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Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor

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Induction of apoptosis in lymphocytes, which may account for the therapeutic effects of glucocorticoids in various diseases including leukemia, depends on the glucocorticoid receptor. However, the events leading from the activated receptor to cell lysis are not understood. A prevailing hypothesis postulates induction of so-called 'lysis genes' by the activated receptor. In this study, we show that an activation-deficient glucocorticoid receptor mutant is as effective as the wild-type receptor in repression of AP-1 activity, inhibition of interleukin-2 production, inhibition of c-*myc* expression and induction of apoptosis. Furthermore, we show that retinoic acid can also induce apoptosis in these cells through the retinoic acid receptor, whose repressive functions but not target site specificity, are similar to those of the glucocorticoid receptor. Therefore, the primary effect of the receptor in glucocorticoid-mediated apoptosis correlates with transcriptional repression rather than activation and could be mediated by interference with other transcription factors required for cell survival.

Key words: apoptosis/glucocorticoid/glucocorticoid receptor/leukemia

Introduction

Glucocorticoids have wide medical applications for their immunosuppressive, anti-inflammatory and, in some cells, cytostatic effects. Glucocorticoids are key elements in the therapy of rheumatoid arthritis, collagen diseases, lymphatic leukemias and lymphomas (Parillo and Fauci, 1979; Haynes, 1990). Despite their importance, use of glucocorticoids in these conditions is largely empirical and the mechanisms mediating the beneficial effects are poorly understood (Haynes, 1990). One relevant mechanism may be the induction of cell death. Glucocorticoids kill immature thymocytes by activating a suicide process referred to as apoptosis or programmed cell death (Wyllie, 1980; Ucker, 1987; Cohen, 1989). Likewise, several leukemic cell lines undergo this type of cell death in response to glucocorticoids (Baxter *et al.*, 1971; Sibley *et al.*, 1974; Bourgeois and Newby, 1977; Norman *et al.*, 1977). In addition, glucocorticoids were reported to induce apoptosis in mature, activated, peripheral T lymphocytes (Galili, 1983). Therefore, induction of apoptosis may be one of the key mechanisms mediating the therapeutic effect of glucocorticoids in the treatment of leukemias, lymphomas and various autoimmune disorders.

The glucocorticoid receptor (GR) is required for glucocorticoid-induced apoptosis (Baxter *et al.*, 1971; Sibley *et al.*, 1974), but the GR domains necessary for induction of apoptosis have been controversial (Nazareth *et al.*, 1991; Dieken and Miesfeld, 1992), as have been the relevant events following GR activation by the ligand (Caron-Leslie *et al.*, 1991; Dowd and Miesfeld, 1992; Shi *et al.*, 1992; Thulasi *et al.*, 1993). Based on the ability of activated steroid receptors to stimulate gene expression (Yamamoto and Alberts, 1975), it was suggested that glucocorticoid-dependent apoptosis is mediated by induction of so-called 'lysis genes' (Gasson and Bourgeois, 1983). However, despite major efforts, such glucocorticoid-inducible 'lysis genes' have not yet been identified. Other relevant mechanisms that could mediate the therapeutic effects of glucocorticoids are inhibition of cell proliferation (reviewed by Cupps and Fauci, 1982) and inhibition of interleukin-2 (IL-2) production by activated T cells (Gillis *et al.*, 1979; Arya *et al.*, 1984). The latter mechanism is of importance for the immunosuppressive effects of glucocorticoids because IL-2 production is critical for clonal expansion of T cells and other immune functions (Gillis *et al.*, 1979; Cantrell and Smith, 1984).

It was suggested that the inhibition of cell proliferation and IL-2 production is mediated by the recently discovered ability of activated GR to interfere with transcriptional activation by other transcription factors, especially AP-1 (Jonat *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990). This hypothesis was based not only on the ability of activated GR to inhibit AP-1 activity, but also on the known involvement of AP-1 in cell proliferation (Kovary and Bravo, 1991; reviewed by Angel and Karin, 1991) and IL-2 induction (Serfling *et al.*, 1989; Jain *et al.*, 1992). Indeed, analysis of the *cis* elements through which glucocorticoids inhibit IL-2 production failed to identify GR binding sites (GREs) and implicated sites recognized by AP-1 as mediators of this effect (Vacca *et al.*, 1990; Northrop *et al.*, 1992). Such findings raised the possibility that induction of T cell apoptosis may also be mediated by interference with transcription factors, such as AP-1, required for cell survival rather than direct induction of 'lysis genes'. To test this possibility we introduced into a GR-deficient T cell leukemia cell line, expression vectors encoding either a wild type (wt) GR or a mutant derivative that is defective in gene activation but fully competent in interference with AP-1 activity. We found that clones expressing this mutant GR were fully sensitive to glucocorticoids, both in respect of inhibition of IL-2 production

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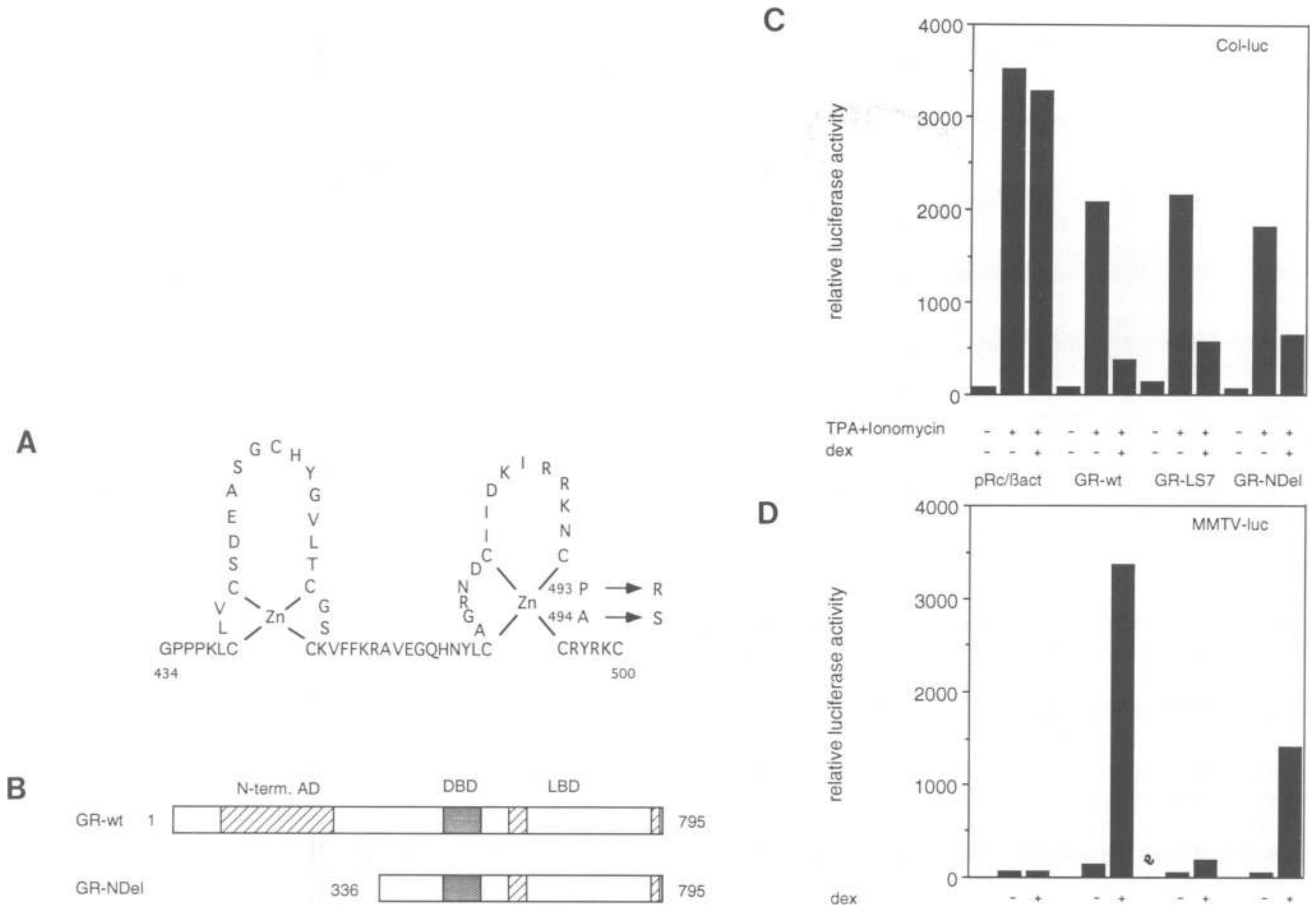

Fig. 1. Transcriptional repression and activation by GR mutants. (**A**) Primary structure of the Zn-finger region of wt GR and LS7 (Godowski *et al.*, 1989). (**B**) Schematic representation of wt GR and the N-terminal deletion mutant GR-NDel. AD, activation domain; DBD, DNA binding domain; LBD, ligand binding domain. (**C**) Dex-dependent repression of the collagenase promoter in TAg-Jurkat cells (Clipstone and Crabtree 1992). Cells were cotransfected with −73Col-LUC and the respective GR expression vectors or the empty expression vector pRC/βact, grown overnight and treated for 8 h with vehicle alone or TPA (50 ng/ml) and ionomycin (4 μM) in the absence or presence of 10^{-8} M dex. Dex was added 1 h previous to TPA and ionomycin. (**D**) Dex-dependent induction of a MMTV-LTR containing two consensus GREs. TAg-Jurkat cells were cotransfected with MMTV-LUC and the same expression vectors as in (A). 10^{-8} M dex or vehicle alone was added 1 h after transfection and cells were harvested after 14 h.

and in undergoing apoptosis. Another GR mutant with a deletion of the N-terminal activation domain was also still able to interfere with AP-1 activity and mediate glucocorticoid-dependent apoptosis. Likewise, stable expression of the retinoic acid receptor alpha (RARα), which binds to different response elements than those recognized by the GR (reviewed by Glass and Rosenfeld, 1991), but has similar AP-1 interfering activity (Schüle *et al.*, 1991; Yang-Yen *et al.*, 1991) conferred upon Jurkat cells susceptibility to all-*trans* retinoic acid (RA)-induced apoptosis.

Results

To stably express various versions of the rat GR in a subclone of the human T cell leukemia-derived Jurkat cell line, which does not express a functional endogenous GR, we constructed expression vectors containing wt or mutant GRs under control of a β-actin promoter. Mutant LS7 (Godowski *et al.*, 1989) contains two adjacent amino acid substitutions in the second half of the second zinc finger, replacing Pro493 and Ala494 by Arg and Ser, respectively (Figure 1A). LS7 was reported to have little or no transactivating potential, while retaining suppressing activity on the bovine prolactin promoter (Godowski *et al.*, 1989). Likewise, the ability of LS7 to interfere with AP-1-mediated transactivation of the collagenase promoter was comparable with that of wt GR (Yang-Yen *et al.*, 1990). Mutant NDel encodes a truncated receptor lacking the amino terminal activation domain (Figure 1B). Transient transfections were performed to check the function of the constructs and to determine the relative ability of the receptor mutants to repress AP-1 activity and to transactivate in Jurkat cells (Figure 1C and D). −73Col-LUC, containing a truncated collagenase promoter (Deng and Karin, 1993), was used as an AP-1-dependent reporter to examine the ability of the various GRs to interfere with AP-1 activity. AP-1 activity, as measured by −73Col-LUC expression, was not detected in non-activated Jurkat cells, but was strongly induced upon cell activation with TPA and the Ca^{2+}-ionophore ionomycin. All GR types, but not the empty expression vector, markedly suppressed this induction in response to the synthetic glucocorticoid dexamethasone (dex) (Figure 1C). No considerable differences in their AP-1 interfering activity were observed. GRE-mediated transactivation was determined by a MMTV-LUC reporter containing two consensus GREs (Drouin *et al.*, 1993) (Figure 1D). Consistent with previous

Table I. Inhibition of cell proliferation by dex

dex (M)	% Thymidine incorporation									
	GR⁻ Jurkat	wt		LS7					N-Del	
		D1-1	A11-1	C3-2	F6-1	A5-1	B12-1	F10-1	#10	#5
0	100.0 ± 9.2	100.0 ± 2.0	100.0 ± 05.0	100.0 ± 08.7	100.0 ± 4.2	100.0 ± 3.3	100.0 ± 5.0	100.0 ± 4.2	100.0 ± 08.2	100.0 ± 3.0
5×10^{-10}	104.1 ± 4.1	95.0 ± 5.0	93.3 ± 15.2	90.4 ± 10.5	61.0 ± 6.2	88.8 ± 4.0	82.9 ± 4.7	101.8 ± 8.4	71.9 ± 14.4	85.8 ± 3.3
1×10^{-9}	104.5 ± 4.9	81.0 ± 2.9	68.1 ± 02.3	66.8 ± 04.9	23.7 ± 0.2	90.6 ± 1.6	60.0 ± 4.1	89.9 ± 3.0	41.9 ± 03.4	73.9 ± 1.9
2×10^{-9}	101.9 ± 2.3	50.8 ± 1.6	27.8 ± 00.3	19.0 ± 00.7	3.4 ± 0.2	70.1 ± 5.1	32.6 ± 1.2	50.4 ± 3.0	17.0 ± 01.0	61.2 ± 3.1
$3.3\times10^{--9}$	105.1 ± 0.7	31.5 ± 0.4	5.3 ± 00.1	5.9 ± 00.3	1.4 ± 0.0	55.6 ± 2.3	19.8 ± 0.7	31.6 ± 2.8	7.7 ± 00.2	47.5 ± 2.1
5×10^{-9}	99.1 ± 2.9	15.0 ± 0.2	1.3 ± 00.1	2.0 ± 00.1	1.1 ± 0.0	29.6 ± 1.7	11.5 ± 0.6	15.1 ± 0.8	4.9 ± 00.2	37.9 ± 1.0
1×10^{-8}	82.5 ± 3.7	5.2 ± 0.3	0.7 ± 00.0	1.3 ± 00.4	1.2 ± 0.3	12.3 ± 0.3	7.0 ± 0.5	6.8 ± 0.5	3.0 ± 00.1	22.4 ± 0.8
1×10^{-7}	86.2 ± 2.1	1.6 ± 0.2	0.5 ± 00.1	0.9 ± 00.3	1.1 ± 0.4	3.5 ± 0.3	3.0 ± 2.2	1.7 ± 0.2	1.5 ± 00.1	9.1 ± 0.9
Rec./cell	–	43 000	72 000	78 000	78 000	32 000	66 000	29 000	ND	ND

Parental GR⁻ Jurkat cells and cells stably transfected with GR-wt, GR-LS7 or GR-NDel were seeded in microtiter plates in the presence of the indicated concentrations of dex and grown for 42 h, followed by 8 h of [^{3}H]thymidine incorporation. Numbers are percent thymidine incorporation ± 1 standard deviation as compared with the vehicle control for each clone. For wt and LS7 clones, receptor numbers per cell as determined by Scatchard analysis are listed below the thymidine incorporation data. ND, not determined.

results in CV-1 cells, LS7 exhibited minimal transactivating potential, while GR-NDel demonstrated intermediate dex-dependent transactivation amounting to 40% of GR-wt, mirroring the activity of the similarly structured mutant Δ77-262 in CV-1 cells (Yang-Yen *et al.*, 1990).

Using these plasmids, several stably transfected cell clones were isolated that constitutively expressed either wt GR or one of the mutants. GRE-mediated transactivation in GR-wt, GR-LS7 clones and in parental Jurkat cells is shown in Figure 2. The difference in activation of transiently transfected MMTV-LUC between GR-wt and GR-LS7 clones was found to be ~10- to 20-fold at maximum receptor occupancy, at and above 10^{-8} M dex.

Dex concentrations considerably below the one commonly used (10^{-6} M) efficiently inhibited proliferation of clones expressing either GR type (Table I). Inhibition of cell proliferation, measured by thymidine incorporation, was virtually identical for GR-wt, GR-LS7 and GR-NDel-expressing clones. Dex concentrations of 10^{-8} M or higher, completely abrogated proliferation, whereas the responses were more gradual in the nanomolar range for each receptor type. Although clonal variability was observed, no consistent differences were detected between clones expressing GR-wt, GR-LS7 or NDel. The parental cells were insensitive to dex. Scatchard analyses were performed for GR-wt, GR-LS7 and the parental cells to determine receptor numbers of individual clones (Table I), using [^{3}H]dex binding to whole cells as described previously (Helmberg *et al.*, 1990). The observed receptor numbers ranged between 30 000 and 80 000 per cell and were somewhat higher than the 14 000 endogenous receptors found in the lymphoblastic leukemic CEM-C7 cell line (Harbour *et al.*, 1990), but lower than the 125 000 endogenous receptors in the P388D1 cell line (Helmberg *et al.*, 1990). Clones with higher receptor numbers tended to be more sensitive to dex. Receptors from all clones bound dex with virtually the same affinity (K_d = 3–5 nM). No specific binding of dex was detected in parental cells.

Similar results were obtained by measuring the effect of dex on cell viability using trypan blue exclusion (Figure 3A). These assays revealed massive cell death in the presence of dex beginning after ~24 h. DNA fragmentation

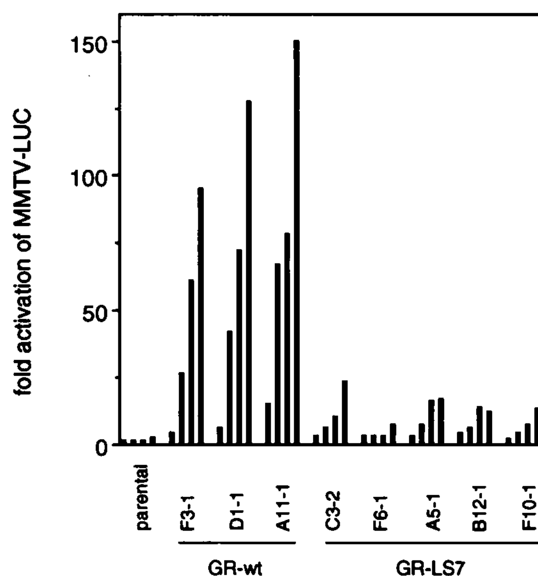

Fig. 2. GR-mediated transactivation in parental and stably transfected Jurkat cells. Parental GR⁻ Jurkat cells, three clones expressing GR-wt and five clones expressing GR-LS7 were transfected by electroporation with MMTV-LUC reporter. Cells were divided into five batches and treated for 16 h with either vehicle (ethanol) alone or 3×10^{-10}, 1×10^{-9}, 3×10^{-9} and 10^{-8} M dex. For each clone, the four bars indicate luciferase activity in dex treated wells divided by luciferase activity in the vehicle control.

by internucleosomal cleavage, a hallmark of apoptosis (Wyllie, 1980), was detected after a 40 h incubation with dex in GR-wt, GR-LS7 and GR-NDel-expressing clones, but not in the parental cells (Figure 3B). Attempts to block this effect of dex with cycloheximide or actinomycin D (Cohen and Duke, 1984), revealed that either agent induces DNA fragmentation by itself, as shown for other lymphoid cells (Martin *et al.*, 1990; Bazar and Deeg, 1992). Induction of apoptosis was also confirmed by morphological criteria, such as cell shrinkage and breakdown of the cell into apoptotic bodies (not shown).

Most importantly, the inhibition of cell proliferation and eventually cell killing did not correlate with transcriptional activation by the GR. For example, GR-wt clone D1-1 (43 000 receptors/cell) responds to 10^{-9} M dex with a

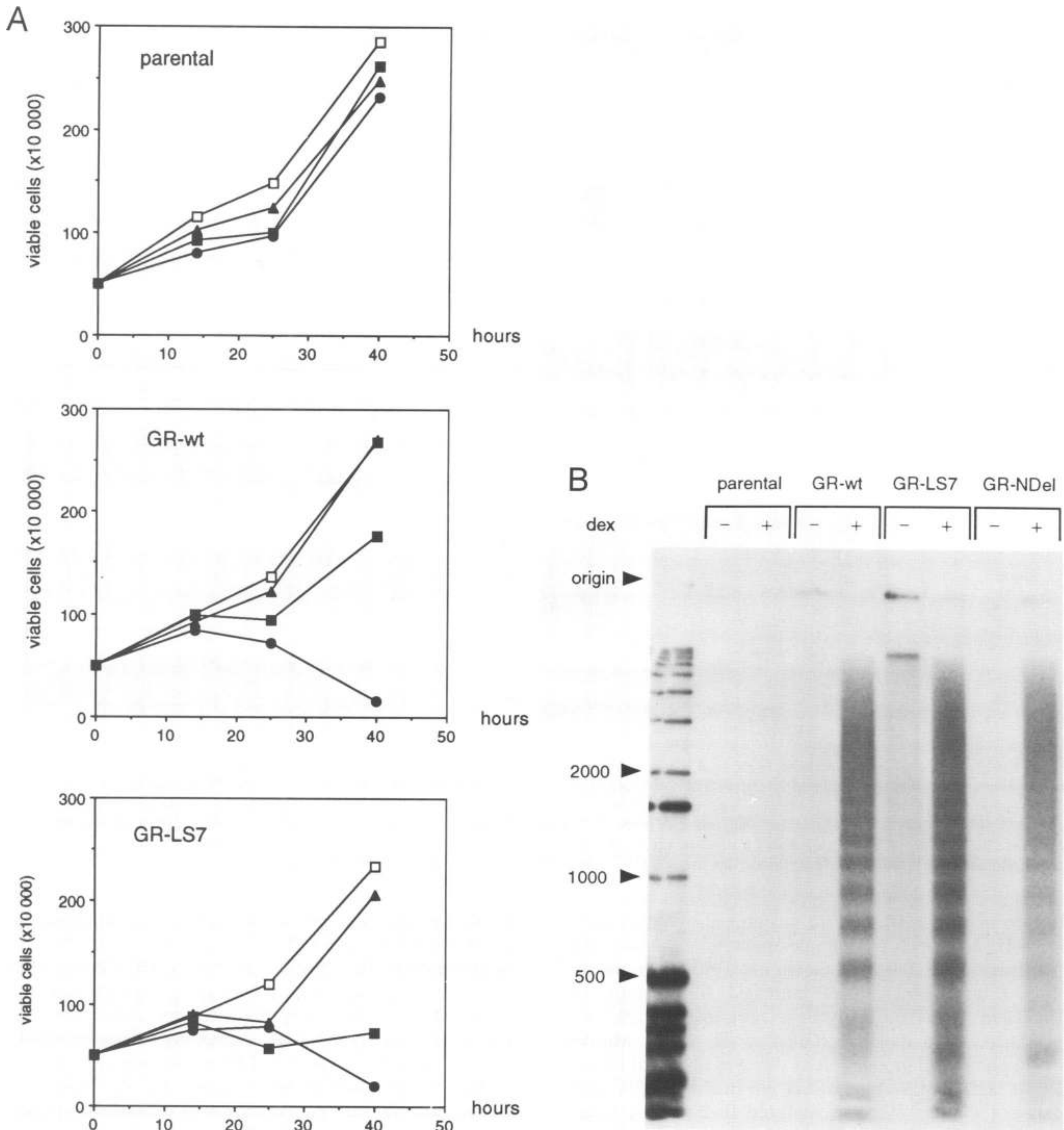

Fig. 3. Dex-mediated apoptosis of GR-expressing Jurkat cells. (**A**) Cell kinetics assayed by trypan blue exclusion of parental cells, GR-wt clone D1-1 and GR-LS7 clone F6-1 in the presence of vehicle alone (□), 10^{-10} M dex (▲), 10^{-9} M dex (■), and 10^{-8} M dex (●). Non-stained cells were regarded as viable cells, even if the beginning of cell fragmentation was already clearly visible. (**B**) DNA fragmentation associated with glucocorticoid-induced apoptosis. Parental, GR-wt, GR-LS7 and GR-NDel cells were incubated with 10^{-8} M dex. After 40 h, DNA was extracted, end-labeled with [α-^{32}P]ddATP, electrophoresed through a 2% agarose gel and autoradiographed.

40-fold activation of MMTV-LUC (Figure 2), but with little inhibition of cell proliferation (81% thymidine incorporation) (Table I) and loss of viable cell count (Figure 3A). Conversely, GR-LS7 clone F6-1 (78 000 receptors/cell) demonstrates only 3-fold activation of MMTV-LUC at 10^{-9} M dex (Figure 2), but considerable inhibition of thymidine incorporation (23% incorporation) and loss of viable cell count (Figure 3A).

To compare the activating and suppressive activities of the wt GR and the LS7 mutant on transcription of endogenous genes in our stably transfected clones, we chose glutamine synthase and IL-2 expression as indicators. The glutamine synthase promoter contains a GRE and is glucocorticoid inducible (Pu and Young, 1989). IL-2 induction is inhibited by glucocorticoids (Gillis *et al.*, 1979; Arya *et al.*, 1984), and the *cis* elements mediating this inhibition were identified (Vacca *et al.*, 1990; Northrop *et al.*, 1992). As in the case of the collagenase promoter,

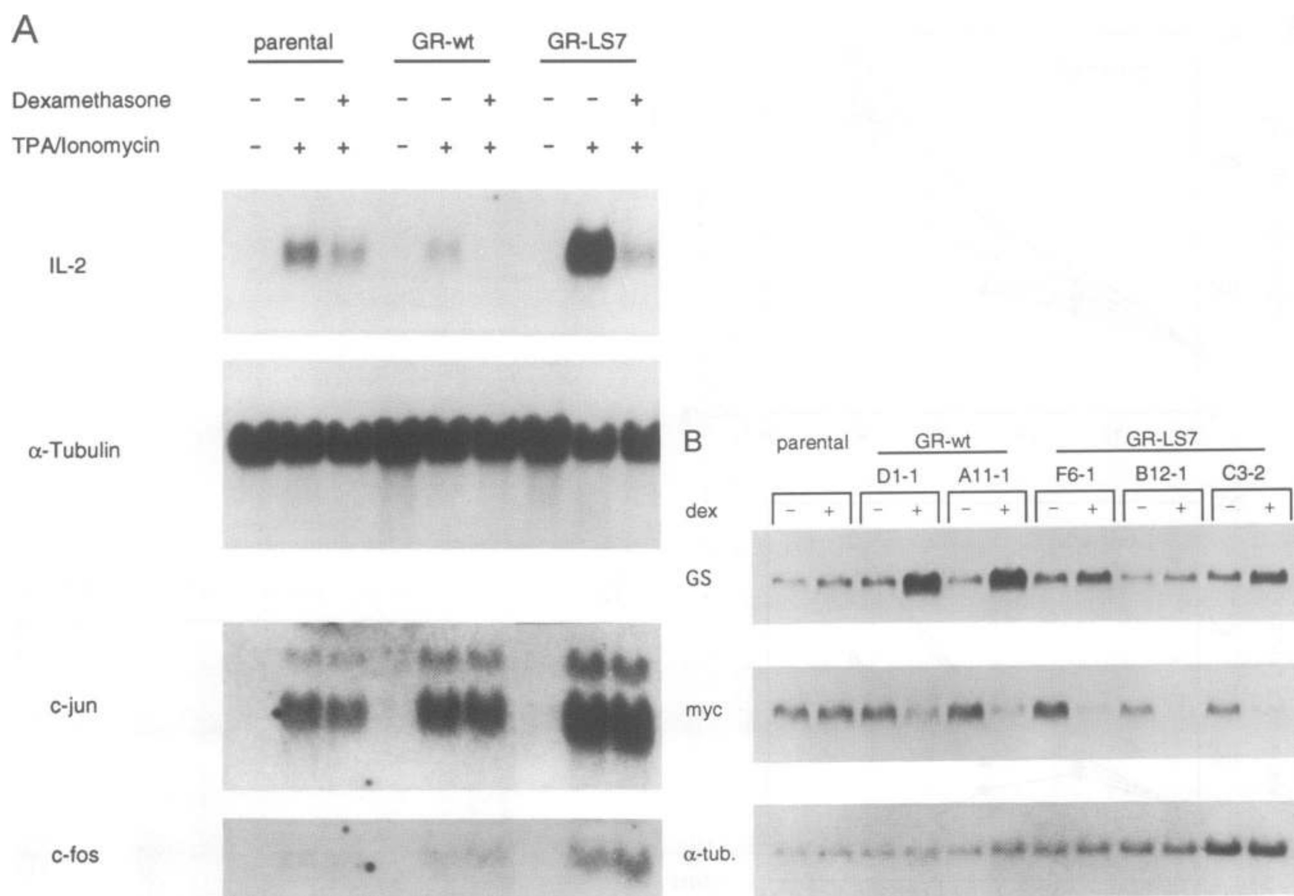

Fig. 4. Effects of dex on gene expression in parental, GR-wt and GR-LS7 Jurkat cells. (**A**) Cells were treated for 8 h (IL-2) or 1 h (c-*jun*, c-*fos*) as indicated, total RNA was extracted and 20 μg per lane were separated on a 1% agarose gel, transferred to a nylon membrane and analyzed by hybridization using probes specific for human IL-2, c-*jun*, α-tubulin and murine c-*fos*. (**B**) Cells were grown for 3 h in the presence or absence of dex (10^{-8} M), and RNA was extracted and analyzed with probes for human glutamine synthase (Van den Hoff *et al.*, 1991), c-*myc* and α-tubulin. Note that GR-LS7 clone C3-2, which shows a small induction of glutamine synthase mRNA is also the clone with the highest residual transcriptional activity shown in Figure 2, and that the two C3-2 lanes contain more RNA than the other lanes.

they do not bind GR, but are recognized by either AP-1 or NF-IL-2A, which is a complex between AP-1 and Oct1 (Northrop *et al.*, 1992; Ullman *et al.*, 1993). The amount of IL-2 in supernatants of untreated, TPA/ionomycin-stimulated or TPA/ionomycin + dex-treated cells was quantitated by a bioassay (Gillis *et al.*, 1978). Marked inhibition of IL-2 production by dex was observed in both GR-wt and GR-LS7 clones, but not in the parental cells (data not shown). We also analyzed IL-2 mRNA steady state levels after 8 h of stimulation to avoid non-specific effects due to cell killing. Induction of IL-2 mRNA was strongly suppressed by dex in both GR-wt and GR-LS7 cells, but not in parental cells (Figure 4A). The same result was found when the cells were stimulated by CD3-crosslinking (Weiss and Stobo, 1984), instead of TPA/ionomycin (data not shown). On the other hand, dex did not inhibit induction of c-*jun* or c-*fos* mRNAs by TPA and ionomycin (Figure 4A). This pattern is identical to that described previously for HeLa cells (Jonat *et al.*, 1990; Yang-Yen *et al.*, 1990). Although the activated GR interferes with AP-1 activity, interference is not due to inhibition of *jun* and *fos* gene expression. Recently, Thulasi *et al.* (1993) reported that glucocorticoid treatment of a leukemic cell line repressed c-*myc* expression. We checked c-*myc* expression in our system and found that as early as 90 min after dex application, c-*myc* was repressed in all of the GR-wt and GR-LS7-expressing clones but not in the parental GR^- Jurkat cells (Figure 4B). On the other hand, dex-mediated induction of the glutamine synthase gene, which is positively regulated by glucocorticoids, was observed only in wt GR-expressing clones and not in the parental GR^- cells or in LS7-expressing clones.

As a more comprehensive screen for the effects of GR-wt and GR-LS7 on expression of endogenous genes, we used differential mRNA display by polymerase chain reaction (PCR) as described by Liang and Pardee (1992). To exclude secondary effects on gene expression, parental GR^-, GR-wt and GR-LS7 cells were grown for 3 h in the presence of cycloheximide, with and without 10^{-8} M dex. Total RNA was purified, reverse transcribed and the resulting cDNAs amplified and separated over a sequencing gel. Whereas the vast majority of cDNAs appeared in equal abundance in all three clones, independently of hormone treatment, several examples of induced and repressed genes were detected (Figure 5). The dex-induced cDNA species shown in the upper panel of Figure 5 was reproducibly detected several times and was consistently found to be induced only in GR-wt-expressing cells, but not in GR-LS7-expressing cells or in parental GR^- cells. On the other hand, the cDNAs whose abund-

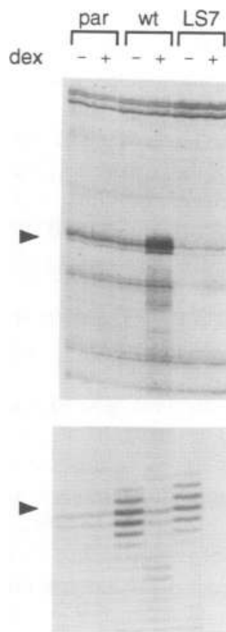

Fig. 5. Global effects of dex on gene expression revealed by differential mRNA display. Parental, GR-wt and GR-LS7 cells were grown for 3 h in the presence of cycloheximide, with and without 10^{-8} M dex. Total RNA was purified and reverse transcribed using anchored primers, followed by sets of PCRs using the anchored primer and different 10mers in the presence of [^{35}S]dATP. The resulting amplified cDNAs were separated on a sequencing gel and and autoradiographed. Due to the ragged ends of PCR products, copies from the same cDNA can differ in size by four to six nucleotides. Shown are examples of one dex-inducible cDNA product which is induced only in wt-GR-expressing cells and one dex-repressible cDNA product that is repressed in both wt-GR and LS7-expressing cells.

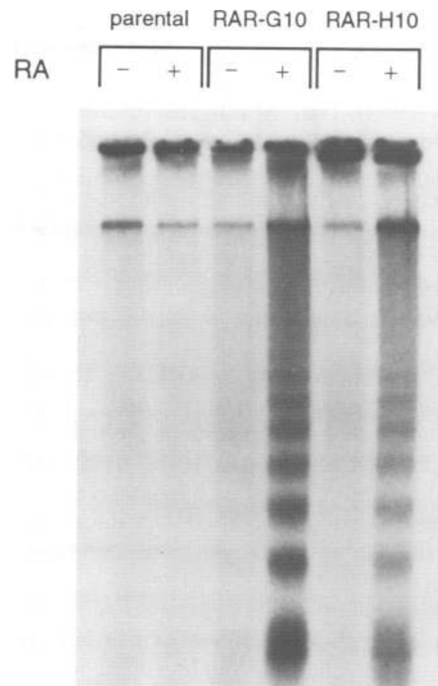

Fig. 6. DNA fragmentation associated with RA-induced apoptosis. Parental Jurkat cells that are RAR$^-$ and RARα transfected clones G10 and H10 were incubated with 10^{-6} M RA. After 48 h, DNA was extracted, end-labeled with [α-^{32}P]ddATP, electrophoresed through a 2% agarose gel and autoradiographed.

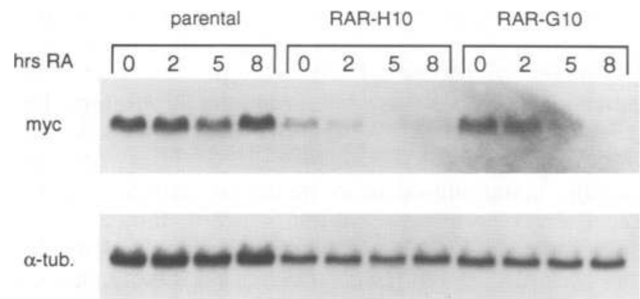

Fig. 7. Effects of RA on c-*myc* expression in parental and RARα-transfected Jurkat cells. Cells were grown in the presence of 10^{-6} M RA for the indicated intervals and RNA was extracted and analyzed with a human c-Myc probe as described in Figure 4.

ance was decreased following dex treatment were detected only in GR-wt and GR-LS7-expressing cells, but not in parental GR$^-$ cells. Only 15 randomly chosen primer combinations have been analyzed.

If the repressive function of the GR, exemplified by its interference with AP-1 activity, was necessary and sufficient to induce apoptosis, this would predict that the same result should be obtained using another nuclear receptor having similar transcription interfering capabilities. To test this hypothesis, we selected the RA receptor, RARα. To activate transcription in a ligand-dependent manner, RARα binds to specific response elements, RAREs, that are different from GREs (for a review, see Glass and Rosenfeld, 1991). However, like the GR, RARα interferes with AP-1 activity in a ligand-dependent fashion (Schüle *et al.*, 1991; Yang-Yen *et al.*, 1991). This interference also does not require direct binding of RARα to DNA. In many systems, RA induces cellular differentiation (Ragsdale and Brockes, 1991), whereas in a few instances it has been shown to induce cell death (Alles and Sulik, 1990; Piacentini *et al.*, 1991). Since no RARα mRNA was detected in parental Jurkat cells, we established Jurkat cells expressing stably transfected RARα using a pRc/βact-RARα expression vector. When exposed to 10^{-6} M RA, cells from RARα-expressing clones died within 72 h, showing the typical internucleosomal DNA degradation pattern, whereas parental Jurkat cells did not (Figure 6). Subsequently, we assayed the effect of RA on c-*myc* expression. c-*myc* mRNA levels did not change following RA treatment of the parental cells, but decreased markedly in RARα-expressing cells over a period of 8 h (Figure 7). The lack of RA sensitivity of the parental cells suggests that they do not express functional RA receptor.

Discussion

Since the classical mode of action of the ligand-activated GR is induction of gene transcription (Yamamoto and Alberts, 1975), it has been assumed for many years that T cell apoptosis is mediated by induction of 'lysis genes' (Gasson and Bourgeois, 1983). However, despite the isolation of two cDNAs for mRNAs whose expression correlates with induction of apoptosis (Owens *et al.*, 1991), no functional glucocorticoid-inducible 'lysis genes' were identified. The work described above casts serious doubts on the 'lysis-gene' hypothesis and strongly suggests that an alternative mechanism is at par. As we show, a variant

GR that is severely compromised in its ability to activate GRE-mediated gene transcription (Godowski *et al.*, 1989), yet fully capable of interfering with AP-1 activity (Yang-Yen *et al.*, 1990), confers full glucocorticoid sensitivity when expressed at normal levels in GR-deficient Jurkat cells. Analysis of five different cell clones expressing this mutant receptor, LS7, indicates that they all undergo apoptosis in response to the same concentration of dex that induces lysis of clones expressing the wt GR, even though they exhibit highly compromised GRE-mediated gene induction, amounting to 5–10% of wt activity. Likewise, expression of RARα, which shares only its AP-1 interfering effect with the GR, was found to confer RA-mediated apoptosis on these cells. These results strongly suggest that glucocorticoids are unlikely to cause apoptosis, at least in Jurkat cells, by direct induction of 'lysis genes'. A more plausible hypothesis consistent with our findings is that glucocorticoids induce apoptosis by inhibiting the expression of yet-to-be identified 'survival genes' (Raff, 1992).

Previous mapping of GR domains required for lysis of the human lymphoblastic leukemia CEM-ICR 27 cell line suggested that only the DNA binding domain was sufficient and that none of the transcriptional activation domains was required (Nazareth *et al.*, 1991). These results, however, are based on transient transfection of GR expression vectors and only a fraction of the cells are therefore susceptible to lysis under such conditions. In addition, transient transfection usually results in vast overexpression of the relevant protein. In direct contrast to this study, Dieken and Miesfeld (1992), using stably transfected S49 mouse lymphoma cells, reported that the N-terminal activation domain of the GR is absolutely required for induction of apoptosis. We find the mutant that lacks the N-terminal activation domain of the rat GR is, as expected, partially compromised in its ability to activate a GRE-dependent reporter gene, but has nearly normal AP-1 interfering activity. When stably expressed in Jurkat cells this construct confers glucocorticoid inducible apoptosis as efficiently as the wt GR. This discrepancy may be due to the use of a slightly larger N-terminal deletion by Dieken and Miesfeld (1992), which may have attenuated the transcriptional interfering activity of the receptor in addition to its activation ability. Some contribution of N-terminal sequences to transcriptional interference was previously reported (Jonat *et al.*, 1990; Yeng-Yen *et al.*, 1990).

Repression of potential 'survival genes' is likely to be mediated through transcriptional interference between the activated GR and transcription factors such as AP-1. As we show here and previously (Yang-Yen *et al.*, 1990), the LS7 mutant is fully capable of interfering with AP-1 activity. Furthermore, induction of *IL-2*, an AP-1 target gene (Serfling *et al.*, 1989; Jain *et al.*, 1992; Ullman *et al.*, 1993; Su *et al.*, 1994), is fully susceptible to repression by dex in LS7-expressing clones. c-*myc* expression, which as we show is repressed by liganded GR-wt, GR-LS7 and RARα, was suggested to be regulated by AP-1 (Hay *et al.*, 1989). So far, our attempts to prove that AP-1 is the relevant target with whose function the activated GR interferes to induce T cell lysis have failed, due to the toxic effect of c-Jun or c-Fos overexpression on lymphoid cells (A.Helmberg and N.Auphan, unpublished results). Thus, it has not been possible to obtain glucocorticoid-resistant clones by overexpression of AP-1 constituents. Although AP-1 is a reasonable candidate for regulating expression of 'survival genes', the activated GR can also interfere with the activity of other transcription factors, including octamer binding proteins (Weiland *et al.*, 1991).

Although IL-2-dependent cells undergo apoptosis in response to IL-2 withdrawal, we do not think that glucocorticoid-induced apoptosis can be reduced to IL-2 withdrawal. Our Jurkat lines are not IL-2 dependent and do not transcribe the *IL-2* gene unless activated (Figure 4A). Addition of recombinant IL-2 did not protect the cells from glucocorticoid-induced apoptosis (data not shown).

Could c-*myc* be a survival gene? Recently, Thulasi *et al.* (1993) reported that repression of c-*myc* correlated with induction of apoptosis by glucocorticoids in a human leukemic cell line and that transient transfection with c-*myc* expression vectors provided some protection against the lethal effects of glucocorticoids. In our system, inhibition of c-*myc* expression preceded induction of apoptosis following activation of either GR-wt, GR-LS7 or RARα. In an attempt to protect the cells from glucocorticoid-induced apoptosis, we generated subclones of GR-wt-expressing cells that constitutively overexpressed c-*myc* mRNA after stable transfection with a c-Myc expression vector. In response to dex, these clones still underwent apoptosis. However, immunoblot analysis of the c-*myc*-overexpressing cells indicated that dex still downregulated c-*myc* expression, suggesting an additional level of interference with c-*myc* expression (A.Helmberg, unpublished results). We therefore can neither confirm nor exclude the requirement for repression of c-*myc* expression in glucocorticoid-induced apoptosis. c-*myc* downregulation in response to glucocorticoids or RA is not T-cell specific, since glucocorticoids reduce c-*myc* mRNA levels in C127 mouse fibroblasts (O'Benion *et al.*, 1992) and RA reduces c-*myc* mRNA-levels in HL-60 promyelocytic cells (Kizaki *et al.*, 1993) without inducing apoptosis. On the other hand, expression of c-*myc* in the absence of a mitogenic signal was shown to induce the apoptosis of rat-1 fibroblasts (Evan *et al.*, 1992).

While the 'survival genes' whose expression is inhibited by the activated GR remain to be identified, our work strongly suggests that interference with the expression of such genes is sufficient for induction of apoptosis. We suggest that the function of such 'survival genes' is cell cycle progression. Glucocorticoids are known to lead to G_1 cell cycle arrest in human leukemic T-cells (Harmon *et al.*, 1979) and many other cell types (Goya *et al.*, 1993; Sanchez *et al.*, 1993). AP-1 has been reported to be required for cell cycle progression (Kovary and Bravo, 1991), and c-*myc* expression has been shown to be necessary for S to G_2/M transition in the lymphoid BAF-B03 cell line (Shibuya *et al.*, 1992). Likely, the derangement of these parameters caused by glucocorticoid action cannot be made up for by constitutive overexpression of a single proto-oncogene. Yet, it is possible that by interfering with normal cell cycle progression, glucocorticoids or any other agent having the same effect, such as RA, may trigger a default pathway resulting in activation of the constitutively expressed, but inactive, nuclease that causes the internucleosomal cleavage of chromatin. Other

processes involved in programmed cell death are likely to be activated through the same pathway.

Materials and methods

Cell culture

Normal Jurkat cells and TAg Jurkat cells (Clipstone and Crabtree, 1992) were grown in RPMI 1640 containing 10% fetal calf serum, 10 mM HEPES and 50 μM β-mercaptoethanol.

Plasmids

The expression vector for stable transfection, pRc/βact, was constructed by exchanging the *Nru*I−*Hin*dIII fragment containing the CMV promoter of pRc/CMV (InVitrogen, La Jolla, CA) with the 1.3 kb *Xho*I−*Hin*dIII fragment of pAGS (Miyazaka *et al.*, 1989). The *Xba*I fragments containing the full length coding sequences of either GR-wt or GR-LS7 were subcloned from p6R (Godowski *et al.*, 1989) into pRc/CMV, followed by ligating the large *Pvu*I−*Not*I fragment of the resultant plasmids, containing the cDNA, to the small *Pvu*I−*Not*I fragment of pRc/βact, containing the β actin promoter, to generate the expression vectors pRc/βact-GR-wt and pRc/βact-GR-LS7. pRc/βact-GR-NDel was derived from pRC/βact-GR-wt by deletion of the 1050 bp *Hin*dIII fragment containing the 324 N-terminal codons. This eliminates the N-terminal activation domain located at codons 77–262 (Hollenberg and Evans, 1988). Translation of this mutant probably starts at Met336. pRc/βact-RARα was constructed by subcloning the cDNA encoding human RARa (Glass *et al.*, 1989) into the *Hin*dIII and *Not*I sites of pRc/βact. The reporter plasmids used were a MMTV-LUC reporter containing two consensus GREs (Drouin *et al.*, 1993) and the AP-1-dependent −73Col-LUC (Deng and Karin, 1993) derived from the human collagenase promoter (Angel *et al.*, 1987).

Isolation of stable transfectants

10^7 Jurkat cells in 0.5 ml of fresh medium were electroporated at 960 μFD, 280 V with 20 μg of *Pvu*I-linearized pRc/βact-GR-wt, pRc/βact-GR-LS7, pRc/βact-NDel or pRc/βact-RARα, respectively. Subsequently, the cells were aliquotted into 96-well plates and, beginning with the next day, kept at 1.5 mg/ml G418 (60% active compound). G418-resistant clones developed that were replicated and examined by thymidine incorporation assays in the presence and absence of 10^{-6} M dex or RA. Some of the clones demonstrating glucocorticoid-dependent inhibition of thymidine incorporation were recloned by limiting dilution. Northern analysis of the resulting clones revealed high expression levels of the transfected GR or RARα that were not influenced by cell treatment with TPA/ionomycin or dex (not shown). No endogenous GR or RARα message was detected.

Transient transfections

Logarithmically growing cells were centrifuged, resuspended in fresh medium at 10^7/0.5 ml, electroporated at 960 μF, 320 V with 10 μg of reporter plasmid and, in the cases of TAg Jurkat cells, with 5 μg of expression vector. Electroporated cells were then diluted to 10 ml with fresh medium. Luciferase activity was normalized to the protein concentration of extracts.

Thymidine incorporation assays

Approximately 1.25×10^5 cells/ml were seeded in microtiter plates and grown for 42 h in the absence or presence of dex, followed by addition of methyl-[^{3}H]thymidine (Amersham, 5 Ci/mmol) to a final activity of 4 μCi/ml for an additional 8 h. Subsequently, cells were harvested and counted.

DNA fragmentation assay

After having been grown for 24 or 48 h in the presence or absence of the appropriate hormone, ~2×10^6 cells were lysed in 400 μl of DNA isolation buffer (10 mM Tris−HCl pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS and 0.5 mg/ml proteinase K). After overnight incubation at 37°C, DNA was ethanol precipitated, resuspended in 400 μl of TE buffer (10 mM Tris−HCl, pH 8.0, 1 mM EDTA) containing 10 mg/ml RNase A and incubated at 37°C for 2 h. Samples were extracted with phenol/chloroform followed by chloroform, and ethanol precipitated. DNA was resuspended in TE buffer and quantified. Equal amounts of DNA were either directly resolved on a 2% agarose gel and visualized by ethidium bromide or first end-labeled using terminal transferase and [α-^{32}P]ddATP as described (Tilly *et al.*, 1993). In the latter case, the gel was dried under vacuum at room temperature and autoradiographed.

Differential mRNA display

This was done using total cellular RNA exactly as described by Liang and Pardee (1992).

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References

Alles,A.J. and Sulik,K.K. (1990) *Development*, **108**, 73–81.
Angel,P. and Karin,M. (1991) *Biochim. Biophys. Acta*, **1072**, 129–157.
Angel,P. *et al.* (1987) *Cell*, **49**, 729–739.
Arya,S.K., Wong-Staal,F. and Gallo,R.C. (1984) *J. Immunol.*, **133**, 273–276.
Baxter,J.D., Harris,A.W., Tomkins,G.M. and Cohn,M. (1971) *Science*, **171**, 189–191.
Bazar,L.S. and Deeg,H.J. (1992) *Exp. Hematol.*, **20**, 80–86.
Bourgeois,S. and Newby,R.F. (1977) *Cell*, **11**, 423–430.
Cantrell,D.A. and Smith,K.A. (1984) *Science*, **224**, 1312–1316.
Caron-Leslie,L.A., Schwartzman,R.A., Gaido,M.L., Compton,M.M. and Cidlowski,J.A. (1991) *J. Steroid Biochem. Mol. Biol.*, **40**, 661–671.
Clipstone,N.A. and Crabtree,G.R. (1992) *Nature*, **357**, 695–697.
Cohen,J.J. and Duke,R.C. (1984) *J. Immunol.*, **132**, 38–42.
Cohen,J.J. (1989) In Schleimer,R.P., Claman,H.N. and Oronsky,A. (eds), *Anti-inflammatory Steroid action: Basic and Clinical Aspects.* Academic Press, San Diego, CA, pp. 110–131.
Cupps,T.R. and Fauci,A.S. (1982) *Immunol. Rev.*, **65**, 133–155.
Deng,T. and Karin,M. (1993) *Genes Devel.*, **7**, 479–490.
Dieken,E.S. and Miesfeld,R.L. (1992) *Mol. Cell. Biol.*, **12**, 589–597.
Dowd,D.R. and Miesfeld,R.L. (1992) *Mol. Cell. Biol.*, **12**, 3600–3608.
Drouin,J., Sun,Y.L., Chamberland,M., Gauthier,Y., De Lean,A., Nemer,M. and Schmidt,T.J. (1993) *EMBO J.*, **12**, 145–156.
Evan,G.I *et al.* (1992) *Cell*, **69**, 119–128.
Galili,U. (1983) *J. Steroid Biochem.*, **19**, 483–490.
Gasson,J.C. and Bourgeois,S. (1983) *J. Cell Biol.*, **96**, 409–415.
Gillis,S., Ferm,M.M., Ou,W. and Smith,K.A. (1978) *J. Immunol.*, **120**, 2027–2032.
Gillis,S., Crabtree,G.R. and Smith,K.A. (1979) *J. Immunol.*, **123**, 1624–1631.
Glass,C.K. and Rosenfeld,M.G. (1991) In Foulkes,G. and Cohen,P. (eds), *The Hormonal Control of Gene Transcription.* Elsevier, Amsterdam, Vol. 6, pp. 299–327.
Glass,C.K., Lipkin,S.M., Devary,O.V. and Rosenfeld,M.G. (1989) *Cell*, **54**, 313–323.
Godowski,P.J., Sakai,D.D. and Yamamoto,K.R. (1989) In Gralla,J.D. (ed.), *DNA−Protein Interactions in Transcription.* UCLA Symposia on Molecular and Cellular Biology, New Series, Alan R.Liss, New York, Vol. 95, pp. 197–210.
Goya,L., Maiyar,A.C., Ge,Y., Firestone,G.L. (1993) *Mol. Endocrinol.*, **7**, 1121–1132.
Harbour,D.V., Chambon,P. and Thompson,E.B. (1990) *J. Steroid Biochem.*, **35**, 1–9.
Harmon,J.M., Norman,M.R., Fowlkes,B.J. and Thompson,E.B. (1979) *J. Cell. Physiol.*, **98**, 267–278.
Hay,N., Takimoto,M. and Bishop,J.M. (1989) *Genes Dev.*, **3**, 293–303.
Haynes,R.C.,Jr. (1990) In Gilman,A.G., Roll,T.W., Nies,A.S. and Taylor,P. (eds), *Goodman and Gilman's The Pharmacological Basis of Therapeutics.* 8th edn. Pergamon, New York, pp. 1452–1457.
Helmberg,A., Fässler,R., Geley,S., Jöhrer,K., Kroemer,G., Böck,G. and Kofler,R. (1990) *J. Immunol.*, **145**, 4332–4337.
Hollenberg,S.M., and Evans,R.M. (1988) *Cell*, **55**, 899–906.
Jain,J., Valge-Archer,V.E. and Rao,A. (1992) *J. Immunol.*, **148**, 1240–1250.

Jonat,C., Rahmsdorf,H.J., Park,K.K., Cato,A.C.B., Gebel,S., Ponta,H., and Herrlich,P. (1990) *Cell*, **62**, 1189–1204.
Kizaki,M., Ikeda,Y., Tanoseki,R., Nakajima,H., Moridawa,M., Sakashita,A., and Koeffler,H.P. (1993) *Blood*, **82**, 3592–3599.
Kovary,K. and Bravo,R. (1991) *Mol. Cell. Biol.*, **11**, 4466–4472.
Liang,P and Pardee,A.B. (1992) *Science*, **257**, 967–970.
Martin,S.J., Lennon,S.V., Bonham,A.M. and Cotter,T.G. (1990) *J. Immunol.*, **145**, 1859–1867.
Miyazaki,J., Takaki,S., Araki,K., Tashiro,F., Tominaga,A., Takatsu,K. and Yamamura,K. (1989) *Gene*, **79**, 269–277.
Nazareth,L.V., Harbour,D.V. and Thompson,E.B. (1991) *J. Biol. Chem.*, **266**, 12976–12980.
Norman,M.R. and Thompson,E.B. (1977) *Cancer Res.*, **37**, 3785–3791.
Northrop,J.P., Crabtree,G.R. and Mattila,P.S. (1992) *J. Exp. Med.*, **175**, 1235–1245.
O'Banion,M.K., Levenson,R.M., Brinckmann,U.G. and Young,D.A. (1992) *Mol. Endocrinol.*, **6**, 1371–1380.
Owens,G.P., Hahn,W.E. and Cohen,J.J. (1991) *Mol. Cell. Biol.*, **11**, 4177–4188.
Parillo,J.E. and Fauci,A.S. (1979) *Annu. Rev. Pharmacol. Toxicol.*, **19**, 179–209.
Piacentini,M., Fesus,L., Farrace,M.G., Ghibelli,L., Piredda,L. and Melino,G. (1991) *Eur. J. Cell Biol.*, **54**, 246–54.
Pu,H. and Young,A.P. (1989) *Gene*, **81**, 169–175.
Raff,M.C. (1992) *Nature*, **356**, 397–400.
Ragsdale,C.W. and Brockes,J.P. (1991) In Parker,M.G. (ed.), *Nuclear Hormone Receptors*. Academic Press, New York, pp. 235–259.
Sanchez,I., Goya,L., Vallerga,A.K., and Firestone,G.L. (1993) *Cell Growth Differ.*, **4**, 215–225.
Schüle,R., Rangarajan,P., Kliewer,S., Ransone,L.J., Yang,N., Verma,I.M. and Evans,R.M. (1990) *Cell*, **62**, 1217–1226.
Schüle,R., Rangarajan,P., Yang,N., Kliewer,S., Ransone,L.J., Bolado,J Verma,I.M. and Evans,R.M. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 6092–6097.
Serfling,E., Barthelmäs,R., Pfeuffer,I., Schenk,B., Zarius,S., Swoboda,R., Mercurio,F. and Karin,M. (1989) *EMBO J.*, **8**, 465–473.
Shi,Y., Glynn,J.M., Guilbert,L.J., Cotter,T.G., Bissonnette,R.P. and Green,D.R. (1992) *Science*, **257**, 212–214.
Shibuya,H., Yoneyama,M., Ninomiya-Tsuji,J., Matsumoto,K. and Taniguchi,T. (1992) *Cell*, **70**, 57–67.
Sibley,C.H. and Tomkins,G.M. (1974) *Cell*, **2**, 213–220.
Su,B., Jacinto,E., Hibi,M., Kallunki,T., Karin,M. and Ben-Neriah,Y. (1994) *Cell*, **77**, 727–736.
Thulasi,R., Harbour,D.V. and Thompson,E.B. (1993) *J. Biol. Chem.*, **268**, 18306–18312.
Tilly,J.L. and Hsueh,A.J. (1993) *J. Cell. Physiol.*, **154**, 519–526.
Ucker,D.S. (1987) *Nature*, **327**, 62–64.
Ullman,K.S., Northrop,J.P., Admon,A. and Crabtree,G.R. (1993) *Genes Dev.*, **7**, 188–196.
Vacca,A. *et al.* (1990) *J. Biol. Chem.*, **265**, 8075–8080.
Van den Hoff,M.J.B., Geerts,W.J.C., Das,A.T., Moorman,A.F.M. and Lamers,W.H. (1991) *Biochim. Biophys. Acta*, **1090**, 249–251.
Weiland,S., Dobbeling,U. and Rusconi,S. (1991) *EMBO J.*, **10**, 2513–2521.
Weiss,A. and Stobo,J.D. (1984) *J. Exp. Med.*, **160**, 1284–1299.
Wyllie,A.H. (1980) *Nature*, **284**, 555–556.
Yamamoto,K.R. and Alberts,B.M. (1976) *Annu. Rev. Biochem.*, **45**, 722–746.
Yang-Yen,H.F., Chambard,J.C., Sun,Y.L., Smeal,T., Schmidt,T.J., Drouin,J. and Karin,M. (1990) *Cell*, **62**, 1205–1215.
Yang-Yen,H.F., Zhang,X.K., Graupner,G., Tzukerman,M., Sakamoto,B., Karin,M. and Pfahl.M. (1991) *New Biol.*, **3**, 1206–1219.

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