Distinct Domains of the RelA NF-κB Subunit Are Required for Negative Cross-talk and Direct Interaction with the Glucocorticoid Receptor*

(Received for publication, April 4, 1997, and in revised form, June 10, 1997)

Sacha Wissink‡, Erika C. van Heerde‡, M. Lienhard Schmitz§&¶, Eric Kalkhoven‡**, and Paul T. van der Saag‡ ‡‡

From the ‡Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, The Netherlands and the ¶Institute of Biochemistry and Molecular Biology, Albert-Ludwigs-Universitat, Freiburg, Germany

The RelA subunit of NF-κB and the glucocorticoid receptor mutually repress each others transcriptional activity, thus providing a mechanism for immunosuppression. Deletion analysis of the glucocorticoid receptor has shown that the DNA binding domain and the ligand binding domain are essential components for repression. Here, we show by deletions and point mutations that both the Rel homology domain and the transactivation domains of RelA are required for repression of the transcriptional activity of the glucocorticoid receptor in intact cells. However, only the Rel homology domain of RelA was found to associate with the glucocorticoid receptor in vitro. RelA mutants, not able to repress glucocorticoid receptor activity, but still able to dimerize, behaved as transdominant inhibitors of the repressive activity of wild type RelA. Furthermore, we show that the 135 E1A protein is able to interfere with the transrepressive activity of RelA. We propose that negative cross-talk between the glucocorticoid receptor and RelA is due to direct interaction via the Rel homology domain of RelA and the DNA binding domain of the glucocorticoid receptor in combination with interference by the transactivation domains of RelA with the transcriptional activity of the glucocorticoid receptor.

The NF-κB/Rel family of transcription factors regulates the expression of a variety of genes involved in immune and inflammatory responses. Presently, five members of the NF-κB/Rel family have been identified in mammals including NF-κB1, NF-κB2, RelA, c-Rel, and RelB. These proteins share homology in their 300-amino acid N-terminal regions. This region of sequence similarity, which is designated the Rel homology domain (RHD), functions in DNA binding, dimerization, and interaction with IκB (1, 2). NF-κB was originally identified as a heterodimer of NF-κB1 and RelA (3, 4), but a variety of other homo- and heterodimers have been described. NF-κB is present in an inactive form in the cytoplasm, associated to an inhibitor protein, IκB. Upon exposure of the cells to inflammatory cytokines, like tumor necrosis factor-α and interleukin-1, or lipopolysaccharide, UV radiation, or viral infection, IκB becomes phosphorylated, ubiquitinated, and subsequently degraded (2). As a result, NF-κB is translocated to the nucleus, where it binds to specific DNA sequences and activates transcription.

Transactivation functions have been located in the C-terminal regions of RelA (5), c-Rel (6, 7), and in both the C-terminal and N-terminal region of RelB (8, 9). RelA contains at least two strong transactivation domains (TADs) within its C terminus; activation domain TA1, consisting of the 30 C-terminal amino acids, and TA2, located within the 90 amino acids next to TA1 (5, 10). At the N-terminal part of TA2, a mini-leucine zipper motif is present composed of three leucines arranged in a heptad repeat (5). Both TADs contain a common sequence motif (11).

Cross-talk between transcription factors of distinct families is an important phenomenon in regulating gene transcription and has recently become the subject of intensive investigation. NF-κB, and particularly RelA, has been shown to interact functionally and physically with numerous other transcription factors, including members of the AP-1 family, resulting in enhanced biological activity of these transcription factors (12). Previously, we and others have reported that steroid receptors, including the glucocorticoid receptor (GR) (13–15), the estrogen receptor (16), the progesterone receptor (17), and the androgen receptor (18), are able to inhibit NF-κB activity and can physically interact with NF-κB proteins in vitro. Since RelA represses ligand-dependent activation of steroid receptor-regulated promoters, a mutually inactive complex formed either by a direct protein-protein interaction of the receptor and RelA or via a third partner has been proposed (13–18).

Steroid receptors belong to the superfamily of steroid/thyroid hormone receptors, and their modular structure, consisting of a DNA binding domain (DBD) and a ligand binding domain (LBD), is highly conserved. Transactivation domains have been located N-terminally to the DBD, designated AF-1, and within the LBD, named AF-2. Whereas AF-1 is a hormone-independent activation domain, AF-2 functions hormone dependently (19). Recently, several cofactors interacting with AF-2 have been described to inhibit (corepressors) or enhance (coactivators) transcription by nuclear receptors (20).

The domains involved in interaction between steroid receptors and NF-κB have not been mapped in much detail. For steroid receptors, analysis of deletion mutants has revealed that both the DBD and the LBD are necessary for repression of NF-κB activity (13, 16, 17). So far, the domain(s) of NF-κB
involved in inhibition of steroid receptor activity have not been determined. Therefore, we investigated the importance of different regions in RelA in repressing GR activity. Our results show that both the RHD and the TADs of RelA are required for repression of hormone-dependent activation of GR, while only the RHD and not the TADs were found to directly interact with GR in vitro. Furthermore, cotransfection of the co-factor 13 S E1A resulted in a decrease in repressive activity of RelA in COS-1 cells. These data suggest that the mutual repression between GR and RelA is due to complex formation via the RHD of RelA, while the TADs of RelA, able to interact with cofactors, are required to repress the transcriptional activity of GR.

EXPERIMENTAL PROCEDURES

**Special Reagents and Antibodies**—Dexamethasone was obtained from Sigma. Polyclonal antibodies against the N-terminal domain of RelA (SC-109) and against the C-terminus of RelA (SC-372) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody against Lebα was from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Cell Culture and Transient Transfections**—Monkey COS-1 cells and human 293 embryonal kidney cells were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (Life Technologies, Inc.), buffered with bicarbonate and supplemented with 7.5% fetal calf serum from Integro (Linz, Austria). For transient transfections, the cells were cultured in six-well tissue culture plates. Cells were transfected using calcium-phosphate coprecipitation with 2 μg of luciferase reporter, 3 μg of pDMHαZ, and the indicated amount of expression plasmids. pBlueScript SK+ plasmid was added to obtain a total amount of 10 μg of DNA/well. After 16 h, the medium was refreshed and hormone was added. Cells were harvested 24 h later and assayed for luciferase activity using the luciferase reporter gene assay kit (Packard). Values were corrected for transfection efficiency by measuring β-galactosidase activity (21).

**Plasmids**—Details about the construction of the clones presented in this report can be obtained from the authors upon request. The luciferase reporter plasmid containing three NF-κB sites from the ICAM-1 promoter and the reporter plasmid 2 × GREtk luc were described elsewhere (22, 23). The CMV4 expression vectors containing human RelA and GR, the 12 S E1A, 13 S E1A, and RKKo expression plasmids, and glutathione S-transferase (GST)-NF-κB and -RARR have been described previously (13, 17, 24). pCAB and p300 were kind gifts from Drs. R. H. Goodman (Portland, OR) and R. Ecker (Boston, MA).

**In Vitro Protein Binding Assay**—Proteins were synthesized in vitro using the TnT-coupled rabbit reticulocyte lysate system (Promega) in the presence of [35S]methionine according to the manufacturer’s description. GST fusion proteins were expressed in *Escherichia coli* (BL21 [pLysS]). Expression and purification with glutathione-coated beads (Pharmacia) was performed as described previously (17). The fusion proteins loaded on Sepharose beads were subsequently incubated with in vitro synthesized proteins in 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin (NETN) for 1 h at room temperature. Beads were washed four times with NETN, resuspended in sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis.

**Electrophoretic Mobility Shift Assay**—293 cells were grown in 10-cm dishes and transfected as described above with 20 μg of expression plasmid and 20 μg of both plasmids when combinations are used. Cells were harvested in Dignam C (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin), incubated for 30 min at 4°C, and membranes were pelleted. The protein concentration of the supernatant was determined by the Bio-Rad protein assay according to the manufacturer’s protocol. Double-stranded oligonucleotides containing the κB site from the ICAM-1 promoter (5′-agctctGGAAATTCCggagc-3′) were labeled with [32P]dCTP using the Klenow fragment of DNA polymerase I. Whole cell extracts (5 μg) were incubated with 10,000 cpm of probe (0.1–0.5 ng) and 1 μg of poly(dI-dC) for 30 min at room temperature in a total reaction mixture of 20 μl containing 20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 1 μg/ml bovine serum albumin. Samples were loaded on a 5% polyacrylamide (29:1) gel, containing 0.25 × TBE (90 mM Tris borate, 2 mM EDTA) as running buffer.

**Western Blot Analysis**—COS-1 cells and 293 cells were transfected as described above. Subsequently cells were harvested directly in sample buffer. Samples were separated on SDS-polyacrylamide gels and transferred to Immobilon (Millipore). Blots were blocked with Blotto (phosphate-buffered saline containing 4% non-fat milk powder and 0.05% Tween 20) for 30 min. All subsequent steps were carried out in Blotto: phosphate-buffered saline (1:1). Blots were probed with the polyclonal antibody SC-109 or SC-372 against RelA. After washing, blots were incubated with peroxidase-conjugated antibodies (1:10,000, Amersharm Corp.). Blots were washed again, and immunoreactive bands were visualized with ECL according to the manufacturer’s instructions (Amersham Corp.).

**RESULTS**

**Regions of RelA Required for Transactivation and Transpression**—To determine the domain(s) in RelA involved in repression of GR activity, several deletion constructs, lacking (part of) the TADs or (part of) the RHD were used. Furthermore, a mutant was used containing a point mutation in the RHD (Fig. 1A). Expression levels of these RelA constructs in 293 cells were similar, as detected on a Western blot (Fig. 1B). To detect the RelA proteins in lanes 1–12, a polyclonal antibody against the N-terminal region of RelA was used. A different antibody, directed against the C-terminal region of RelA, was used to detect RelA 1–551 [22–248] protein (lane 13). This antibody shows an aspecific signal as indicated in Fig. 1B. Similar levels of expression were observed in COS-1 cells (data not shown).

To study the ability of the different proteins to activate transcription from an NF-κB reporter, the constructs encoding the different RelA mutants were cotransfected with this reporter into 293 cells. As shown in Fig. 1C, full-length RelA (1–551) strongly activated the reporter (~80-fold), while deletions in transactivation domains 1 and 2 (TA1 and TA2) drastically decreased the transactivation potential. As expected, no activity was observed when both activation domains were deleted (RelA 1–431 and RelA 1–305). Also the constructs containing either deletions or mutations in the RHD, which are therefore no longer able to bind DNA (see Fig. 4B and data not shown), could not activate the NF-κB reporter (RelA 1–551 [222–248], RelA 1–551 [179–357], and RelA 1–222) (E301). Similar results were obtained in COS-1 cells (data not shown). For all constructs that were able to transactivate the NF-κB reporter (Fig. 1C), repression of their transcriptional activity could be observed upon cotransfection of expression vector encoding GR (1 μg) in the presence of dexamethasone (results not shown). This indicates that not a specific part of the transactivation domain of RelA is involved in the repression of RelA activity by GR.

To examine whether the RelA mutants were able to repress GR activity, 293 cells and COS-1 cells were transfected with a reporter construct containing two glucocorticoid response elements (GREs) in front of the thymidine kinase promoter coupled to the luciferase gene. Cotransfection with GR expression vector (1 μg) resulted in a hormone-dependent induction of luciferase activity, which could be clearly repressed by the presence of full-length RelA (1–551) (Fig. 2), as described previously (13). Deletion of either TA1 (1–521), or the TA2-like domain in TA1 (1–551 [443–476]) (11), hardly affected the repression activity of RelA, while deletion of both the leucine zipper-like structure and part of the TA2-like domain in TA2 (RelA 1–551 [443–470]) did have a small effect. In 293 cells, further deletion of the TADs and combinations of deletions in TA1 and TA2 resulted in a decrease in repressive activity and RelA 1–431, lacking both TADs was no longer able to repress GR activity (Fig. 2A). However, in COS-1 cells, these deletions in the TADs had only minor effects, and RelA 1–431 was still able to repress GR activity to around 35% (Fig. 2B). RelA 1–305, containing only the RHD, no longer showed repressive activity in both cell lines. These results indicate that, in 293 cells and in
COS-1 cells, the TADs of RelA are necessary for repression of GR activity. In addition, there is no strict correlation between the transactivation function and the transrepression function of the RelA mutants.

When (part of) the RHD of RelA was deleted (RelA 1–551 D 22–248, RelA 1–551 D 79–95), the resulting mutants were no longer able to bind DNA. Cotransfection of these constructs did not result in repression of GR activity in 293 cells and hardly showed repressive activity in COS-1 cells, whereas point mutant E39I, also defective in DNA binding (25) was able to repress GR activity in COS-1 cells but not in 293 cells. The DNA binding-defective mutants that still contained intact TADs, able to interact with transcription intermediary factors, were not able to repress GR activity. This indicates that negative cross-talk between GR and RelA is not only the result of competition for common coactivators, a process named squelching (26, 27). It is clear that, although there are cell-type specific differences in repressive activity of RelA, both the RHD and the TADs of RelA are required for repression of GR activity.

In Vitro Association of the RHD of RelA with GR—To investigate the possible function of the RHD and the TADs of RelA in the interaction with GR, reciprocal binding assays were performed. First, the cDNAs of RelA 1–551, RelA 1–431, lacking the TADs, RelA 1–305, containing only the RHD, and RelA 1–551 D 22–248, lacking the RHD, were fused in-frame to the GST gene. GST-NF-κB1 and GST-RARβ were used as controls. GST fusion proteins were expressed in bacteria, purified with glutathione-coated agarose beads, and subsequently incubated with equivalent amounts of [35S]methionine-labeled GR or RXRa protein, synthesized by in vitro transcription-translation. As shown in Fig. 3 A, GR could not be precipitated by GST alone and hardly precipitated by GST-NF-κB1 and GST-RARβ, whereas GST-RelA 1–551, GST-RelA 1–431, and GST-RelA 1–305 efficiently bound GR protein. However, GST-RelA 1–551 D 22–248, lacking the RHD, was not able to precipitate GR protein. Furthermore, the GST-RelA proteins were incubated with [35S]methionine-labeled RXRa, and it was found that only GST-RARβ was able to precipitate RXRa protein, clearly demonstrating the specificity of the interaction between the RelA proteins and GR.

Next, in an alternative approach to determine the domain in RelA involved in interaction with GR, the cDNA encoding GRΔAB (amino acids 420–779) was fused to the GST gene and tested for its ability to bind in vitro synthesized, [35S]methionine-labeled mutant RelA proteins. As shown in Fig. 3 B, the labeled RelA proteins could not be precipitated by GST alone, whereas GST-NF-κB1, known to associate with RelA (4), and GST-GRΔAB clearly precipitated RelA 1–551, RelA 1–431, and RelA 1–305. RelA 286–551 could not be precipitated by both GST-NF-κB1 and GST-GRΔAB, confirming our previous results shown in Fig. 3 A. In all cases, the additional presence of

**FIG. 1.** Deletion analysis of RelA. A, schematic representation of RelA deletion constructs. Wild type RelA (1–551) consists of two transcription activation domains (TA1 and TA2) and a domain involved in DNA binding and dimerization (RHD). Numbers refer to original amino acid sequence (35). B, Western blot analysis of the RelA deletion constructs. 293 cells were transiently transfected with 10 μg of the indicated RelA plasmid. Extracts were fractionated by SDS-polyacrylamide gel electrophoresis, blotted, and immunostained with antibodies specific for the N-terminal part of RelA (lanes 1–12) or the C-terminal part of RelA (lane 13). Numbers on the left indicate molecular mass markers (kDa). The arrowhead on the right indicates the position of the aspecific protein in lane 13. C, 293 cells were transfected with 2 μg of 3 x NF-κBkluc reporter and 100 ng of empty expression vector or 100 ng of RelA expression construct as indicated. Fold induction of luciferase activity by wild type RelA (1–551) over empty expression vector is set at 100%. Bars represent the mean of at least three independent experiments ± S.D.
A

![Graph A](image)

B

![Graph B](image)

**FIG. 2. Transrepression of GR activity by mutant RelA proteins.** A, 293 cells were transiently transfected with 100 ng of GR and 500 ng of empty expression vector or 500 ng of the indicated deletion constructs. 2 × GREtkluc (2 μg) was used as reporter construct. The relative induction of GR, indicating luciferase activity in cells treated with dexamethasone over untreated cells, is set at 100%. Bars represent the mean of at least three independent experiments ± S.D. B, COS-1 cells were transiently transfected as in A.

hormone had no effect (data not shown). These data indicate that, although negative cross-talk between GR and RelA in intact cells requires both the RHD and the TADs, physical association between GR and RelA involves the RHD only.

**Dominant Inhibition of the Repressive Activity of RelA—**RelA mutants in which the domain involved in repression is mutated, but which are still able to dimerize, can associate with wild type RelA protein. The potential effect of these complexes on the repressive activity of RelA was tested in transfection experiments. Several RelA mutants were analyzed by transfection of 293 cells with a GRE reporter construct and expression constructs encoding GR (100 ng) and RelA 1–551 (100 ng). RelA can readily repress GR activity to ~55% when cotransfected in amounts as low as 100 ng (Fig. 4A). Cotransfection of wild type RelA (1 μg) resulted in an increased repression of GR activity, whereas cotransfection of RelA 1–431, RelA 1–305, or RelAE39I (1 μg), showed a decrease in the repressive activity of RelA, and therefore these constructs could be considered to act as transdominant negative mutants. A deletion construct that was no longer able to dimerize with wild type RelA did not influence this repressive activity (RelA 1–551E39I).

To verify that the transdominant mutants indeed dimerized with RelA, electrophoretic mobility shift assays were performed. Therefore RelA and the transdominant mutants were overexpressed in 293 cells, and their ability to form heterodimers was examined. As shown in Fig. 4B, heterodimer formation could be observed between RelA 1–551 and the mutants, resulting in either the formation of a complex with intermediate mobility in the case of RelA 1–431 and the mutants, or resulting in a decrease in DNA binding of wild type RelA when RelAE39I was co-transfected (lane 8). Coexpression of RelA 1–551 and RelA 1–305 (lane 6), or resulting in a decrease in DNA binding of wild type RelA protein (lane 8). These results indicate that RelA deletion mutants, which were not able to repress GR activity (but still able to dimerize), can act as transdominant negative inhibitors of repressive activity of the wild type RelA protein.

**Effect of 13 S E1A on the Transrepression Function of RelA—**As we showed (Fig. 2), there appeared to be cell type-specific differences in repressive activity of RelA in COS-1 cells and 293 cells, which were most pronounced in the case of RelA 1–521, RelA 1–431, and RelA 1–305 (lane 6). However, the transactivation potential of these RelA mutants was identical in both cell types (Fig. 1C). The difference in repressive activity between both cell types could be due to the presence of cell type-specific cofactors. Nuclear receptors have been described to inhibit or enhance transcription by recruiting specific coactivator or corepressor proteins to the transcription complex (20). These cofactors might also interfere with the cross-talk between GR and RelA. An important difference between COS-1 cells and 293 cells is that 293 cells contain E1A protein (28). Furthermore, it has previously been described that 13 S E1A was able to associate with the C-terminal part of RelA and to stimulate the transcriptional activity of RelA (29). Therefore, we investigated the possible role of E1A in the transrepression potential of RelA. To study this, COS-1 cells and 293 cells were transiently transfected with a GRE reporter construct (2 μg), an expression construct for GR (100 ng) and expression vectors encoding RelA (500 ng) and 13 S E1A (1 μg) as indicated. Fig. 5 clearly demonstrates that cotransfection of 13 S E1A in COS-1 cells results in a decrease in repressive activity of RelA and RelA 1–521 (lane 8). This resembles the lower transrepression potential of RelA in 293 cells, in which cotransfection of 13 S E1A had no effect. Similar results were obtained when RelA 1–431 and RelA 1–551E39I were used (not shown). No effect was observed on the transcriptional activity of GR itself or on the repressive activity of RelA 1–305 (results not shown). Of the two known splice variants of E1A, only the 13 S form, and not 12 S E1A, was able to influence the transrepressive activity of RelA (data not shown). These results suggest that cofactors, such as 13 S E1A might be able to modify the negative cross-talk between GR and RelA.

**DISCUSSION**

In this report, we demonstrate that both the RHD and the TADs of RelA are required for repression of GR activity in
intact cells. Furthermore, a physical interaction between GR and RelA requires the presence of the RHD of RelA, which has already been shown to be sufficient for binding to estrogen receptor (16) and to AP-1 (12). Since the DBD of GR is essential for repression of RelA activity (13, 15), this domain in GR may directly interact with the RHD of RelA. Both the DBD of GR and the RHD of RelA are essential for the mutual repression but not sufficient. In addition, the LBD of GR (13) as well as the TADs of RelA are required for the functional repression. Both AF-2 in the LBD of steroid receptors and the TADs of RelA are known to interact with cofactors, which inhibit or enhance their transcriptional activity (20, 29, 30). Together with the fact that 13 S E1A is able to decrease the repressive activity of RelA, these findings suggest that cofactors might be involved in cross-talk between GR and RelA.

Our data show a difference in the functional interaction between GR and RelA, requiring both the RHD and the TADs, and the physical interaction, requiring the RHD only. One possible explanation for this difference is that the RHD may bind to GR first and in this way facilitates subsequent binding of the TADs to GR. However, this explanation seems unlikely because our results suggest that not a specific part of the C terminus of RelA determines its ability to repress, but rather the length of the C terminus or the presence of a functional TAD. Therefore, an alternative model could be that the RHD accounts for the interaction with GR, whereas the C-terminal part of RelA interferes with transcriptional activation by GR. This could be attained by either masking the domain(s) in GR necessary for interaction with the basal transcription machinery and/or coactivators, or through binding of the TADs of RelA to these cofactors themselves. This latter model is in agreement with the findings of several groups that show that NF-κB1, lacking the C-terminal extension present in RelA, can interact in vitro with GR, estrogen receptor, and androgen receptor, but is, in contrast to RelA, not able to repress the transcriptional activity of these receptors (15, 16, 18).

We found that mutations in RelA leading to a loss of repressive activity, but still allowing protein dimerization, resulted in transdominant inhibition of the repressive activity of wild type RelA. These mutants dimerized with RelA in mobility shift assays, suggesting that interaction between the RelA mutants and wild type RelA results in a heterodimer, unable to repress GR activity. It remains unclear whether dimerization of RelA is required for the transrepression. The dominant negative effect of the RelA mutants could also be due to competition with wild type RelA for the interaction domain in GR, since both RelA 1–431 and RelA 1–305 were found to bind to GR in vitro.

By using both 293 cells and COS-1 cells to study the repressive activity of RelA, it became clear that there were cell type-specific differences. Although the same pattern of repressive activity of the RelA mutants was observed in both cell types, generally the RelA constructs were more active in repression in COS-1 cells. Furthermore, some deletion constructs, which did not display any repressive activity in 293 cells, were still able to repress GR activity in COS-1 cells. This could be due to a differential regulation of IκB in these cells. Besides complex formation between GR and RelA, resulting in the mutual repression, a second mechanism has been proposed in which

Fig. 3. The RHD of RelA interacts with GR in vitro. A, [35S]methionine-labeled GR (lanes 1–8) and RXRa (lanes 9–16) proteins, synthesized in rabbit reticulocyte lysate, were incubated with bacterially expressed GST fusion proteins as indicated. After extensive washing, proteins bound to glutathione-coated agarose beads were analyzed by SDS-polyacrylamide gel electrophoresis (8% gel). In lanes 1 and 9, 1/10 of the total input of in vitro synthesized protein was loaded. B, [35S]methionine-labeled RelA 1–551 (lanes 1, 5, 9, and 13), RelA 1–431 (lanes 2, 6, 10, and 14), RelA 1–305 (lanes 3, 7, 11, and 15) and RelA 286–551 (lanes 4, 8, 12, and 16), synthesized in rabbit reticulocyte lysate, were incubated with bacterially expressed GST fusion proteins, bound to beads, as indicated. After extensive washing, proteins were analyzed by SDS-polyacrylamide gel electrophoresis (10% gel). In lanes 1–4, 1/10 of the total input of in vitro synthesized protein was loaded. Numbers on the right indicate molecular mass markers (kDa).
glucocorticoids induce I\textsubscript{KB} synthesis and thereby inhibit NF-\textkappa B activity (31, 32). Up-regulation of I\textsubscript{KB} by dexamethasone in 293 cells could therefore result in an inhibition of RelA translocating to the nucleus and in this way decrease the ability of RelA to repress GR activity. However, Western blot analysis showed no up-regulation of I\textsubscript{KB} protein after transfection with an expression vector for GR and treatment with dexamethasone in both COS-1 cells and 293 cells.\textsuperscript{2} Therefore, differential regulation of I\textsubscript{KB} seems not to account for the differences observed between the two cell lines.

Recently it has been reported that inhibition of AP-1 activity by GR in HeLa cells is mediated by competition for limiting amounts of CREB-binding protein or p300, which serve as coactivators for both GR and AP-1 transcriptional activity (33). The fact that the TADs are required for the repression function of RelA could also indicate a role for cell type-specific coactivators in the negative cross-talk between GR and RelA. However, we have been unable to observe an effect of cotransfection of CREB-binding protein or p300 on RelA transcriptional activity or on the cross-talk between GR and RelA in COS-1 cells and 293 cells.\textsuperscript{2}

In addition, RelA has been shown to interact with components of the basal transcription machinery, such as TFIIH and TATA-binding protein, and some cofactors have been described to enhance the transcriptional activity of RelA, such as PC1 (30) and E1A (29). E1A is expressed in 293 cells (28) and not in COS-1 cells, possibly explaining some of the differences in repressive activity of RelA in these cells. Our data show that cotransfection of 13 S E1A and not 12 S E1A in COS-1 cells results in a decrease in repressive activity of RelA, thus resembling the repression potential of RelA in 293 cells. Possibly, 13 S E1A interferes with the transrepressive activity of RelA via binding to RelA and thereby preventing interaction of RelA with GR or coactivators for GR. It has been described previously that 13 S E1A but not 12 S E1A activates NF-\kappa B and interacts with RelA at the C terminus (29). In conclusion, it seems likely that some of the many coregulatory proteins, which function between transcription factors and the basal transcription machinery and among transcription factors of distinct families, may influence the effectiveness of the interaction between GR and NF-\kappa B. However, the fact that this

\textsuperscript{2}S. Wissink, E. C. van Heerde, B. van der Burg, and P. T. van der Saag, unpublished data.
response occurs in many different cell types (13–15, 34), despite differences in the magnitude of the negative cross-talk between GR and RelA, supports a mechanism of repression involving direct protein-protein interaction.

Acknowledgments—We thank Dr. R. H. Goodman for the CREB-binding protein cDNA and Dr. R. Eckner for the p300 cDNA. We thank G. Folkers and Drs. C. L. Mummery and C. Kuil for useful discussions and suggestions and critical reading of the manuscript. We also thank J. Heinen and F. Vervoordeldonk for photographic reproductions. The work was carried out in the Graduate School for Developmental Biology, Utrecht.

REFERENCES

1. Baeuerle, P. A., and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179
2. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
3. Grimm, S., and Baeuerle, P. A. (1995) Biochem. J. 300, 297–308
4. Urban, M. B., Schreck, R., and Baeuerle, P. A. (1991) EMBO J. 10, 1817–1825
5. Schmitz, M. L., and Baeuerle, P. A. (1991) EMBO J. 10, 3805–3817
6. Bull, P., Morley, K. L., Hockstra, M. F., Hunter, T., and Verma, I. M. (1990) Mol. Cell. Biol. 10, 5473–5485
7. Ishikawa, H., Asano, M., Kanda, T., Kumar, S., Gélinas, C., and Ito, Y. (1993) Oncogene 8, 2889–2896
8. Dohrnanski, P., Ryseck, R.-P., and Brava, R. (1993) Mol. Cell. Biol. 13, 1572–1582
9. Ryseck, R.-P., Bull, P., Takmiya, M., Bours, V., Siebenist, U., Dohrhanski, P., and Brava, R. (1993) Mol. Cell. Biol. 13, 674–684
10. Moore, P. A., Ruben, S. M., and Rosen, C. A. (1993) Mol. Cell. Biol. 13, 1666–1674
11. Schmitz, M. L., dos Santos Silva, M. A., and Baeuerle, P. A. (1995) J. Biol. Chem. 270, 15576–15584
12. Stein, B., Baldwin, A. S., Jr., Ballard, D. W., Greene, W. C., Angel, P., and Herrich, P. (1993) EMBO J. 12, 3879–3881
13. Caldenhoven, E., Lider, J., Wissink, S., van de Stolpe, A., Raaijmakers, J., Koenderman, L., Ökret, S., Gustafsson, J.-A., and van der Saag, P. T. (1995) Mol. Endocrinol. 9, 401–412
14. Ray, A., and Prefontaine, K. E. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 752–756
15. Scheinman, R. I., Gualberto, A., Jewell, C. M., Cidlowski, J. A., and Baldwin, A. S., Jr. (1995) Mol. Cell. Biol. 15, 945–953
16. Stein, B., and Yang, M. X. (1995) Mol. Cell. Biol. 15, 4971–4979
17. Kalkhoven, E., Wissink, S., van der Saag, P. T., and van der Burg, B. (1996) J. Biol. Chem. 271, 6217–6224
18. Palvimo, J. J., Reinikainen, P., Ikonen, T., Kailing, P. J., Mollians, A., and Junne, O. A. (1996) J. Biol. Chem. 271, 24153–24156
19. Beato, M., Herrlich, P., and Schütz, G. (1995) Cell 83, 851–857
20. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) Mol. Endocrinol. 10, 1167–1177
21. Pfahl, M., Trukenmann, M., Zhang, X.-K., Lehmann, J. M., Hermann, T., Wills, K. N., and Graupner, G. (1990) Methods Enzymol. 189, 256–270
22. van de Stolpe, A., Caldenhoven, E., Stade, B. G., Koenderman, L., Raaijmakers, J. A. M., Johnson, J. P., and van der Saag, P. T. (1994) J. Biol. Chem. 269, 6185–6192
23. Schüle, R., Muller, M., Kaltschmidt, C., and Renkawitz, R. (1988) Science 242, 1418–1421
24. Falkers, G. E., and van der Saag, P. T. (1995) Mol. Cell. Biol. 15, 5868–5878
25. Toledano, M. B., Ghosh, D., Trinh, F., and Leonard, W. J. (1993) Mol. Cell. Biol. 13, 852–860
26. Gill, G., and Pashme, M. (1988) Nature 334, 721–724
27. Meyer, M. E., Gromeyer, H., Turcotte, B., Bocquel, M. T., Tasset, D., and Champon, P. (1989) Cell 57, 433–442
28. Graham, F. L., Smiley, J., Russel, W. C., and Nairn, R. (1977) J. Gen. Virol. 36, 59–72
29. Schmitz, M. L., Lindor, A., Limbourg, F. P., Studler, H., Traenckner, E. B. M., and Baeuerle, P. A. (1992) Mol. Cell. Biol. 15, 4052–4063
30. Schmitz, M. L., Stelzer, G., Allmann, H., Meisterernst, M., and Baeuerle, P. A. (1995) J. Biol. Chem. 270, 7219–7226
31. Auphan, N., Didonato, J. A., Rosette, C., Helmbarg, A., and Karin, M. (1995) Science 270, 286–290
32. Scheinman, R. I., Cogswell, P. C., Loquist, A. K., and Baldwin, A. S., Jr. (1995) Science 270, 283–286
33. Kamei, Y., Xu, L., Heinzel, T., Torchio, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) Cell 85, 405–414
34. Mukaida, N., Morita, M., Ishikawa, Y., Rice, N., Okamoto, S., Kasahara, T., and Matushima, K. (1994) J. Biol. Chem. 269, 13289–13295
35. Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C.-H., Maher, M., Baeuerle, P. A., and Rosen, C. A. (1993) Science 251, 1490–1493