin since it would involve a modification rather than a change in the base sequence of the DNA. In contrast, the variety of receptor-defective variants generated from W7 and S49 cell lines by mutagenic treatments (5) appear to be the result of rare genetic (mutational) events.

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