

Negative cross-talk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells

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The tumour suppressor p53 and the glucocorticoid receptor (GR) respond to different types of stress. We found that dexamethasone-activated endogenous and exogenous GR inhibit p53-dependent functions, including transactivation, up- (Bax and p21^{WAF1/CIP1}) and down- (Bcl2) regulation of endogenous genes, cell cycle arrest and apoptosis. GR forms a complex with p53 *in vivo*, resulting in cytoplasmic sequestration of both p53 and GR. In neuroblastoma (NB) cells, cytoplasmic retention and inactivation of wild-type p53 involves GR. p53 and GR form a complex that is dissociated by GR antagonists, resulting in accumulation of p53 in the nucleus, activation of p53-responsive genes, growth arrest and apoptosis. These results suggest that molecules that efficiently disrupt GR–p53 interactions would have a therapeutic potential for the treatment of neuroblastoma and perhaps other diseases in which p53 is sequestered by GR.

Keywords: apoptosis/cell cycle/cellular localization/GR antagonists/neuroblastoma

Introduction

The tumour suppressor p53 is frequently inactivated in human cancers, most commonly by mutation (Hollstein *et al.*, 1991), but also by other mechanisms including overexpression of inhibitory proteins and sequestration in the cytoplasm (Steele *et al.*, 1998). p53 is an ephemeral protein with a half life of 10–15 min that is stabilized and activated by diverse signals (Giaccia and Kastan, 1998). The physiological function of p53 is to mediate cell cycle arrest or apoptosis in response to various stresses, such as DNA damage (Harris, 1996; Ko and Prives, 1996; Levine, 1997). p53 is a transcription factor that regulates many different genes (Elkeles *et al.*, 1999; Yu *et al.*, 1999; Zhao *et al.*, 2000), in particular, the pro-apoptotic *bax* (Miyashita *et al.*, 1994b; Miyashita and Reed, 1995), the cell cycle inhibitor p21^{WAF1/CIP1} (Brugarolas *et al.*, 1995; Waldman *et al.*, 1996) and its auto-regulator *mdm2* (Barak *et al.*, 1993). Mdm2 binds to p53, inhibits its transcriptional activity (Oliner *et al.*, 1993) and increases its degradation (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). p53 represses various promoters lacking p53 binding sites. Repression results from interactions with the basal tran-

scription machinery (Ragimov *et al.*, 1993), the corepressor mSin3A and histone deacetylases (Murphy *et al.*, 1999), or other unidentified factors that negatively regulate the *bcl2* promoter (Miyashita *et al.*, 1994a).

p53 interacts physically with many proteins. These physical interactions modulate the activity of p53 and integrate its functions into other cellular circuits. p53 is negatively regulated by binding to viral factors, and cellular proteins such as Mdm2. p53 and Mdm2 form an auto-regulatory loop that is modulated by c-Abl (Sionov *et al.*, 1999), pRb (Hsieh *et al.*, 1999) and p14^{Arf} (Sherr and Weber, 2000). p53 also interacts with the Werner Syndrome protein (Blander *et al.*, 1999), Ref-1 (Gaiddon *et al.*, 1999), HIF-1 α (An *et al.*, 1998) and JNK (Fuchs *et al.*, 1998). p53 cross-talks with different steroid-hormone receptors. p53 represses and interacts physically with the human thyroid receptor β -1 (Yap *et al.*, 1996), the orphan receptor TR2 (Lin and Chang, 1996) and the oestrogen receptor (Yu *et al.*, 1997b). Oestrogens and anti-oestrogens regulate the levels and activity of p53 and Rb (Hurd *et al.*, 1997; Saji *et al.*, 1999). Cofactors for steroid receptors physically and functionally interact with p53 (Lee *et al.*, 1999). Here, we describe p53 interactions with the glucocorticoid receptor (GR).

The primary physiological function of glucocorticoids (GCs) in humans is to maintain homeostasis in response to internal or environmental changes (Tronche *et al.*, 1998; Kellendonk *et al.*, 1999). GCs are involved in differentiation, nervous system functions and intermediary metabolism. GCs regulate the activity of GR, a transcription factor that is ubiquitously expressed in human tissues. GR is indispensable, since targeted disruption of the gene in mice leads to death within a few hours after birth (Cole *et al.*, 1995). GR has a modular structure, including two transactivation domains, a DNA binding domain (DBD) and a ligand binding domain (LBD). In the absence of GCs, GR remains in the cytoplasm in complex with proteins such as Hsp90. GC binding to the LBD results in GR translocation to the nucleus, where it modulates transcription. GR homodimers interact with palindromic glucocorticoid response elements (GREs) in the regulatory regions of target genes. They activate transcription through interactions with co-activators and the basic transcription machinery (Beato *et al.*, 1995). GR represses transcription of certain genes by direct binding to negative response elements (Drouin *et al.*, 1993) and composite elements (Diamond *et al.*, 1990). GR cross-talk with AP-1 and NF- κ B signalling leads to repression of pro-inflammatory cytokines (Barnes and Adcock, 1993). Although the mechanism of this cross-talk is still elusive, it is a major regulatory function of GR. Loss of the ability of GR to dimerize in mice blocks transactivation of canonical GREs, but does not compromise the viability or the

capacity to trans-repress AP-1-regulated genes (Reichardt *et al.*, 1998).

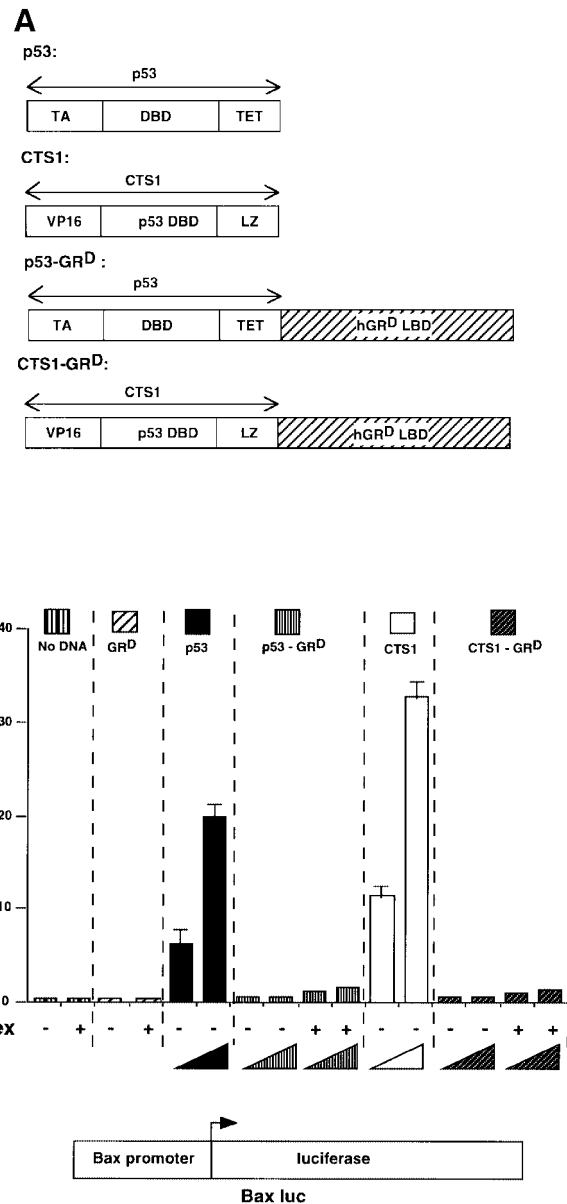
We have previously described tamoxifen-inducible chimeric tumour suppressors (LI-CTS), which regress tumours in a ligand-dependent manner (Sengupta *et al.*, 2000). We found that similar dexamethasone-inducible chimeras did not have the expected properties, most probably due to negative interactions between p53 and GR. p53 and GR interact physically and functionally, and these interactions lead to inactivation of p53 by cytoplasmic sequestration in neuroblastoma cells.

Results

DITS do not transactivate and suppress cell growth in a dexamethasone-dependent manner

We constructed Dexamethasone-inducible Tumour Suppressors (DITS), which contain a mutated LBD from human GR fused to the C-terminal region of p53 or CTS1 (p53-GR^D, CTS1-GR^D; Figure 1A). The mutated LBD (I747T) is inducible by the synthetic GC dexamethasone (Dex), but not by natural GCs (Roux *et al.*, 1996). CTS1 is a highly active chimeric tumour suppressor based on p53 (Conseiller *et al.*, 1998). Transactivation by DITS, p53 and CTS1 was studied by transient transfection in p53 null cell lines, HSC-2 (Figure 1B) and Saos-2 (data not shown). p53 and CTS1 transactivated the p53-responsive reporter Bax-luc, whereas both DITS had very little effect with or without ligand (Figure 1B). As expected, GR also had little effect. Similar results were obtained with another p53-inducible reporter [WAF1-luc (el-Deiry *et al.*, 1993) data not shown]. DITS was efficiently expressed, as shown by western blotting (Figure 1C, GFP is the internal control for transfection efficiency). We found that the activity of DITS was not induced by Dex in a variety of other assays (long-term colony formation and apoptosis) and in different cell lines (HSC-2 and Saos-2; data not shown). These results were surprising since similar molecules with an oestrogen receptor ligand-binding domain (CTS-ER^T) were inducible (Sengupta *et al.*, 2000). They raised the possibility that GR inhibits the activity of p53.

Fig. 1. DITS have attenuated transactivation and growth suppressive functions. (A) Schemes. hGR^D-LBD was fused to the C-terminal end of p53 or CTS1 (Conseiller *et al.*, 1998) to generate p53-GR^D and CTS-GR^D, respectively. CTS1, chimeric tumour suppressor 1; VP16, herpes simplex virus 1 VP16 transcription activation domain; DBD, DNA binding domain; LZ, leucine zipper; LBD, ligand binding domain; TET, tetramerization domain of p53; TA, transactivation domain of p53; hGR, human glucocorticoid receptor. (B) HSC-2 cells were transfected with GR^D, p53, p53-GR^D, CTS1 or CTS1-GR^D expression vectors (250 and 500 ng/ml), the p53-responsive reporter (Bax-luc), and CMV lacZ (500 ng/ml) to measure transfection efficiencies. Activity represents the luciferase values measured 24 h after the end of transfection and corrected for transfection efficiency. The values were similar in three independent transfections. (C) Transfections were carried out as in (B) using 500 ng/ml of the expression plasmids and 500 ng/ml of pEGFP. Cell lysates (50 µg) were analysed by 10% SDS-PAGE and western blotting with (a) a monoclonal antibody that recognizes the DBD of p53 (pAb 240) or (b) anti-GFP. MW = molecular weight markers. Lane 1, mock transfection; lane 2, p53; lane 3, CTS1; lane 4, p53-GR^D; lane 5, p53-GR^D + Dex; lane 6, CTS1-GR^D; lane 7, CTS1-GR^D + Dex.



p53 and GR inhibit each other's transactivation properties

We investigated whether GR affects p53 activity and the converse in HSC-2 cells (Figure 2). Dexamethasone (Dex) and cortisol (Cort) have been shown to activate the transcriptional activity of GR to similar extents (Roux *et al.*, 1996). Vehicle alone (ethanol), Cort (a natural glucocorticoid), Dex (an agonist) and RU-486 (an antagonist) did not change the basal activity of the p53 CON reporter (Figure 2A, lanes 1–4). Increasing amounts of p53 stimulated reporter activity in a dose-dependent manner (Figure 2A, lanes 5–7). p53 was inhibited ~3-fold by endogenous GR activated with either Cort or Dex, but was not affected by RU-486 (Figure 2A, lanes 8–16). With a constant level of transfected p53, exogenous GR decreased p53 activity to a small extent (Figure 2A, lanes 18–20), whereas GR + Dex or GR + Cort efficiently inhibited p53 (Figure 2A, lanes 21–26). In contrast, GR + RU-486 had little effect (Figure 2A, lanes 27–29). Similar results were obtained in Saos-2 and SiHa cells (data not shown). Western blots showed that the amounts of expressed protein increased as increasing amounts of expression vectors for GR and p53 were transfected (Figure 2B, lanes 1–4 and 8–11). Co-transfection of GR did not affect p53 levels (lanes 5–7) showing that the inhibition did not result from decreased expression of p53. In the converse experiments with the GR-responsive reporter GRE-tk-luc, Dex-dependent activation by exogenous GR was also inhibited by p53 (Figure 2C).

We investigated the mechanism of repression of p53 by GR. Antisense GR RNA (ASGR) expressed by a vector was used to down-regulate GR. We verified that ASGR inhibited transactivation of the GRE-tk-luc reporter by exogenous GR in the presence of Dex (Figure 2D, panel a), and that it reduced endogenous GR protein levels, as shown by western blotting (Figure 2D, panel b). We tested whether down-regulation of endogenous GR would stimulate p53 transactivation. Indeed, Dex inhibited p53-dependent transactivation from the p53 CON reporter and ASGR relieved this repression (Figure 2E). These results show that down-regulation of endogenous GR relieves repression of p53 by Dex.

We studied GR + Dex repression of natural p53-responsive promoters in HSC-2 cells. Dex had little effect on bax-, p21- and bcl2-luc in the absence of p53. Dex inhibited p53-mediated activation of bax- and p21-luc, and relieved repression of bcl2-luc (Figure 3A). We also studied expression of the endogenous genes by western blotting. Dex or Cort inhibited p53 induction of Bax and p21^{WAF1/CIP1}, and relieved repression of Bcl2, without affecting expression of p53 (Figure 3B). These results show that activated GR regulates the expression of several p53-responsive genes in a p53-dependent manner.

GR inhibits p53-induced cell cycle arrest and apoptosis

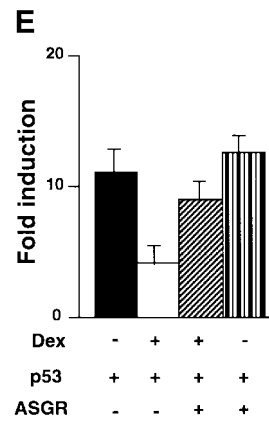
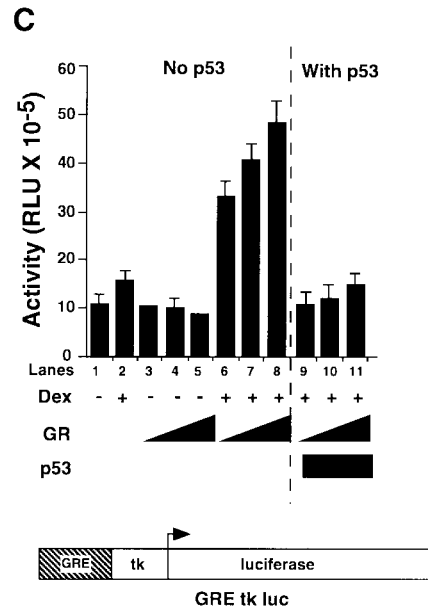
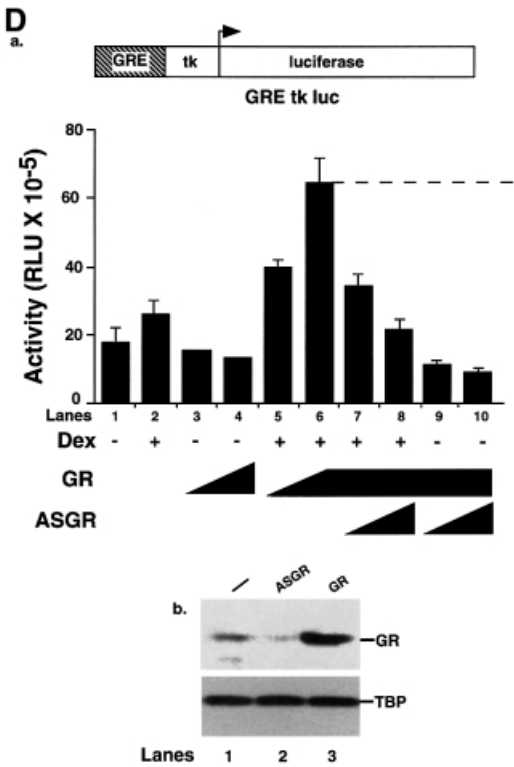
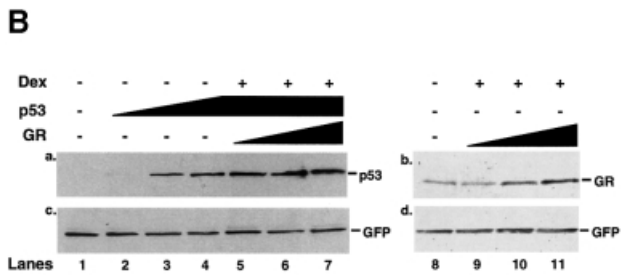
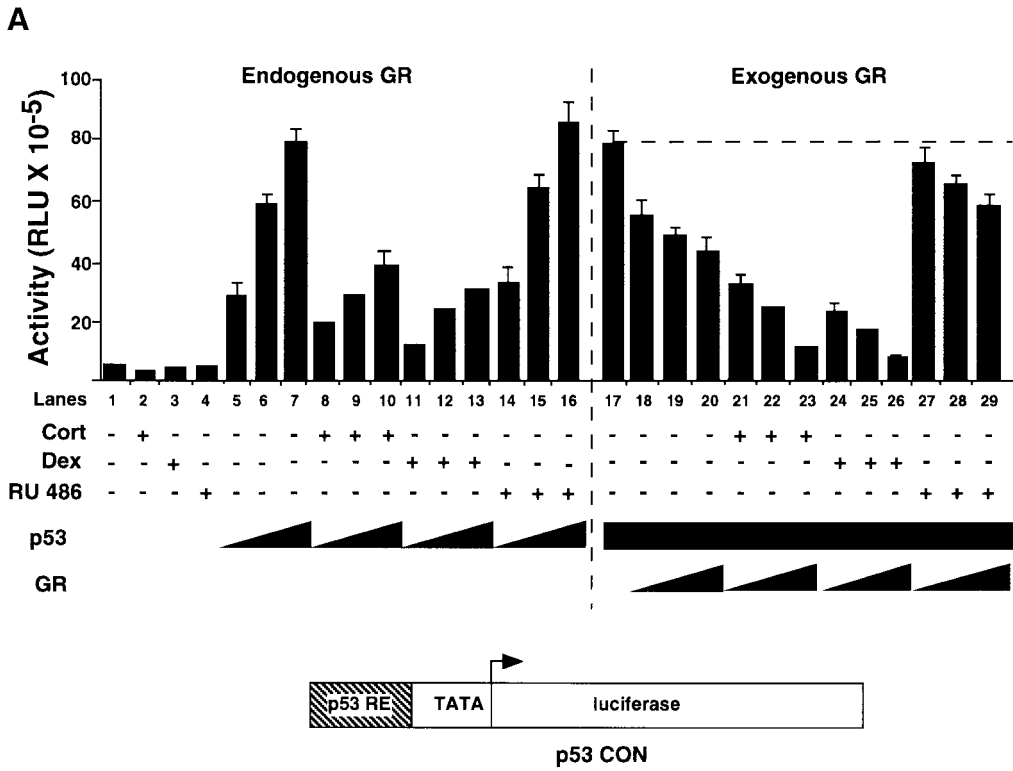
We tested whether GR + Dex reduced p53-mediated cell cycle arrest, apoptosis and inhibition of colony formation. Apoptosis was measured by PARP cleavage (Figure 4A), DNA fragmentation (Figure 4B) and sub-G₁ cells in flow

cytometry (Figure 4C). CD20 co-expression was used to select transfected cells. PARP cleavage, which is an early event in apoptosis, showed that apoptosis induced by p53 expression was partially inhibited by exogenous GR, and strongly inhibited by exogenous GR with Dex (Figure 4A). DNA fragmentation, which is a later event, gave similar results (Figure 4B). p53-induced cell cycle arrest, as measured by the increase in G₀-G₁ cells by flow cytometry, was inhibited partially by GR and totally by GR + Dex (Figure 4C). Activation of endogenous GR by Dex also rescued the cells from p53-mediated apoptosis (Figure 4C). p53-mediated inhibition of colony formation was relieved by Dex (Figure 4D). These results show that endogenous and exogenous GR inhibit p53-mediated functions. The degree of inhibition depends on the amount of GR and is enhanced when GR is activated by ligands.

GR forms a complex with p53 in the cytoplasm

p53 and GR have been reported to interact physically *in vitro* (Yu *et al.*, 1997a). We investigated whether they interact *in vivo*. Immunoprecipitation assays were performed with extracts of p53 null HSC-2 cells co-transfected with expression plasmids encoding p53 and GR, in the presence of Dex. Complexes were immunoprecipitated with p53- or GR-specific antibodies and probed by western blotting (Figure 5A). GR was present in p53 antibody immunoprecipitates, whether or not GR was co-expressed with p53 (Figure 5A, lanes 3 and 4, panel a), showing that both exogenous and endogenous GR bind to p53. There was no GR in the p53 antibody immunoprecipitates when p53 was not expressed (Figure 5A, lane 2, panel a), showing that the immunoprecipitations were specific. As expected, p53 was only detected when it was transfected (Figure 5A, lanes 1–4, panel b). In the converse experiments, p53 was detected in the GR antibody immunoprecipitates only when p53 was expressed (Figure 5A, lanes 6–8, panel c). GR was detected in all the immunoprecipitates (Figure 5A, panel d), even when GR was not co-transfected, as expected from the presence of endogenous GR.

GR is a cytoplasmic protein that translocates to the nucleus upon ligand binding. We investigated how GR-p53 interactions affect their localization by immunocytochemistry (IHC) in HSC-2 (Figure 5B, panel a) and Saos-2 (panel b) cells. Typical cells are shown in Figure 5B, and the quantification in Figure 5C. In HSC-2 cells expressing low amounts of exogenous GR (determined in titration experiments), the receptor is almost exclusively cytoplasmic (96%) and predominantly nuclear in the presence of Dex. p53 is predominantly nuclear (60%) but in many cells it is both cytoplasmic and nuclear (C+N, 38%). When the two proteins are co-transfected in the absence of ligand, GR remains cytoplasmic whereas the number of cells with p53 in both the cytoplasm and nucleus, or the cytoplasm alone, increases slightly. Strikingly, in the presence of Dex, p53 and GR co-localize in the cytoplasm in >90% of the transfected cells. Similar cytoplasmic co-localization was observed in Saos-2 (Figure 5B, panel b) and PAP (data not shown) cells, indicating the generality of the phenomenon. Endogenous GR can also affect the localization of p53. The addition of Dex alone decreased the proportion of cells with exclusively nuclear p53, and increased the proportion with both



cytoplasmic and nuclear or just cytoplasmic p53 (Figure 5C, compare c and f). Down-regulation of endogenous GR with ASGR in the presence of Dex markedly increased the number of cells with exclusively

nuclear p53 (Figure 5C, compare g with f). These results show that the interaction between p53 and GR in the presence of ligand results in efficient mutual sequestration in the cytoplasm.

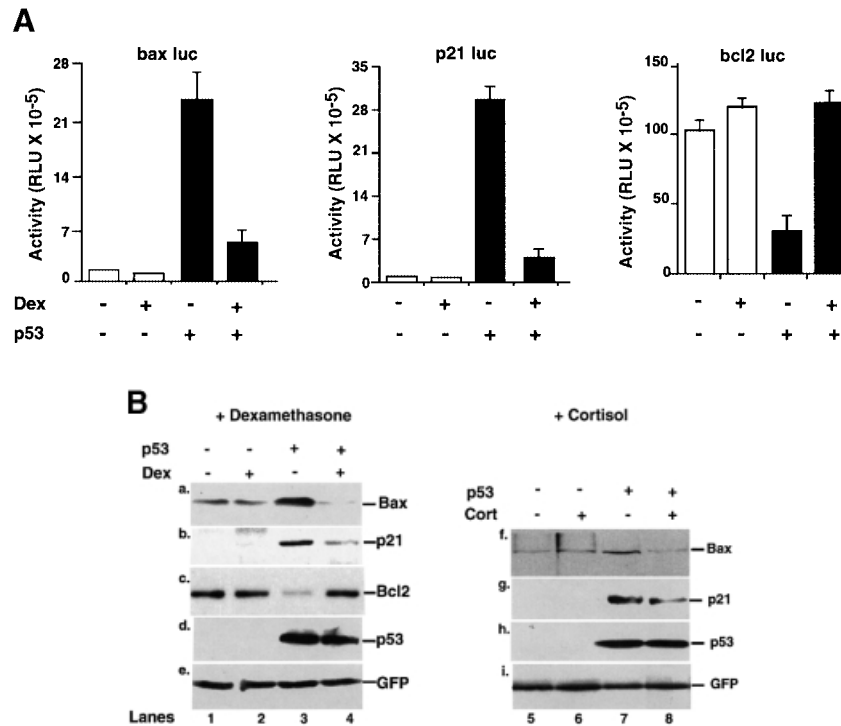


Fig. 3. p53-responsive genes are modulated by Dex. (A) HSC-2 cells were transfected with an expression vector for p53 (filled bars) or the corresponding empty vector (open bars), and reporters containing the natural p53-responsive promoters *bax*, *p21^{WAF1/CIP1}* and *bcl2* linked to luciferase, and CMV *lacZ* to measure transfection efficiency. Luciferase activity was assayed 24 h post-transfection and corrected for transfection efficiency. The values are from two independent experiments. (B) Transfections were carried out as in (A). Additional plates with Cort (10^{-7} M) were included. pEGFP (500 ng/ml) was used to measure transfection efficiency. Cell lysates (50 μ g) were analysed by SDS-PAGE and western blotting with antibodies to: (a and f) Bax (N-20, polyclonal); (b and g) *p21^{WAF1/CIP1}* (1WA-IC581, monoclonal); (c) *bcl2* (100, monoclonal); (d and h) p53 (DO-1, monoclonal); (e and i) GFP (monoclonal). Lanes 1 and 5, mock transfection; lane 2, mock + Dex; lanes 3 and 7, p53; lane 4, p53 + Dex; lane 6, Cort; lane 8, p53 + Cort. -, vehicle alone; +, 10^{-7} M Dex.

Fig. 2. The human glucocorticoid receptor and p53 inhibit each other's transactivation properties. (A) HSC-2 cells were transfected with increasing concentrations of either p53 or GR with a constant amount of p53, a minimal p53 reporter (*p53 CON*) and CMV *lacZ* to measure transfection efficiency. Vehicle alone (ethanol, -), Cort (10^{-7} M), Dex (10^{-7} M) or RU-486 (10^{-7} M) were added as indicated. Lane 1, mock transfection; lane 2, + Cort; lane 3, + Dex; lane 4, + RU-486; lanes 5-7, p53 (125, 250, 500 ng/ml); lanes 8-10, p53 (125, 250, 500 ng/ml) + Cort; lanes 11-13, p53 (125, 250, 500 ng/ml) + Dex; lanes 14-16, p53 (125, 250, 500 ng/ml) + RU-486; lane 17, p53 (500 ng/ml); lanes 18-20, p53 (500 ng/ml) + GR (250, 500, 1250 ng/ml); lanes 21-23, p53 (500 ng/ml) + GR (250, 500, 1250 ng/ml) + Cort; lanes 24-26, p53 (500 ng/ml) + GR (250, 500, 1250 ng/ml) + Dex; lanes 27-29, p53 (500 ng/ml) + GR (250, 500, 1250 ng/ml) + RU-486. Luciferase activity was measured 24 h post-transfection and corrected for transfection efficiency. The values shown are from four independent experiments. p53 RE, p53-responsive element. TATA, minimal promoter element. (B) Transfections were carried out as in (A), except that 500 ng/ml of pEGFP was included to control for transfection efficiency. Cell lysates (50 μ g) were analysed by 10% SDS-PAGE and western blotting with antibodies to: (a) p53 (DO-1), (b) GR (E-20 and P-20) and (c and d) GFP. Lanes 1 and 8, mock transfection with vehicle alone (ethanol); lanes 2-4, p53 (125, 250, 500 ng/ml) with vehicle alone; lanes 5-7, p53 (500 ng/ml) and GR (250, 500 and 1250 ng/ml) + Dex; lanes 9-11, GR (250, 500 and 1250 ng/ml) + Dex. (C) HSC-2 cells were transfected with increasing concentrations of the GR expression vector alone or with a constant amount of the p53 expression vector, the GR reporter (*GRE-tk-luc*) and CMV *lacZ* to measure transfection efficiency. Twenty-four hours post-transfection luciferase activities were measured and corrected for transfection efficiencies. The values were similar in three independent experiments. Lane 1, mock transfection; lane 2, mock + Dex; lanes 3-5, GR (250, 500, 1250 ng/ml); lanes 6-8, (250, 500, 1250 ng/ml) + Dex; lanes 9-11, (250, 500, 1250 ng/ml) + p53 (500 ng/ml). -, vehicle alone; +, 10^{-7} M Dex; GRE, glucocorticoid response element; tk, HSV tk minimal promoter. (D) (a) Transfections with expression vectors for GR or ASGR (500, 1250 ng/ml), alone or in combination, the *GRE-tk-luc* reporter and CMV *lacZ* for transfection efficiency. Luciferase was measured 24 h post-transfection and corrected for transfection efficiency. The values are from two independent experiments. Lane 1, mock transfection; lane 2, mock + Dex; lanes 3 and 4, GR (500, 1250 ng/ml); lanes 5 and 6, GR (500, 1250 ng/ml) + Dex; lanes 7 and 8, GR (1250 ng/ml) + ASGR (500, 1250 ng/ml) + Dex; lanes 9 and 10, GR (1250 ng/ml) + ASGR (500, 1250 ng/ml). (b) Cell lysates (50 μ g) were prepared 48 h post-transfection and analysed by 10% SDS-PAGE and western blotting with antibodies to TBP (3G3) or GR (E-20 and P-20). Lane 1, untransfected; lane 2, ASGR; lane 3, GR. -, vehicle alone; +, 10^{-7} M Dex. (E) Transfections contained expression vectors for p53 (250 ng/ml), ASGR (1250 ng/ml), the p53 CON reporter and CMV *lacZ* to measure transfection efficiency. Luciferase activities were measured after 48 h, corrected for transfection efficiency and expressed as fold induction relative to the control (reporter in the absence of p53). The values represented are from two independent experiments. -, vehicle alone; +, 10^{-7} M Dex.

(<10% of the cells had exclusively nuclear GR). In the presence of RU-486, a dramatic redistribution of both proteins was apparent, with >80% of the cells containing p53 and GR in the nucleus as well as in the cytoplasm. RU-486 can also function as a weak agonist for GR (Baulieu, 1997) and an antagonist for the progesterone receptor (PR) (Meyer *et al.*, 1990). Another GR-specific antagonist, RU-43,044 (RU-044) (Bocquel *et al.*, 1993), also induced nuclear localization of p53 (data not shown), suggesting that nuclear localization was due to GR antagonism rather than other side-effects of a particular antagonist. Nuclear accumulation was detected with different p53 antibodies (DO1, 421 and 1801; data not shown). Two other neuroblastoma cell lines, SK-SY5Y and SK-N-SH, gave a similar redistribution of p53 and GR in the presence of RU-486 and RU-044 (data not shown). These results show that GR antagonists dissociate the GR-p53 complex and induce nuclear accumulation of p53.

p53 is activated by GR antagonists in neuroblastoma cells

We tested whether nuclear accumulation of p53 in neuroblastoma cells treated with GR antagonists increased p53 activity. When IMR 32 cells were plated in the presence of RU-486 there was a reproducible inhibition of cell growth (Figure 7A). Twenty-four hours of treatment with RU-486 and RU-044 induced expression of the p53 downstream genes, p21^{WAF1/CIP1} and bax, as detected by western blotting (Figure 7B, panels b and c). There was no change in the level of p53 (Figure 7B, panel a) suggesting that p53 stabilization is not involved. Flow cytometry showed that both antagonists affect cell cycle progression (Figure 7C). They increased the proportion of cells in the sub-G₀-G₁ (~5%) and G₀-G₁ (~14%) phases, and decreased S phase (~15%), and G₂-M (~4%). These results are consistent with the model that GR antagonists release p53, which translocates to the nucleus and induces downstream effects, including gene expression, cell cycle arrest and apoptosis.

Nuclear localization alone may not be sufficient to fully activate p53. We tested whether the DNA damaging agent mitomycin C enhances the effects of GR antagonists in IMR 32 cells. Mitomycin C alone increased the proportion of cells in the sub-G₀ and G₀-G₁ phases by 4 and 9%, respectively (Figure 7C). GR antagonists together with mitomycin C had a much greater effect, with an increase of ~9% in sub-G₀ and 26% in G₀-G₁ compared with the control. These results show that GR antagonists can considerably increase the effects of DNA damaging agents on neuroblastoma cells.

Discussion

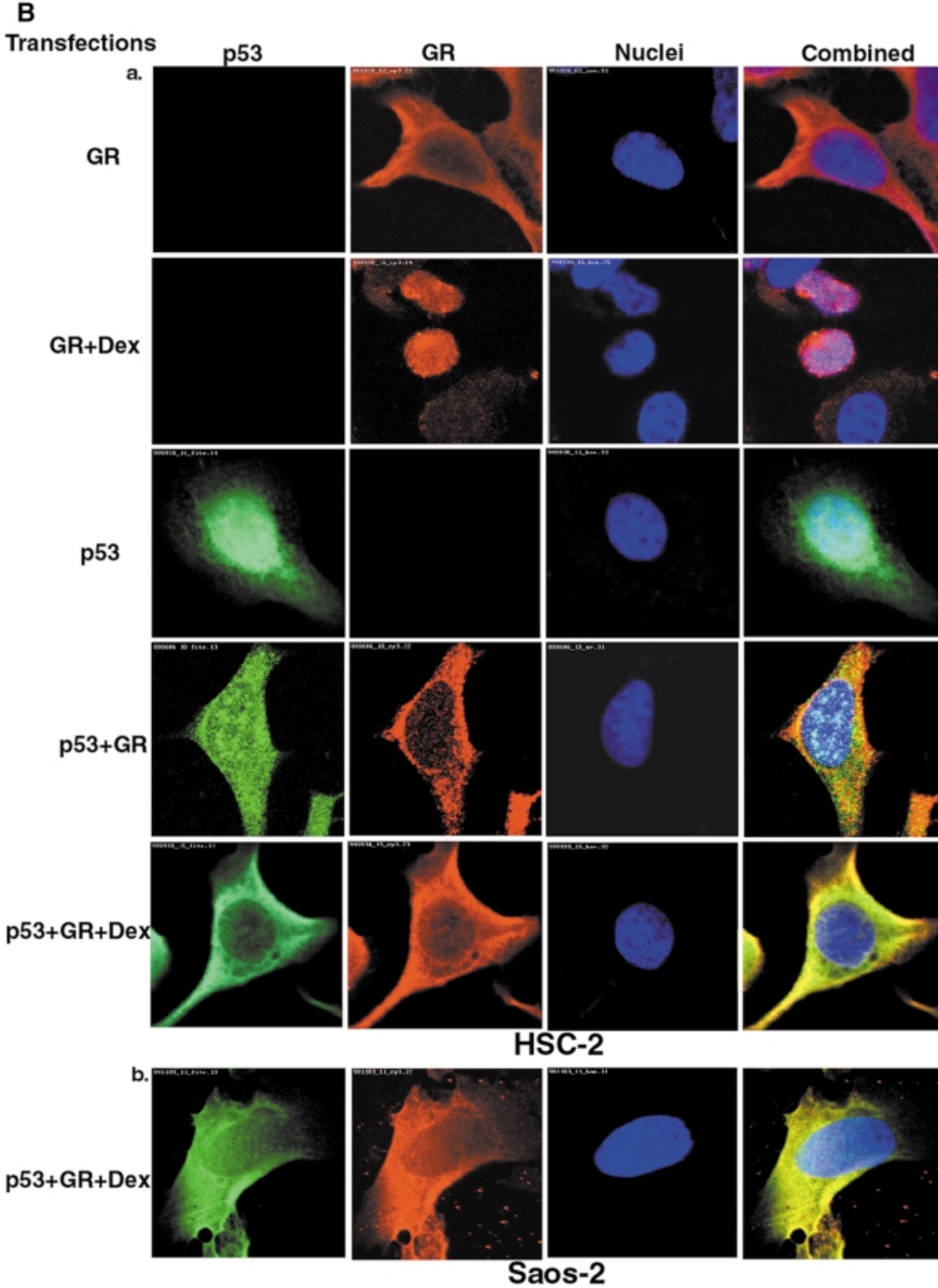
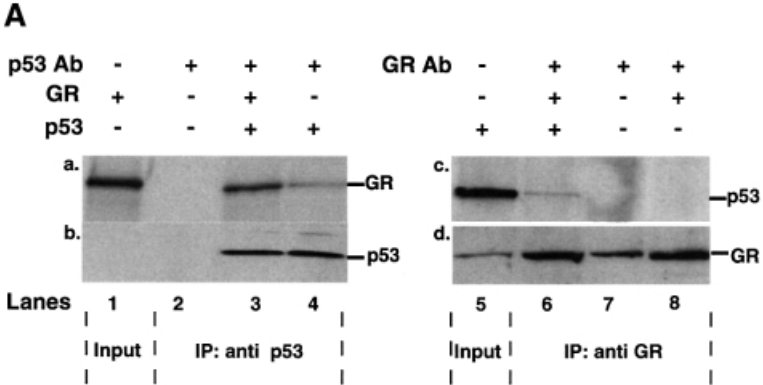
Our attempts to make glucocorticoid-inducible p53 chimeras that resembled the successful tamoxifen-inducible chimeras (Sengupta *et al.*, 2000) apparently failed because of the mutually antagonistic activities of p53 and GR. We found that endogenous and exogenous GR decreased the transcriptional activity of p53 in a dexamethasone-dependent manner. Similar observations have been made in another study (Maiyar *et al.*, 1997). We have extended these observations, by showing that antisense GR RNA blocks endogenous GR + Dex

inhibition of p53. Furthermore, we showed that GR + Dex or Cort antagonize p53's positive and negative effects on the natural downstream targets, p21^{WAF1/CIP1}, bax and bcl2, at the level of both their promoters and expression of the endogenous genes. Finally, we found that GR + Dex antagonize p53-mediated functions, including cell cycle arrest and apoptosis.

GR has been shown to inhibit apoptosis and stimulate cell cycle progression in many contexts, but has the opposite effects in others. Dex induces apoptosis in thymocytes (Cohen *et al.*, 1992), lymphocytes (D'Adamio *et al.*, 1997) and multiple myeloma cells (Chauhan *et al.*, 1997). Dex has been shown to arrest the cell cycle by direct regulation of p21^{WAF1/CIP1} (Cha *et al.*, 1998). In contrast, glucocorticoids repress apoptosis and increase proliferation in cancer cell lines (Chang *et al.*, 1997), neutrophils (Daffern *et al.*, 1999), retinal pigment cells (He *et al.*, 1994), fibroblasts (Li *et al.*, 1998), rat hepatoma (Yamamoto *et al.*, 1998) and serum-depleted T lymphocytes that express Bcl2 (Huang and Cidlowski, 1999). Interestingly, Dex inhibits apoptosis induced by various chemotherapeutic agents more efficiently in cell lines with wild-type as opposed to mutant p53 (Weller *et al.*, 1997). Steroids are known to decrease the efficacy of chemotherapy for some tumours (Green *et al.*, 1983). Our study raises the possibility that GR inhibition of p53 may be involved in these effects.

In vitro studies have shown that GST-p53 interacts with GR (Yu *et al.*, 1997a). We extended these observations by showing that GR and p53 interact *in vivo*. Furthermore, in the presence of Dex, GR and p53 co-localize in the cytoplasm, which could account for their mutually antagonistic activities. This observation raised the possibility that pathological and natural situations in which p53 is cytoplasmic could result from sequestration by GR. This could be the case in various situations. Wild-type p53 is cytoplasmic in inflammatory breast carcinoma (Moll *et al.*, 1992), undifferentiated neuroblastomas (Moll *et al.*, 1995), colon adenocarcinomas (Bosari *et al.*, 1995), differentiated rat oligodendrocytes, neurons and PC12 pheochromocytoma cells (Eizenberg *et al.*, 1996), neuroblastoma cell lines (Moll *et al.*, 1996), Balb/c 3T3 cells in certain stages of the cell cycle (Shaalsky *et al.*, 1990) and normal mouse embryonic stem cells (Aladjem *et al.*, 1998).

In neuroblastoma cell lines we found that GR antagonists dissociate the GR-p53 complex, leading to nuclear accumulation of both p53 and GR, whereas a GR agonist did not have the same effect. The nuclear accumulation of p53 is associated with augmented expression of downstream genes, cell cycle arrest and apoptosis. The antagonist RU-486 has been observed to decrease proliferation of neuroblastoma cells in another report (Maggi *et al.*, 1998), but the mechanism was not studied. Various attempts have been made to understand the mechanism of cytoplasmic accumulation of p53 in NB cell lines. A C-terminal peptide was shown to induce p53 re-localization to the nucleus (Ostermeyer *et al.*, 1996), due to the unmasking of a nuclear export signal (NES) in the tetramerization domain (Stommel *et al.*, 1999). Inhibition of nuclear export by leptomycin B also results in nuclear accumulation of p53 (Stommel *et al.*, 1999). p53 is sequestered in large protein complexes in the cytoplasm



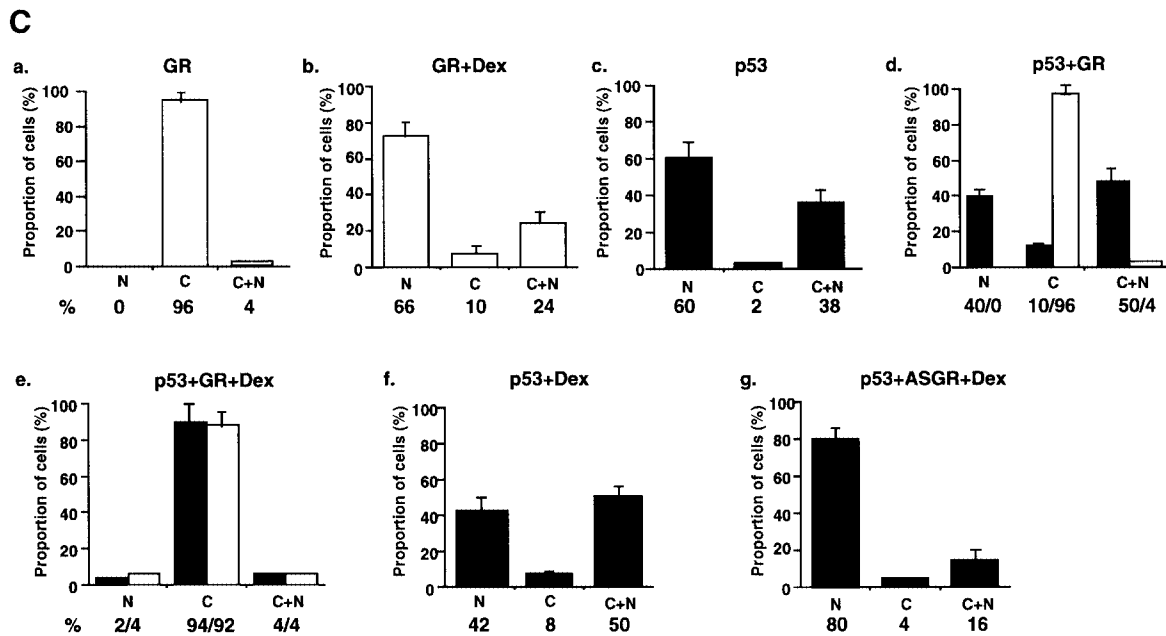


Fig. 5. p53 and GR interact physically and co-localize in the cell. (A) HSC-2 cells were transfected with p53 (500 ng/ml) and/or GR (1250 ng/ml) expression plasmids, as indicated, in the presence of 10^{-7} M Dex. Lysates prepared 24 h post-transfection were immunoprecipitated (IP) with either p53 (DO-1, monoclonal) or GR (E-20 and P-20, polyclonal) antibodies (Ab). After the washes, the immunoprecipitates were analysed by 10% SDS-PAGE and western blotting with antibodies against either GR (p53 IP, panel a) or p53 (GR IP, panel c). The efficiency of immunoprecipitation was checked by western blotting with self antibodies (panels b and d). Lane 1, transfected GR, no IP (20% of p53 input); lane 2, mock transfection, IP with p53 Ab; lane 3, transfected GR and p53, IP with p53 Ab; lane 4, transfected p53, IP with p53 Ab; lane 5, transfected p53, no IP (20% of input for GR IP); lane 6, transfected p53 and GR, IP with GR Ab; lane 7, mock transfection, IP with GR Ab; lane 8, transfected GR, IP with GR Ab. (B) HSC-2 (a) or Saos-2 (b) cells were transfected with p53 (500 ng/ml) and/or GR (1250 ng/ml) expression plasmids in the presence of either vehicle (ethanol) or 10^{-7} M Dex. Twenty-four hours post-transfection the cells were fixed, incubated with antibodies against GR (E-20 and P-20, polyclonal) or p53 (DO-1, monoclonal) and analysed by confocal microscopy. (C) Quantitation of the transfections in (B). At least 500 cells were counted for each transfection. The percentage of cells with the protein in the nucleus (N), the cytoplasm (C) and both (C + N) is shown. (a) GR; (b) GR + Dex; (c) p53; (d) p53 + GR; (e) p53 + GR + Dex; (f) p53 + Dex; (g) p53 + ASGR + Dex. Open bars, GR; filled bars, p53.

(Moll *et al.*, 1996), which was proposed to account for its resistance to Mdm-2-mediated degradation (Zaika *et al.*, 1999). A model taking into account these observations and our results is that p53 is sequestered in the cytoplasm in a complex with GR, which is in equilibrium with molecules free to cycle in and out of the nucleus. In the complex, the nuclear localization signal of either molecule may be masked. The inactive DITS chimeras minimally contain the p53 DNA binding domain and the GR LBD, suggesting that these domains of the two proteins interact. GR antagonists, upon binding to the LBD, presumably alter the conformation and thereby weaken the interaction with p53. Release of p53 would disturb the equilibrium and thereby increase the pool of p53 that can move into the nucleus. Increasing the rate of entry or decreasing the rate of exit from the nucleus of molecules free to cycle would also lead to nuclear accumulation.

The mechanisms that regulate ligand-independent formation of GR-p53 complexes and control the amount of p53 that is in the cytoplasm remain to be established. An interesting possibility is that the extent of p53-GR interaction in the absence of ligand is set at different levels, which are low in most cell types and high in neuroblastoma. In several cell lines other than neuroblastoma we observed that GR inhibits p53 functions to a low extent in the absence of ligand, suggesting that there could be a low level of ligand-independent interaction. The amount of endogenous glucocorticoids in the serum most likely is not enough to activate the receptor.

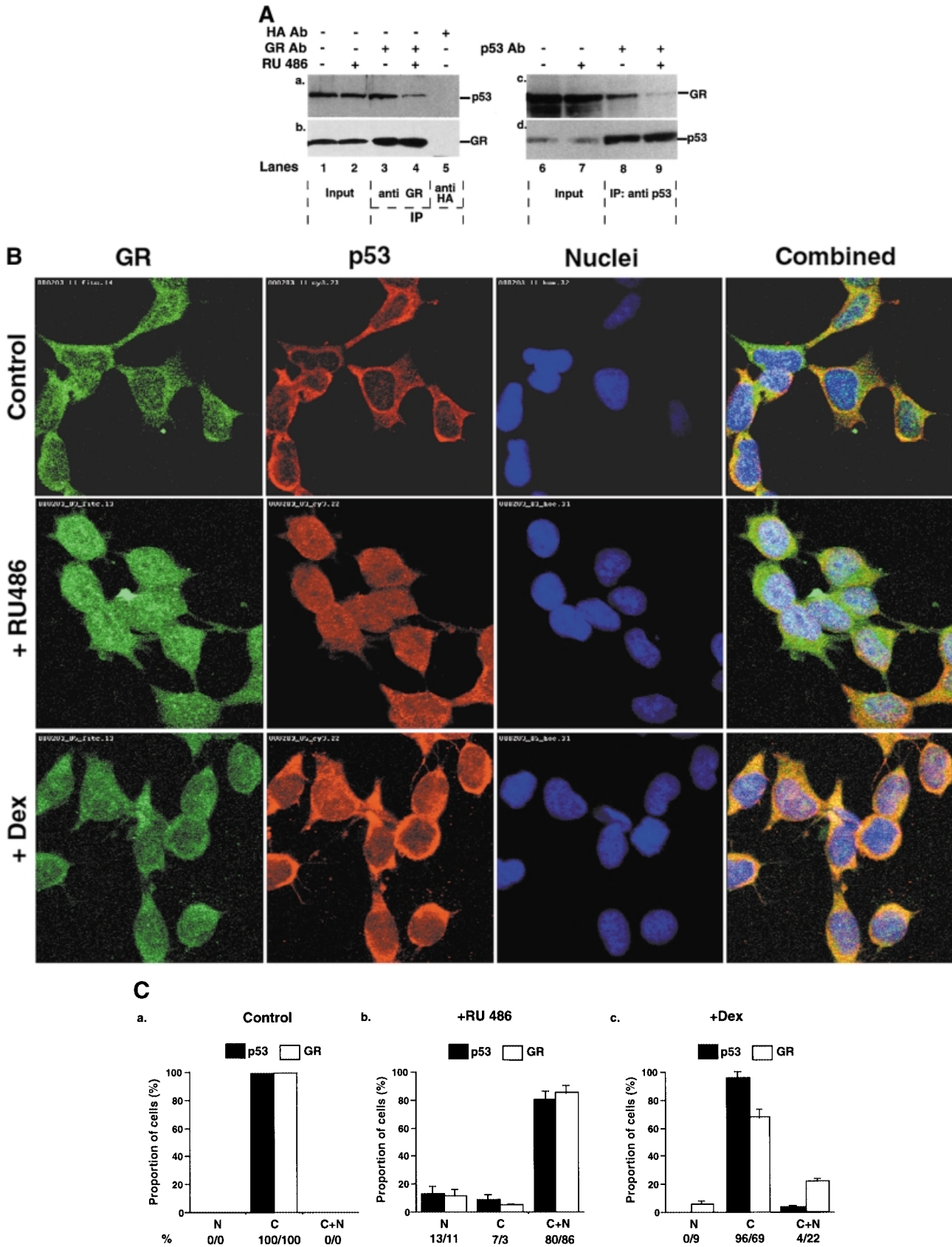
Furthermore, in preliminary experiments, we found that GR inhibits p53 in stripped medium (data not shown). The mechanisms that regulate ligand-independent interactions could involve post-translational modifications and interactions with other proteins. Interestingly, a protein kinase inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), has been demonstrated to induce apoptosis in NB cell lines in a p53-dependent manner (Ronca *et al.*, 1997), raising the possibility that phosphorylation may regulate the GR-p53 interaction. It would also be interesting to determine whether down-regulation of Hsp90, which is known to bind to and inactivate GR, affects p53 function.

The normal physiological function of cross-talk between GR and p53 is an interesting field for further study. Glucocorticoids and p53 have antagonistic effects under various conditions that could be imagined to require cross-talk. One of these is that glucocorticoids increase glucose metabolism in response to psychological stress. Hypoxic stress leads to p53 activation, increased apoptosis and decreased glucose metabolism (Riva *et al.*, 1998). Perhaps the choice between adaptation (increased glucose metabolism) and death in response to hypoxic stress involves cross-talk between GR and p53. Interestingly, the type II hexokinase gene, whose overexpression is associated with increased glucose catabolism in cancer cells, is apparently regulated in opposing manners by p53 and GR (Mathupala *et al.*, 1997).

In summary, we have demonstrated that p53 and GR repress each other's functions. Mutual repression

apparently results from their physical interaction *in vivo* and co-localization in the cytoplasm. However, we cannot exclude that other mechanisms also contribute to p53

inactivation by GR (effects on DNA binding or others). Strikingly, p53 is able to prevent ligand-mediated transport of GR to the nucleus. In a pathological situation,



represented by neuroblastoma cells, endogenous GR and p53 also interact physically. Disruption of this complex results in translocation of wild-type p53 from the cytoplasm into the nucleus, activation of p53-dependent endogenous genes, growth arrest and apoptosis. Thus, this

report provides the first evidence for the inactivation of p53 by a steroid hormone-dependent signalling pathway in a human pathological condition.

Materials and methods

Recombinants

p53-GR^D. PCR amplification of the coding region of p53 was used to remove the stop codon and introduce *EcoRI* and *XhoI* sites at 5' and 3' ends, respectively. p53-GR^D was generated by co-ligating the 1.2 kb *EcoRI-XhoI* digested PCR product, the 5.4 kb *EcoRI-XhoI* fragment from pcDNA3 and the 1 kb *XhoI-SalI* fragment from pCre-GR^D containing GR^D-LBD.

CTS1-GR^D. Initially, modified CTS1 without the stop codon was produced by ligating the 6.4 kb *XhoI-PflMI* fragment from pcDNA3CTS1 (Conseiller *et al.*, 1998) with the *PflMI-XhoI* 100 bp fragment from a PCR product that was amplified with a primer before the *PflMI* site (within CTS1) and a reverse primer excluding the stop codon but incorporating a *XhoI* site. The 6.5 kb *XhoI*-digested modified-CTS1 plasmid was ligated to the 1 kb *XhoI-SalI* fragment from pCre-GR^D containing the GR^D-LBD, to generate CTS1-GR^D.

Antisense GR (ASGR). The 2.7 kb *EcoRI* fragment from HG1 (Kumar *et al.*, 1987) was ligated to *EcoRI*-linearized pcDNA3 to generate pcDNA3ASGR or pcDNA3GR.

Reporters and expression vectors. p53CON, a gift from J.Shay (Funk *et al.*, 1992); p21^{WAF1/CIP1}-luc, a gift from B.Vogelstein (el-Deiry *et al.*, 1993); bax-luc, a gift from J.Reed (Miyashita and Reed, 1995); bcl2-luc, a gift from L.Boxer (Budhram-Mahadeo *et al.*, 1999); GRE-tk-luc, a gift from H.Gronemeyer (Roux *et al.*, 1996); CTS1, a gift from E.Conseiller (Conseiller *et al.*, 1998); pC53-SN3, a gift from A.J.Levine (Hinds *et al.*, 1990); pCre-GR^D, a gift from D.Metzger (Brocard *et al.*, 1998); HG1, a gift from P.Chambon (Kumar *et al.*, 1987); CMV-CD20, a gift from L.Zhu (Zhu *et al.*, 1993); CMV-LacZ, IGBMC core facility.

Cells

HSC-2 (head and neck carcinoma), a gift from N.Tsuchida (Momose *et al.*, 1989); PAP (head and neck carcinoma), IGBMC cell culture facility; Saos-2 (osteosarcoma), SiHa (cervical carcinoma), IMR-32

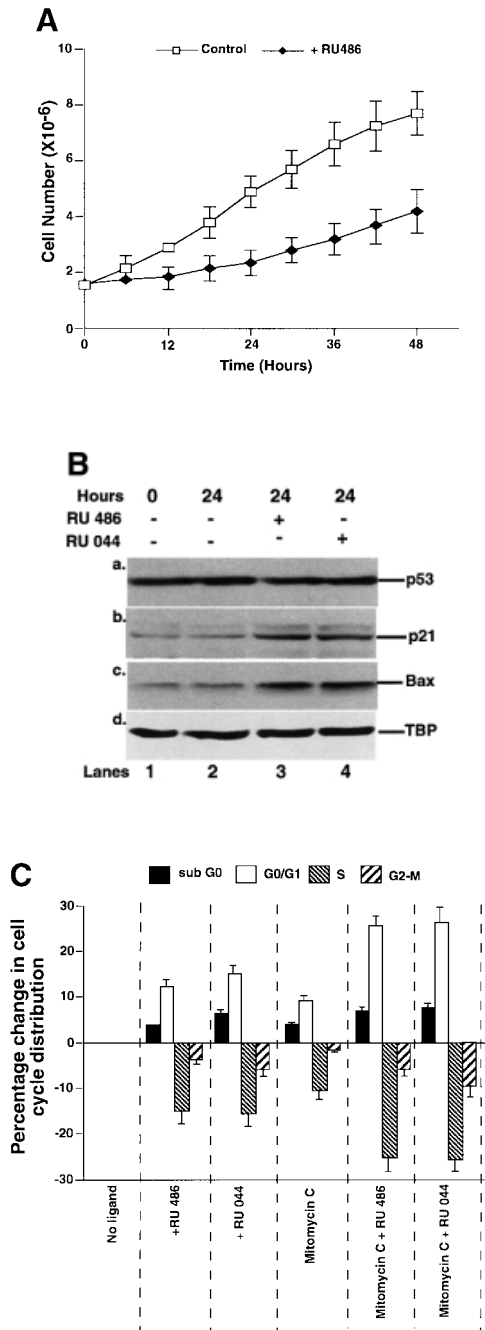


Fig. 6. The GR antagonist RU-486 disrupts the GR-p53 complex and increases p53 localization in the nucleus in neuroblastoma cells. (A) IMR 32 cells were grown for 24 h in the presence of vehicle (ethanol) or 10⁻⁷ M RU-486. Immunoprecipitates (IP) formed with Ab against p53 (DO-1, monoclonal), HA (12CA5, monoclonal) or GR (E-20 and P-20, polyclonal) were washed, subjected to 10% SDS-PAGE and western blotted with Abs against p53 (GR and HA IP, panel a) or GR (p53 IP, panel c). The efficiency of IP was checked by western blotting with self antibodies (panels b and d). Lane 1, IMR 32 cells with vehicle (-, 5% of the lysate used for GR/HA IP); lane 2, IMR 32 cells with RU-486 (+, 5% of input for GR/HA IP); lane 3, vehicle (-), IP with GR Ab; lane 4, RU-486 (+), IP with GR Ab; lane 5, vehicle (-), IP with HA Ab; lane 6, vehicle (-, 20% of input for p53 IP); lane 7, RU-486 (+, 20% of input for p53 IP); lane 8, vehicle (-), IP with p53 Ab; lane 9, RU-486 (+), IP with p53 Ab. (B) IMR 32 cells were cultured with vehicle (control), RU-486 or Dex, and after 24 h they were fixed, incubated with antibodies against GR (E-20 and p20, polyclonal) or p53 (DO-1, monoclonal) and examined by confocal microscopy. (C) Quantification of (B). For each group ~1000 cells were counted. (a) control; (b) RU-486; (c) Dex. N, nuclear; C, cytoplasmic; C + N, both cytoplasmic and nuclear.

Fig. 7. Effect of GR antagonists on the growth, expression of p53-responsive genes and the cell cycle of IMR 32 neuroblastoma cells. (A) Equal numbers (1.7 × 10⁶) of IMR 32 cells were plated in the presence of vehicle (open symbols) or 10⁻⁷ M RU-486 (closed symbols). At different times the number of viable cells was counted. The values are from three independent experiments. (B) IMR 32 cells were cultured with vehicle, 10⁻⁷ M RU-486 or 10⁻⁷ M RU-044 for 24 h. Lysates (100 µg) were analysed by SDS-PAGE and western blotting with antibodies to (a) p53 (DO-1, monoclonal), (b) p21^{WAF1/CIP1} (1WA-IC581, monoclonal), (c) Bax, (N-20, polyclonal), (d) TBP (3G3, monoclonal). Lane 1, cells before ligand addition; lane 2, vehicle (-); lane 3, RU-486 (+); lane 4, RU-044 (+). (C) IMR 32 cells were cultured as in (B) and additionally with mitomycin C (1 µM) as indicated. After 24 h of treatment, the cells were harvested and analysed by flow cytometry. Experimental points were in duplicate and the whole experiment was repeated. The values (with standard deviation) are from four experimental points. The percentage of cells in each stage of the cell cycle was subtracted from the control (2% sub-G₀, 39% G₀-G₁, 48% S, 11% G₂-M). The control, with zero change, is not visible.

(neuroblastoma), SK-SY5Y (neuroblastoma) and SK-N-SH (neuroblastoma), ATCC.

Transfections

Cell lines were maintained in Dulbecco's modified Eagle's medium + 10% serum + antibiotics at 37°C with 5% CO₂. They were transfected using calcium phosphate (Chen and Okayama, 1987) with 4 µg of plasmid, in 36 mm plates (6-well cluster, Costar 3516) in the presence of either the vehicle (ethanol) or the ligands (Dex or RU-486, Sigma; RU-044, a gift from H.Gronemeyer). After 16 h the cells were washed with medium and antibiotics and re-fed with full growth medium ± ligands. Post-transfection is defined as after removal of the precipitate.

Colony formation assays

Forty-eight hours post-transfection the cells were washed and cultured in 1 mg/ml G418 for 3 weeks, with medium changes every 5–6 days. Three independent transfections were performed for each experimental point and the whole experiment was repeated at least twice. Clones were stained with Giemsa and counted.

Transcriptional activity and DNA fragmentation

Cell lysates were analysed for luciferase and β-galactosidase activities, and DNA fragmentation, as described previously (Sengupta *et al.*, 2000).

Immunoprecipitations

Standard procedures were used. The cells were lysed on ice in NP-40 buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl pH 8.0) and the cleared supernatant used for immunoprecipitation. The antigen-antibody complex, formed on ice for 1 h, was immobilized on protein G beads, washed three times, boiled in Laemmli buffer and analysed by SDS-PAGE.

Western blots

The protein content of RIPA buffer lysates was estimated by the Bio-Rad assay. Equal quantities were fractionated by SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell) and processed for western blotting. Primary and secondary antibodies: Bax, N20 (Santa Cruz Biotechnology); p21^{WAF1/CIP1}, 1WA-IC581 (L.Andera and the IGBMC core facility); TBP, 3G3 (Brou *et al.*, 1993); p53, DO-1 (Vojtesek *et al.*, 1992); p53, pAb 240 (Gannon *et al.*, 1990); GFP (Clontech); GR, E-20 and P-20 (Santa Cruz Biotechnology); Bcl2, 100 (Santa Cruz Biotechnology); PARP (Kaufmann *et al.*, 1993); HA, 12CA5 (Boehringer Mannheim); anti-CD20 (Pharmingen); peroxidase-coupled anti-rabbit or mouse antibody (Jackson Laboratories). The blots were revealed by chemiluminescence (Pierce Super Signal).

Flow cytometry

HSC-2 cells were co-transfected with the appropriate vectors and CMV-CD20 in 10 cm dishes (20 µg total plasmid). Twenty-four hours post-transfection the cells were processed as described previously (Wasylyk *et al.*, 1999). About 15 000 CD-20 positive cells for HSC-2 and 50 000 cells for IMR 32 were analysed.

Immunofluorescence

The cells were fixed with acetone:methanol (1:1), blocked with 10% normal goat serum, incubated with primary antibodies (mouse for p53 and rabbit for GR) for 2 h, followed by appropriate secondary antibodies linked either to Cy3 or fluorescein isothiocyanate (Möll *et al.*, 1996), and visualized by confocal microscopy (Leica TCS 4D). Hoechst dye was used to stain nuclei.

Acknowledgements

We would like to thank H.Gronemeyer and P.Anglard for critical reading of the manuscript, P.Chambon, J.Shay, B.Vogelstein, J.Reed, L.Boxer, H.Gronemeyer, E.Conseiller, A.J.Levine, D.Metzger and L.Zhu for the gifts of recombinants, N.Tsuchida for HSC-2 cells, L.Andera and G.de Murcia for the gift of antibodies, the IGBMC core facilities for help and support, CEFIPRA for their financial and scientific contribution to the early stages of this work, BioAvenir (Rhône-Poulenc, now Aventis), the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Hôpital Universitaire de Strasbourg, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Ligue Nationale Française

contre le Cancer, the Ligue Régionale (Haut-Rhin) contre le Cancer and the Ligue Régionale (Bas-Rhin) contre le Cancer for financial help.

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Received June 27, 2000; revised August 8, 2000;
accepted August 10, 2000