Inhibition of Glucocorticoid-induced Apoptosis in 697 Pre-B Lymphocytes by the Mineralocorticoid Receptor N-terminal Domain*

Sonia L. Planey1**, Assia Derfoul1⊥**, Andrzej Steplewski1, Noreen M. Robertson1, and Gerald Litwack1‡

1Department of Biochemistry and Molecular Pharmacology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107, U.S.A.

Running title: The NTD of MR inhibits glucocorticoid-induced apoptosis

*Supported by NIH Grant AI/HL 40976 (GL) and ALA Grant RG-034N (NR)

**Supported by Training grant 5T32 DK07705 from the National Institutes of Health

⊥Present address: National Institutes of Health, National Institute of Arthritis, Musculoskeletal and Skin Diseases, 13 South Drive, Room 3W17, MSC5755, Bethesda, MD 20892, U.S.A.

‡Address correspondence to Dr. Gerald Litwack

Thomas Jefferson University, 233 S. 10th Street, BLSB #350, Philadelphia, PA 19107
TEL: 215-503-4634
FAX: 215-503-5393
Email: Gerry.Litwack@mail.tju.edu
The glucocorticoid and mineralocorticoid receptors (GR, MR) share considerable structural and functional homology and bind as homodimers to hormone response elements. We have shown previously that MR and GR can form heterodimers that inhibit transcription from a glucocorticoid (GC)-responsive gene, and that this inhibition was mediated by the N-terminal domain (NTD) of MR. In this report, we examine the effect of NTD-MR on GC-induced apoptosis in the GC-sensitive pre-B-lymphoma cell line, 697. In GC-treated 697 cells, we demonstrate that stable expression of NTD-MR blocks apoptosis and inhibits proteolytic processing of pro-caspases -3, -8, and -9 and PARP. Importantly, gel shift and immunoprecipitation analyses revealed a direct association between the GR and amino acids 203-603 of NTD-MR. We observed down-regulation of c-myc and of the anti-apoptotic proteins Bcl-2 and Bfl-1 as well as high levels of the pro-apoptotic proteins Bax and Bid. Conversely, cells stably expressing NTD-MR exhibited increased expression of Bcl-2 and Bfl-1 and diminished levels of Bid and Bax. These data provide a potential mechanism for the observed inhibition of cytochrome c and Smac release from the mitochondria of NTD-MR cells and resultant resistance to GC-induced apoptosis. Thus, NTD-MR may mediate GC effects through heterodimerization with GR and ensuing inhibition of GC-regulated gene transcription.
Introduction

The glucocorticoid and the mineralocorticoid receptors (GR and MR) are closely related members of the steroid receptor superfamily, with high sequence homology within the DNA-binding and ligand-binding domains (1). These receptors function as ligand-activated transcription factors that control various aspects of metabolic homeostasis, embryonic development, and physiological stress by binding to specific hormone response elements (HREs) in the regulatory regions of target genes (2). In vitro, both the GR and the MR bind to and are activated by the physiological glucocorticoid (GC), cortisol. And while both receptors, when activated, can bind and transactivate glucocorticoid response elements (GREs) in the promoters of target genes, each has distinct transcriptional activities. (3,4). For instance, the GR, but not the MR, self-synergizes at multimerized HREs (5,6), and only the GR can inhibit induction of AP-1-dependent genes by Fos-Jun heterodimers (7). In addition, the ability to mediate apoptosis in susceptible cells is exclusive to the GR (8).

Induction of apoptosis by GCs occurs in numerous cell types including immature lymphocytes and various malignancies of lymphoid origin (9,10). Evidence that the GR is essential for this process has been provided by studies using GR antagonists, such as RU486, which completely block GC-induced cell death, and by experiments involving GR knockout mice (11-13). The effects of mutations in the GR on its ability to cause apoptosis vary among cell lines. In human CEM and Jurkat lymphoid cells, the DNA Binding Domain (DBD) and the Ligand Binding Domain (LBD) of the GR are essential for GC-induced cell death. Specifically, mutations that deleted either of the zinc fingers of the DBD or substituted amino acids in critical sites within the amino-terminal zinc finger completely blocked the lethal response (14). LBD
The NTD of MR inhibits glucocorticoid-induced apoptosis

mutations also inhibited cell death demonstrating a requirement for hormone binding (15). GR mutants lacking the amino-terminal transactivation domain did not prevent apoptosis in these cell lines, however, in S49 mouse lymphoma cells, this region was essential for steroid-induced lethality (16).

Differences in the transcriptional activities of the GR and the MR, including the ability to mediate apoptosis, may be attributed to structural variability within the N-terminal domain (NTD). This region contains a transactivation function, AF-1, which is involved in the transcriptional transactivation of genes, in receptor heterodimerization, and in binding to other transcription factors (17). Moreover, in the absence of a LBD, this region is constitutively active. The NTDs of GR and MR share only 15% sequence homology and have been shown to have opposite transactivation properties (1). Specifically, chimeric receptor analyses demonstrated that this region of the MR is inhibitory for GR-mediated regulation of the Na/K ATPase β1 gene promoter when MR and GR are coexpressed (18). In addition, several laboratories have shown that GR and MR can form heterodimers capable of inhibiting transcription from a GC responsive gene (5,18-20).

Although the functional consequences of MR/GR heterodimerization are not well understood, the possible physiological significance stems from the observed co-localization of the receptors in various tissues and cells including the brain, heart, vascular smooth muscle, and leukocytes (21-24). For example, in the brain, the MR is involved in the excitability of neurons, whereas the GR opposes these effects. Moreover, in the dentate gyrus of the hippocampus, there is evidence that MR activation can protect neurons against acute GR ligand-mediated apoptosis (25). A recent study has also demonstrated that heterodimerization of MR and GR mediates
The NTD of MR inhibits glucocorticoid-induced apoptosis
direct corticosteroid-induced transrepression of the 5-HT1A receptor promoter (20). These studies have prompted us to examine whether the MR can mediate or abrogate apoptotic cell death in the GC sensitive pre-B lymphoma cell line, 697.
Experimental Procedures

Materials. Restriction enzymes and other reagents were obtained from Promega (Madison, WI), Roche (Indianapolis, IN), or New England Biolabs (Beverly, MA). Horseradish peroxidase (HRP)-conjugated antibodies, ECL reagents, and Hybond membranes were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Triamcinolone acetonide (TA), a synthetic GC analog, was purchased from Sigma Chemical Company (St. Louis, MO). All tissue culture media and supplements were from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Cell Lines and Culture Conditions. The 697 cell line is a cloned human pre-B leukemic cell line derived from childhood acute lymphoblastic leukemia which carries the t(1;19) translocation (26,27). These cells were cultured with RPMI media supplemented with 10% fetal bovine serum, 2mM L-glutamine, penicillin at 100 units/ml, and streptomycin at 100 µg/ml at 37°C under 5% CO₂ in a humidified atmosphere. Exponentially growing cells were used throughout all experiments at a concentration of 5 x 10⁵-1 x 10⁶ cells/ml. The cells were treated with TA at a concentration of 1µM. After steroid treatment, cells were harvested by centrifugation at 1500g for 5 minutes and washed twice with phosphate-buffered saline (PBS).

Generation of Stably Transfected Cell Lines. The N-terminal domain of MR (NTD-MR) or of GR (NTD-GR) was subcloned from the original RshMR or RshGR plasmids (gifts from Dr. R. M. Evans), respectively, into the mammalian expression vector pCMV/myc/nuc (Invitrogen) using the Sal1/Not1 restriction sites. This vector contains a C-terminal c-myc epitope tag and
three tandem nuclear localization signals located downstream of the multiple cloning site. Consequently, the expression of the NTD-MR/c-Myc and NTD-GR/c-Myc fusion proteins, which lack the domains required for translocation, would be efficiently targeted to the nucleus. Following sequence confirmation, these constructs were stably transfected into the 697 cell line.

Cells (6x10^6) were transfected with the Effectene reagent (Qiagen, Valencia, CA) and either 1µg of the NTD-MR plasmid, the NTD-GR plasmid or the empty vector and selected by G418 resistance. G418 resistant cells were subcloned by limiting dilution and fusion protein expression was evaluated by Western blot using a c-myc monoclonal antibody (mAb) (Invitrogen).

The generation of NTD-MR derivatives 1-103, 1-203, 1-303, 1-403, and 1-603 was carried out by PCR amplification of the pRshMR plasmid using the forward primer, MRfor-ATG-Sal1, 5'-CCCCGTCGACATGGAGACCAAAGCACCAC-3' and the reverse primers MRrev103-Not1, 5'-GGGGGGGGCGCCGCCTCAGCTACAGTTGCTGA-3', MRrev203-Not1, 5'-GGGGGGGGCGCCGCCTCAGCTACAGTTGCTGA-3', MRrev303-Not1, 5'-GGGGGGGGCGCCGCCTCAGCTACAGTTGCTGA-3', MRrev403-Not1, 5'-GGGGGGGGCGCCGCCTCAGCTACAGTTGCTGA-3', and MRrev603-Not1, 5'-GGGGGGGGCGCCGCCTCAGCTACAGTTGCTGA-3'. These constructs were subsequently cloned into the pCMV/myc/nuc vector using Sal1/Not1 restriction sites and stably transfected into 697 cells as described above.

**Determination of Cell Number and Viability.** Cells (1 x 10^6 cells/ml) were seeded in 24-well
plates and incubated at 37°C for 2 days in the presence or absence of 1µM TA. Throughout the 48-hour time course, cell viability was determined by trypan blue exclusion using a hemocytometer. For TUNEL staining, cells were treated with 1µM TA for 0, 24 or 48 hours and spun onto slides using a Cytospin centrifuge. The cells were fixed with 4.0% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 15 minutes at room temperature and subsequently washed three times with PBS for 5 minutes. Cells were permeabilized with 0.1% Triton X-100/0.1% sodium citrate, labeled with TUNEL reaction mixture (Roche), and incubated in a humidified chamber for 60 min at 37°C in the dark. After rinsing 3X with PBS, the cells were mounted in Vectashield with DAPI counterstain (Vector Laboratories, Burlingame, CA) and examined with a Zeiss Axiovert 405M microscope using epifluorescence microscopy and photographed using a Phase 3 Imaging System with Cool Spot RT digital camera (Phase 3 Imaging Systems, Glen Mills, PA).

**Gel Electrophoresis and Western Blotting.** Proteins were electrophoresed in 8%, 10% or 12% SDS-polyacrylamide gels and transferred to nitrocellulose for Western blotting. Membranes were blocked overnight with 10% nonfat milk (NFM)/1X PBS/0.1% Tween-20 and incubated with the α-active-caspase-3 rabbit polyclonal antibody (pAb) (1:1000) (BD Biosciences, Palo Alto, CA), α-myc mouse mAb (1:5000) (Invitrogen), α-caspase-8 mouse mAb (.5µg/ml)(BD Biosciences), α-caspase-9 rabbit pAb (1:1000) (BD Biosciences), or the PARP mouse mAb (1:200) (BD Biosciences) in 1X PBS-5% NFM for 1 hour at room temperature, followed by a 1 hour incubation with HRP-labeled donkey anti-rabbit or sheep anti-mouse antibodies (1:2500). Proteins were detected using ECL reagent.
**Immunofluorescence.** NTD-MR (MR-H7) cells were harvested 1 hour after treatment with 1µM TA, spun on to slides, and fixed with 70% methanol/30% acetone for 10 min at -20°C and 5 min at room temperature. Cells were blocked for 30 min with 5% normal goat serum (NGS) in PBS, and then incubated with Avidin D blocking solution (Vector) for 15 min. Slides were washed in PBS/.05%-Tween-20, incubated with biotin blocking solution (Vector) for 15 min, washed again, and incubated overnight at 4°C with preimmune serum or GR pAb (5µg/ml). Slides were washed in PBS/.05%-Tween and incubated for 2 hours at room temperature with biotinylated anti-rabbit IgG (5µg/ml; Vector) diluted in 5%-NGS/PBS. Slides were washed in PBS/.05%-Tween and incubated with Texas Red Avidin (20µg/ml; Vector) diluted in 5%-NGS/PBS for 1.5 hours at room temperature. Slides were washed in PBS prior to air drying, mounted in Vectashield (Vector), and visualized as described previously.

**Electrophoretic Mobility Shift Assay.** A double stranded oligonucleotide corresponding to a GRE from the human Na/K ATPase β1 gene (β wt MRE) at position -662 to -628 GGGTTTGGCAATTGTCCTGCTCGAGGTGGTTCAGG was synthesized. β1 wt MRE was filled using the Klenow fragment (Boehringer Manheim, Indianapolis, IN) and labeled by incorporating 32P αCTP (NEN Du Pont, Boston, MA) for use as a probe. Nuclear extracts of TA-treated 697 cells with vector or NTD-MR (3 to 6µg) were incubated on ice for 10 minutes in 10mM Tris-HCl (pH 7.5), 80mM KCl, 10% glycerol, 1mM dithiothreitol, 1mM of poly (dIdC), in a total volume of 18µl. C-myc mAbs (αmyc) (Invitrogen or CN Biosciences, San Diego, CA) or MR pAbs (αMR recognizing the N-terminus and αhMRsN recognizing a 10 amino acid peptide within the N-terminus) (28), as well as preimmune serum and a GR pAb...
The NTD of MR inhibits glucocorticoid-induced apoptosis

(αGR) were included in reaction mixtures where indicated. 0.1 ng of 5′ ³²P end-labeled β1 wt MRE was added to the reaction and incubation was continued for 10 minutes at room temperature. Reaction mixtures were applied to a 4% non-denaturing polyacrylamide gel and DNA-protein complexes were resolved by electrophoresis (250V) at 4°C, with buffer recirculation in 1X TAE (6.7 mM Tris HCl (pH 7.5), 3.3 mM sodium acetate, 1mM EDTA). The gel was dried under vacuum and autoradiographed at −80°C.

**Immunoprecipitations.** Following a 1-hour treatment with 1 µM TA, 2.0 x 10⁷ 697 cells with either vector, NTD-MR or stably expressed NTD-MR derivatives 1-103, 1-203, 1-303, 1-403, and 1-603 were harvested by low speed centrifugation. Cells were disrupted using ice cold RIPA buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing fresh protease inhibitors and homogenized with passage through a 21-gauge needle. After incubation on ice, the samples were centrifuged at 10,000 g, and the resulting cell lysates were precleared for 1 hour at 4°C with agitation using 2 µg of mouse IgG (Vector) and 20 µl of protein A/G agarose conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The beads were pelleted by centrifugation for 5 min at 2500 rpm at 4°C, and the supernatants were transferred to a fresh tube. C-myc mAb (2 µg/ml) was added to 1 ml of each cell extract, and the mixtures were rocked for 1 hour at 4°C. Then 20 µl of protein A/G agarose conjugate was added to each sample for overnight incubation at 4°C in a rotating device. Precipitates were collected by centrifuging at 2500 rpm for 5 min at 4°C, washed 4 times with RIPA buffer and once with PBS, and resuspended in 40 µl of 1X SDS electrophoresis buffer.
**Isolation and Analysis of RNA.** After steroid treatment, cells were harvested by centrifugation and then frozen immediately on dry ice. Total cell RNA was isolated from frozen samples using Qiagen’s RNeasy® kit. RNA concentrations were determined by measuring the absorbance of each sample at 260 and 280nm. Analysis of RNA for repression of c-myc expression was carried out using RT-PCR and Northern Blot analyses. RT-PCR was performed on 1µg of each RNA sample using the TITANIUM™ One-Step RT-PCR Kit (BD Biosciences) and c-myc- or GAPDH-specific primer pairs (c-myc-forward 51-ATGCCCTCAACGTTAGCTTCACC AACAGG-31, c-myc-reverse 51-TTACGCAACAAGTTCCGTAGCTGTCAAG-31; GAPDH-forward 51-CCACCATGGCAAATTTCCATGGCA-31, GAPDH-reverse 51-TCTAGACGGC AGTTCAGGTCCACC-31). The amplification was performed as follows: 50°C/1hr, 94°C/5min, 30 cycles of 94°C/30s, 65°C/30s, 68°C/1min, and then 68°C/2min. PCR products were separated by electrophoresis in a 1.0% agarose gel and visualized by ethidium bromide staining. Band intensities were quantitated using Kodak 1D Imaging Software (Eastman Kodak Company, Rochester, NY), and fold induction of mRNA expression was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

For Northern Blot analysis RNA samples (15µg each) containing 0.2µg/ml ethidium bromide were fractionated in a 1.2% formaldehyde-agarose gel. The gel was photographed, rinsed in 10X SSC, and transferred by blotting to Hybond-N nylon membrane (Amersham Pharmacia Biotech) with 20X SSC. Accuracy of RNA loading and transfer was confirmed by fluorescence under ultraviolet light after staining with ethidium bromide. The RNA was UV-crosslinked to the membrane, prehybridized, and hybridized at 42°C in 50% formamide-5X wash buffers.

The NTD of MR inhibits glucocorticoid-induced apoptosis.
SSC-0.5% SDS-0.3mg/ml salmon sperm DNA-1X Denhardt’s solution. Radioactive human c-myc DNA probe (100ng) (Geneka Biotechnology Inc., Montreal, Quebec) was used at a concentration of 0.5-1 x 10^6 cpm/ml and was prepared by the random primer labeling method with [α-32P]dCTP. After hybridization, the membranes were washed in 0.1-0.5X SSC-0.1%SDS at 42-68°C and exposed to X-ray film.

Isolation of Mitochondria. 697 cells with vector or NTD-MR were treated with 1μM TA for 0, 24, and 48 hours and harvested by centrifugation at 600g for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended with five volumes of buffer A (20mM Hepes-KOH, pH 7.5, 10mM KCl, 1.5mM MgCl_2, 1mM sodium EDTA, 1mM sodium EGTA, 1mM dithiothreitol, and 0.1mM phenethylsulfonyl fluoride) containing 250mM sucrose. The cells were homogenized for 15 sec, and the homogenates were centrifuged twice at 750g for 10 min at 4°C. The supernatants were centrifuged at 10,000g for 15 min at 4°C, and the resulting mitochondrial pellets were resuspended in buffer A containing 250mM sucrose and frozen in multiple samples at -80°C. The supernatants of the 10,000g spin were further centrifuged at 100,000g for 1 hour or more at 4°C, and the resulting cytosolic fractions were concentrated through a Microcon YM-10 Centrifugal Filter Device (Millipore, Bedford, MA). Mitochondrial and cytosolic fractions were used for Western blot analysis.

The NTD of MR inhibits glucocorticoid-induced apoptosis
Results

We have previously demonstrated that the NTD of MR is inhibitory for GR-mediated gene transcriptional regulation (18,29). To examine the ability of this region to modulate GR-induced apoptosis, the NTD of MR (NTD-MR) comprised of amino acids 1-516, or the NTD of GR (NTD-GR), encompassing amino acids 1-421, were stably expressed in 697 cells. These cells provide a model system for this study because we have previously shown that they express GR, and that they are exquisitely sensitive to GC-induced cell death (30,31). More importantly, immunoblot and RT-PCR analyses of 697 whole cell extracts and of RNA, respectively, have demonstrated that these cells do not express MR (data not shown). As shown in Fig. 1, expression of the NTD-MR/c-Myc or NTD-GR/c-Myc fusion proteins was evaluated by Western blot analysis using a c-myc mAb (Fig. 1). The predicted 59kDa recombinant NTD-MR protein was detected in 5 out of 6 clones examined (Fig. 1A, lanes 1-6), while no c-myc tagged NTD-MR was expressed in the cells transfected with the control vector (lane 7). Expression of the 51kDa recombinant NTD-GR protein was also detected in stably transfected cells (Fig. 1C, lanes 1-6). Following evidence of expression, the clones with the highest level of NTD-MR (MR-H7 or -A2) or NTD-GR (GR-D6) were used for further experimentation.

**NTD-MR Inhibits Glucocorticoid-induced Apoptosis in 697 Cells.** To examine the effects of NTD-MR or NTD-GR on the kinetics of GC-induced cell death, cells were treated with 1µM TA for a 48-hour time course. Fig. 1D shows that 697 cells with vector and NTD-GR cells exhibit declining viability within the first 24 hours of treatment, with complete cell death occurring by 48 hours. Importantly, stable transfection of the NTD-MR in these cells resulted in a strong inhibition of GC-induced cell death (Fig. 1B). To confirm that overexpression of the
The NTD of MR inhibits glucocorticoid-induced apoptosis

MR N-terminus protects 697 cells from GC-induced apoptosis, DNA isolated from cells at indicated time points following treatment with 1µM TA (0, 24, and 48 hours) was examined for nuclear fragmentation using the TUNEL assay. As shown in Fig. 2, 697 cells with vector, treated with TA for 24 hours, exhibited the typical morphologic changes associated with apoptosis such as shrinkage and nuclear blebbing, compared with untreated cells (Fig. 2, Panels A and B). Moreover, the nuclei of these cells exhibited fluorescence indicating the presence of the labeled 3’-OH ends characteristic of apoptotic cells. In contrast, cells stably expressing NTD-MR, which had been treated with TA for the same time period, retained their viability (Fig. 2, Panels C and D).

**NTD-MR Does Not Affect GR Expression or Nuclear Translocation.** To explore the mechanism of inhibition of GC-induced apoptosis by NTD-MR at the receptor level, we first examined GR protein levels by Western blot and GR subcellular localization by immunofluorescence. To compare GR protein levels in 697 and MR-H7 cells, whole cell extracts were prepared from cells harvested at 0, 3, 8, 12, 18, 24, 36, and 48 hours of treatment with 1µM TA. As shown in Fig. 3A, overexpression of NTD-MR did not alter endogenous levels of GR protein, which remained steady over the 48-hour time course and comparable to those levels detected in 697 cells. Immunocytochemical analysis in Fig. 3B revealed that endogenous GR expression in cells stably transfected with NTD-MR (MR-H7) is cytoplasmic. Following treatment of cells with 1µM TA for 1 hour, the GR was detected primarily in the nucleus. These data suggest that the expression of NTD-MR in 697 cells does not prevent translocation of the GR into the nucleus upon hormone binding.

**NTD-MR Does Not Affect the Ability of GR to Bind DNA.** To determine whether the
The NTD of MR inhibits glucocorticoid-induced apoptosis

expression of NTD-MR interferes with the ability of GR to bind DNA, electrophoretic mobility shift analysis (EMSA) was performed using a GR/MR specific response element as a probe and nuclear extracts from 697 cells with vector or MR-H7 cells treated for an hour with 1µM TA. In extracts of 697 cells, we detected protein binding to the β1 MRE/GRE corresponding to endogenous GR (Fig. 4A, lane 1). This binding was reduced when a GR antibody was included in the gel shift reaction (Fig. 4A, lane 2) and was unaffected in the presence of nonspecific serum (Fig. 4A, lane 3) or c-myc mAb (Fig. 4A, lane 4). In extracts of MR-H7 cells, binding of endogenous GR to the β1 MRE/GRE was similar to that seen for 697 cells (Fig. 4B, lanes 1 and 2). To determine whether the protein-DNA complexes contained the N-terminal MR, we used two different c-myc mAbs and MR pAbs. In all cases, the protein-DNA complexes were either ablated or shifted to a higher position when these antibodies were used (Fig. 4B, lanes 3-6) but remained unchanged when a preimmune serum was included in the reaction (Fig. 4B, lane 7). These results indicate that the N-terminus of MR is present with the GR in the protein-DNA complex, suggesting the possibility of MR-GR heterodimer formation.

Interaction between GR and Amino Acids 203-603 of NTD-MR. We have previously shown that when co-expressed, the MR and the GR are able to form heterodimers (18,29). To determine whether the inhibitory effects of NTD-MR on GR function are the result of a direct interaction with the GR, we performed immunoprecipitation analysis. 697 cells stably expressing NTD-MR (MR-H7) or NTD derivatives (1-103, 1-203, 1-303, 1-403, and 1-603) (Fig. 4C) were treated with 1µM TA for 3 hours. Cell lysates were subjected to immunoprecipitation using a c-myc mAb. As shown in Fig. 4D (top), western blot analysis with a GR pAb demonstrated that endogenous GR physically associates with the NTD of the MR.
Specifically, this interaction occurred within the N-terminal region encompassing amino acids 203-603 (lanes 5-9), since no binding was detected with the deletion derivative 1-103 (lane 4). As a control for the amount of immunoprecipitated protein, the membrane was stripped and successively reprobed with a c-myc mAb (bottom).

**Effect of NTD-MR on GR Transcriptional Activity.** To test the effect of GR/NTD-MR heterodimers on GR transcriptional activity in 697 and MR-H7 cells, we evaluated the effect of TA on GR-mediated regulation of the endogenous c-myc gene. Suppression of c-myc mRNA has been reported previously when GR sensitive cells were treated with GC (31). To examine the expression of c-myc at the mRNA level, 697 cells with vector or NTD-MR were cultured in the presence of 1µM TA for 48 hours, and RNA was prepared from cells harvested at time 0, 4, 16, 24, and 48 hours. As determined by semi-quantitative RT-PCR, treatment of 697 cells with GC caused a 7-fold repression of c-myc transcription by 24 hours as compared with untreated cells (time 0) (Fig. 5A). This repression was blocked by NTD-MR in MR-H7 cells as shown in Fig. 5A. GAPDH levels were unaffected by TA treatment in both cell lines. These data were confirmed by Northern blot analysis using a c-myc specific cDNA probe in Fig. 5B, which shows that expression of c-myc mRNA was dramatically reduced in 697 cells treated with TA after 4 hours. Within the time period of the experiment, the level of the c-myc mRNA reached its lowest point at 24 hours after TA treatment. However, in the MR-H7 cells, c-myc mRNA levels remained constant throughout the time course.

**NTD-MR Inhibits Proteolytic Processing of Caspases and PARP.** A common endpoint to apoptosis is a network of caspases whose activation is required for the irreversible commitment to cell death. To determine whether NTD-MR expression inhibits caspase
The NTD of MR inhibits glucocorticoid-induced apoptosis

activation in 697 cells, enzymatic cleavage of endogenous caspases during GC-induced apoptosis was assessed. 697 cells stably expressing NTD-MR (MR-H7) or vector alone were cultured in the presence or absence of 1µM TA during a 48-hour time course and whole cell lysates were examined by Western Blot analysis. As shown in Fig. 6A, proteolytic processing of the proform of the apical caspases -8 (55kDa) and -9 (46kDa) and the downstream effector caspase-3 (32kDa) was observed in 697 cells treated with TA. In contrast, in MR-H7 cells treated with TA, the inactive proenzyme forms of caspases -8, -9, and -3 remained intact, revealing that GC-induced caspase cleavage was abolished. Further evidence of this inhibition was seen when a downstream target of activated caspase-3, poly (ADP)-ribose polymerase (PARP), was examined for enzymatic degradation. Immunoblot analysis in Fig. 6B shows that following treatment of 697 cells with TA, the “death substrate” PARP (116kDa) was proteolyzed with generation of its signature 85kDa cleavage product, while in MR-H7 cells, PARP cleavage was inhibited.

Effect of NTD-MR on Bcl-2 Family Members. It has been shown that GC-induced apoptosis is regulated by members of the Bcl-2 family upstream of caspase activation (32-34). To address potential mechanisms involved in N-terminal MR inhibition of GC-induced apoptosis, we evaluated the expression levels of the anti-apoptotic proteins, Bcl-2 and Bfl-1. As determined by semi-quantitative RT-PCR, treatment of 697 cells with 1µM TA caused a 18-fold repression of Bcl-2 transcription by 16 hours, however, in MR-H7 cells, Bcl-2 mRNA levels increased nearly 2-fold over the 48-hour time course (Fig. 7A). We also observed a 6-fold down-regulation of Bfl-1 mRNA in 697 cells within 4 hours of treatment, whereas Bfl-1 mRNA expression in MR-H7 cells was up-regulated 8-fold (Fig. 7A). These results were
corroborated by Western Blot analyses of cytosolic and mitochondrial extracts. Following GC treatment of 697 cells, mitochondrial levels of Bcl-2 were decreased, while cytosolic levels increased slightly (Fig. 7B). In contrast, mitochondrial Bcl-2 levels were dramatically increased in MR-H7 cells (Fig. 7B). Furthermore, mitochondrial and cytosolic Bfl-1 expression levels were non-detectable in 697 cells following GC treatment whereas in MR-H7 cells, the expression levels of Bfl-1 in the mitochondrial fraction increased slightly at 24 hours and in the cytosolic fraction were increased at 48 hours (Fig. 7B).

A recent study has demonstrated that Bfl-1 can prevent the formation of a pro-apoptotic complex by sequestering BH3 domain-only proteins like Bid and blocking its collaboration with Bax or Bak in the plane of the mitochondrial membrane (35). Thus, we examined the localization and protein levels of the pro-apoptotic proteins Bid and Bax in 697 and MR-H7 cells treated with 1µM TA. Western blot analysis in Fig. 8A shows that in 697 cells, both cytosolic and mitochondrial levels of Bid were dramatically higher than in MR-H7 cells. Moreover, in MR-H7 cells, Bid was located primarily within the mitochondria whereas in 697 cells it was detected in the cytosol as well. Bax protein levels did not seem to be significantly different between the two cell lines, however, Bax was only faintly detectable in the cytosol of MR-H7 cells at 48 hours (Fig. 8A).

**NTD-MR Inhibits Cytochrome c and Smac Release from the Mitochondria.** Bcl-2 has been shown to inhibit cytochrome c (Cyt c) release from mitochondria in pre-apoptotic cells (36). Since we observed reduced expression of Bcl-2 in GC stimulated 697 cells and increased expression in MR-H7 cells, we sought to examine whether Cyt c was released from the mitochondria. As shown in Fig. 8A, GC induced a time-dependent release of Cyt c from the
mitochondria with a concomitant increase of Cyt c into the cytosolic fraction of 697 cells. In contrast, Cyt c was retained in the mitochondria of MR-H7 cells (Fig. 8A). Because Smac/DIABLO is known to promote caspase activation in the Cyt-c/Apaf-1/caspase-9 pathway, we examined whether inhibition of Smac release from the mitochondria plays a role in NTD-MR mediated inhibition of GC-induced apoptosis. As demonstrated in Fig. 8A, the expression level of Smac in the mitochondrial fraction of 697 cells was dramatically reduced within 48 hours following treatment with 1µM TA. This loss of Smac from the mitochondria was accompanied by its presence in the cytosol within 24 hours, an effect that was inhibited in the presence of NTD-MR (Fig. 8A). These results demonstrate that GC-induced apoptosis in 697 cells is accompanied by the release of Cyt c and Smac from the mitochondria into the cytosol, and that the expression of NTD-MR inhibits this process.

Smac/DIABLO, when released from mitochondria into the cytosol, functions by eliminating the inhibitory effects of IAPs (inhibitor of apoptosis proteins), particularly XIAP, on caspases (37-39). We examined the cytosolic fractions from GC treated 697 and MR-H7 cells for the presence of XIAP. As shown in Fig. 8B, XIAP levels were relatively low in 697 cells and completely diminished by 48 hours. However, in MR-H7 cells, levels of XIAP were much higher and remained constant throughout the 48-hour time course (Fig. 8B). These data are important because they support a model in MR-H7 cells that by inhibiting the release of Smac from the mitochondria, XIAP is able to maintain its association with caspases and inhibit their activity as well as cell death.
The NTD of MR inhibits glucocorticoid-induced apoptosis

**Discussion**

GC-induced lymphocytic cell death is one of the earliest recognized forms of apoptosis (8), yet progress in understanding this process has lagged behind significant advances in understanding other forms of apoptosis, such as that induced by death receptors (40). In this report, we examined the ability of the NTD of MR to modulate GR-induced apoptosis in the GC-sensitive cell line, 697, based on our previous findings that this region is inhibitory for GR-mediated gene transcriptional regulation (18,29). Our data show that when treated with the GC analog, TA, parental 697 cells undergo apoptosis. However, in 697 cells stably expressing the NTD-MR, GC-induced apoptosis was dramatically inhibited, indicating that the N-terminal region of MR induces a dominant negative effect and potently inhibits GR function.

Inhibition of GC-induced apoptosis in 697 cells by NTD-MR may reflect interactions occurring at the receptor level. In its inactivated form, the GR exists in the cytoplasm of cells complexed with heat-shock proteins, immunophilins and other inhibitory proteins (41). Upon binding ligand, the GR sheds these proteins and translocates to the nucleus, where it can regulate the transcription of GC-responsive genes by binding to specific glucocorticoid response elements (GREs) within DNA, either enhancing or repressing gene transcription (42). We show that overexpression of NTD-MR does not prevent translocation of the GR into the nucleus upon hormone binding, nor does it alter endogenous GR protein levels. However, EMSA and immunoprecipitation analyses demonstrate that there is a direct interaction between the GR and amino acids 203-603 of the NTD-MR. An evaluation of GR transcriptional activity in both 697 and MR-H7 cells further revealed that NTD-MR expression interfered with the GR-mediated suppression of endogenous c-myc. These results suggest that the observed inhibitory activity of
NTD-MR on GR-mediated transcription and ensuing apoptosis in 697 cells is likely mediated through heterodimerization of the NTD-MR with GR. Importantly, these data are in agreement with several studies substantiating the potential for transcriptional regulation via heteromeric complexes of the GR and MR (18-20,43).

In mammals, the initiation of apoptosis is controlled by two major signaling pathways: the receptor-mediated “extrinsic” pathway and the mitochondrial-mediated “intrinsic” pathway. Caspase-8 is the key initiator of the extrinsic pathway where it is activated in response to death receptor engagement by ligands belonging to the tumor necrosis factor (TNF) superfamily (44,45). The intrinsic pathway involves mitochondrial disruption by pro-apoptotic Bcl-2 family members and consequent release of factors such as cytochrome c that promote caspase-9 activation (46-49). Both pathways culminate in the activation of downstream effector caspases -3, -6 and -7 and can cooperate to enhance apoptosis through caspase-8 mediated cleavage of Bid (50-52). Here we provide evidence that treatment of 697 cells with GC mediates the efficient proteolytic processing of caspases -8, -9, and -3 as well as cleavage of the death substrate, PARP. Remarkably, this processing is completely abolished in 697 cells expressing NTD-MR, suggesting that the survival function of NTD-MR is upstream of caspase activation. Our observation that GC-induced apoptosis in 697 cells is accompanied by the cleavage of caspases -9 and -3 as well as the release of Cyt c from the mitochondria, provides strong evidence that apoptosis in pre-B lymphocytes proceeds mainly through the intrinsic cell death pathway.

Members of the Bcl-2 family, including death repressor (e.g., Bcl-2, Bcl-xL, Bfl-1) and death inducer (e.g. Bax, Bcl-xS, Bak) proteins, are major regulators of mitochondrial apoptotic...
events and act in part by controlling Cyt c release from the mitochondria (53). For example, expression of Bcl-2 and Bcl-xL prevents the redistribution of Cyt c in response to multiple death inducing stimuli (54-56) whereas Bid, Bax, and Bak promote Cyt c release (36,57,58). Bcl-2 family members also form homo- or heterodimers with one another and, depending on the ratio of inhibitor to activator, can either inhibit or activate cell death (59,60). In the present study, we observed that stimulation of 697 cells with GC triggered the loss of Bcl-2 and Bfl-1 expression and the release of cytochrome c and Smac from the mitochondria. Moreover, levels of the pro-apoptotic proteins Bax and Bid remained elevated, supporting destructive alterations in the mitochondria. In sharp contrast, 697 cells stably expressing NTD-MR exhibited increased expression levels of Bcl-2 and Bfl-1 and inhibition of Cyt c and Smac release from the mitochondria. The protein levels of Bid and Bax in these cells were either significantly reduced or not sustained over the 48-hour treatment period with TA, supporting the inhibition of mitochondrial disruption in these cells. Furthermore, the elevated levels of XIAP observed in MR-H7 cells suggest that XIAP would be allowed to maintain its association with caspases and prevent apoptosis.

The observed up-regulation of the anti-apoptotic proteins, Bcl-2 and Bfl-1, in MR-H7 cells sheds light not only on the mechanism by which NTD-MR inhibits the pro-apoptotic action of GCs, but also on the process by which GCs induce apoptosis in pre-B lymphocytes. Recent findings indicate that Bcl-2 family members function upstream of caspase activation to inhibit commitment to cell death. Bcl-2 is thought to act on the outer mitochondrial membrane to prevent mitochondrial permeability transition pore opening and the subsequent release of apoptogenic proteins from the mitochondria (53,61). Bcl-2 can also inhibit the action of
Bax/Bak by forming inactivating heterodimers (57). More recently, it has been shown that overexpression of Bcl-2 in dexamethasone-treated thymocytes inhibits proteasome activation (62). Involvement of the proteasome in the induction of apoptosis is a distinguishing characteristic of the corticosteroid-induced death pathway versus the Fas-mediated cell death pathway where the proteasome appears dispensable (62). Degradation of the cell cycle proteins, Cdk2 and p27Kip1 (33,63), the pro-survival transcription factors, c-Fos and NF-κB (34,64), and the IAPs, c-IAP1 and XIAP (60), by the proteasome have been described. Hence, higher levels of Bcl-2 in MR-H7 cells may act not only to preserve mitochondrial function, but may also function by regulating proteasome-mediated degradation of cell cycle and pro-survival proteins.

Bfl-1 is an anti-apoptotic Bcl-2 family member whose preferential expression in hematopoietic cells and endothelium is controlled by inflammatory stimuli. Cellular expression of Bfl-1 has been shown to confer protection against CD95- and Trail receptor-induced Cyt c release as well as p53- and etoposide-induced apoptosis (65-67). However, a protective function of Bfl-1 in GC-induced apoptosis has not been documented. We observed localization of Bfl-1 and Bid primarily to the mitochondria in MR-H7 cells, but not in 697 cells undergoing apoptosis. Bfl-1 has been shown to prevent the formation of a pro-apoptotic complex by sequestering BH3 domain-only proteins at the mitochondria. Thus, up-regulation of Bfl-1 in MR-H7 cells may serve to bind and sequester tBid at the mitochondria, and prevent its association with Bax, thereby inhibiting mitochondrial disruption and Cyt c release.

A possible mechanism for the inhibitory effect of NTD-MR on GC-induced apoptosis may involve the disruption of an interaction between GR and a specific cellular factor or...
competition between the GR and NTD-MR for limited amounts of mutual coactivators. A coactivator that clearly distinguishes between the GR and MR has not been identified; however, it is likely that coactivators interacting differentially with the AF-1 transactivation domain of these receptors may play an important role in conferring specific glucocorticoid/mineralocorticoid-mediated regulation. A recent study by Sadar et al, has demonstrated that IL-6 mediates activation of the androgen receptor (AR) NTD by a mechanism dependent upon MAPK and STAT3 signal transduction pathways in LNCaP prostate cancer cells (68). Furthermore, STAT3 was shown to interact specifically with fragments of the AR NTD containing all or part of the AF-1 site.

In summary, we have demonstrated that GC-induced apoptosis of 697 cells, the NTD of MR inhibited apoptosis prior to commitment to cell death by interfering with GR-mediated changes in gene transcription. Specifically, we show that GR-mediated repression of c-myc and of the anti-apoptotic proteins Bcl-2 and Bfl-1 is abrogated in cells expressing NTD-MR. The up-regulation of these anti-apoptotic proteins in MR-H7 cells as well as the diminished levels of Bax and Bid provide a potential mechanism for the observed inhibition of Cyt c and Smac release from the mitochondria and resultant resistance to GC-induced apoptosis. Thus, in normal physiology, in cells where MR and GR are coexpressed, the MR may function to counteract the role of activated GR by increasing the ratio of anti-apoptotic molecules relative to pro-apoptotic molecules. Indeed, such a scenario has been observed in the rat hippocampus where the opposing actions of MR and GR on neuronal survival were shown to result from their ability to differentially influence the expression of members of the bcl-2 gene family (69). Further studies are underway to identify GR-induced pro-apoptotic genes that are repressed by
The NTD of MR inhibits glucocorticoid-induced apoptosis

NTD-MR and pro-survival genes that are up-regulated by NTD-MR. The elucidation of GR-regulated apoptotic genes may offer a molecular basis for the treatment of inflammatory diseases and cancer.
The NTD of MR inhibits glucocorticoid-induced apoptosis

References

1. Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987) *Science* **237**, 268-275.
2. Perlmann, T., and Evans, R. M. (1997) *Cell* **90**, 391-397.
3. Arriza, J. L., Simerly, R. B., Swanson, L. W., and Evans, R. M. (1988) *Neuron* **1**, 887-900.
4. Alnemri, E. S., Maksymowych, A. B., Robertson, N. M., and Litwack, G. (1991) *J Biol Chem* **266**, 18072-18081.
5. Liu, W., Wang, J., Sauter, N. K., and Pearce, D. (1995) *Proc Natl Acad Sci U S A* **92**, 12480-12484.
6. Rupprecht, R., Arriza, J. L., Spengler, D., Reul, J. M., Evans, R. M., Holsboer, F., and Damm, K. (1993) *Mol Endocrinol* **7**, 597-603.
7. Pearce, D., and Yamamoto, K. R. (1993) *Science* **259**, 1161-1165.
8. Wyllie, A. H. (1980) *Nature* **284**, 555-556.
9. Thompson, E. B. (1994) *Mol Endocrinol* **8**, 665-673.
10. Evans-Storms, R. B., and Cidlowski, J. A. (1995) *J Steroid Biochem Mol Biol* **53**, 1-8.
11. Thompson, E. B., Thulasi, R., Saeed, M. F., and Johnson, B. H. (1995) *Ann N Y Acad Sci* **761**, 261-275.
12. Caron-Leslie, L. A., and Cidlowski, J. A. (1991) *Mol Endocrinol* **5**, 1169-1179.
13. Tronche, F., Kellendonk, C., Reichardt, H. M., and Schutz, G. (1998) *Curr Opin Genet Dev* **8**, 532-538.
14. Harbour, D. V., Chambon, P., and Thompson, E. B. (1990) *J Steroid Biochem* **35**, 1-9.
15. Nazareth, L. V., Harbour, D. V., and Thompson, E. B. (1991) *J. Biol. Chem.* **266**, 12976-12980.
16. Dieken, E. S., and Meisfeld, R. L. (1992) *Mol. and cell. biol.* **12**, 589-597.
17. Hollenberg, S. M., and Evans, R. M. (1988) *Cell* **55**, 899-906.
18. Derfoul, A., Robertson, N. M., Hall, D. J., and Litwack, G. (2000) *Endocrine* **13**, 287-295.
19. Trapp, T., Rupprecht, R., Castren, M., Reul, J. M., and Holsboer, F. (1994) *Neuron* **13**, 1457-1462.
20. Ou, X. M., Storring, J. M., Kushwaha, N., and Albert, P. R. (2001) *J Biol Chem* **276**, 14299-14307.
21. Trapp, T., and Holsboer, F. (1996) *Trends Pharmacol Sci* **17**, 145-149.
22. Zennaro, M. C., Farman, N., Bouvalet, J. P., and Lombes, M. (1997) *J Clin Endocrinol Metab* **82**, 1345-1352.
23. Wickert, L., Watzka, M., Bolkenius, U., Bidlingmaier, F., and Ludwig, M. (1998) *Eur J Endocrinol* **138**, 702-704.
24. van Steensel, B., van Binnendijk, E. P., Hornsby, C. D., van der Voort, H. T., Krozowski, Z. S., de Kloet, E. R., and van Driel, R. (1996) *J Cell Sci* **109**, 787-792.
25. Hassan, A. H., von Rosenstiel, P., Patchev, V. K., Holsboer, F., and Almeida, O. F. (1996) *Exp Neurol* **140**, 43-52.
26. Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C. M. (1985) *Science* **228**, 1440-1443.
27. Williams, D. L., Look, A. T., Melvin, S. L., Roberson, P. K., Dahl, G., Flake, T., and Stass, S. (1984) *Cell* **36**, 101-109.
The NTD of MR inhibits glucocorticoid-induced apoptosis

28. Robertson, N. M., Schulman, G., Karnik, S., Alnemri, E., and Litwack, G. (1993) Mol Endocrinol 7, 1226-1239.
29. Derfoul, A., Robertson, N. M., Lingrel, J. B., Hall, D. J., and Litwack, G. (1998) J Biol Chem 273, 20702-20711.
30. Robertson, N. M., Zangrilli, J., Fernandes-Alnemri, T., Friesen, P. D., Litwack, G., and Alnemri, E. S. (1997) Cancer Res 57, 43-47
31. Alnemri, E. S., Fernandes, T. F., Haldar, S., Croce, C. M., and Litwack, G. (1992) Cancer Res 52, 491-495
32. Brunet, C. L., Gunby, R. H., Benson, R. S., Hickman, J. A., Watson, A. J., and Brady, G. (1998) Cell Death Differ 5, 107-115.
33. Hakem, A., Sasaki, T., Kozieradzki, I., and Penninger, J. M. (1999) J Exp Med 189, 957-968.
34. Feinman, R., Koury, J., Thames, M., Barlogie, B., Epstein, J., and Siegel, D. S. (1999) Blood 93, 3044-3052.
35. Werner, A. B., de Vries, E., Tait, S. W., Bontjer, I., and Borst, J. (2002) J Biol Chem 277, 2227-2233.
36. Srinivasula, S. M., Datta, P., Fan, X. J., Fernandes-Alnemri, T., Huang, Z., and Alnemri, E. S. (2000) J Biol Chem 275, 36152-36157.
37. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) Cell 102, 33-42.
38. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000) Cell 102, 43-53.
39. Distelhorst, C. W. (2002) Cell Death Differ 9, 6-19
40. Pratt, W. B., and Toft, D. O. (1997) Endocr Rev 18, 306-360
41. Truss, M., and Beato, M. (1993) Endocr Rev 14, 459-479.
42. Liu, W., Hillmann, A. G., and Harmon, J. M. (1995) Mol Cell Biol 15, 1005-1013.
43. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305-1308.
44. Nunez, G., Benedict, M. A., Hu, Y., and Inohara, N. (1998) Oncogene 17, 3237-3245.
45. Hu, Y., Ding, L., Spencer, D. M., and Nunez, G. (1998) J Biol Chem 273, 33489-33494.
46. Saleh, A., Srinivasula, S. M., Acharya, S., Fishel, R., and Alnemri, E. S. (1999) J Biol Chem 274, 17941-17945.
47. Zou, H., Li, Y., Liu, X., and Wang, X. (1999) J Biol Chem 274, 11549-11556.
48. Green, D. R. (2000) Cell 102, 1-4.
49. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) Cell 94, 491-501.
50. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) Cell 94, 481-490.
51. Roy, S., and Nicholson, D. W. (2000) J Exp Med 192, F21-25.
52. Kroemer, G., and Reed, J. C. (2000) Nat Med 6, 513-519
53. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) Science 275, 1132-1136
54. Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. (1997) Cell 91, 627-637
55. Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S. J. (1999) J Biol Chem 274, 1156-1163
56. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993) Cell 74, 609-619
57. Yin, X. M. (2000) J Mol Med 78, 203-211
The NTD of MR inhibits glucocorticoid-induced apoptosis

59. Oltvai, Z. N., and Korsmeyer, S. J. (1994) *Cell* **79**, 189-192.
60. Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. (2000) *Science* **288**, 874-877.
61. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) *Genes Dev* **13**, 1899-1911.
62. Dallaporta, B., Pablo, M., Maisse, C., Daugas, E., Loeffler, M., Zamzami, N., and Kroemer, G. (2000) *Cell Death Differ* **7**, 368-373.
63. Gil-Gomez, G., Berns, A., and Brady, H. J. (1998) *Embo J* **17**, 7209-7218.
64. He, H., Qi, X. M., Grossmann, J., and Distelhorst, C. W. (1998) *J Biol Chem* **273**, 25015-25019.
65. Wang, C. Y., Guttridge, D. C., Mayo, M. W., and Baldwin, A. S., Jr. (1999) *Mol Cell Biol* **19**, 5923-5929.
66. D’Sa-Eipper, C., Subramanian, T., and Chinnadurai, G. (1996) *Cancer Res* **56**, 3879-3882.
67. D’Sa-Eipper, C., and Chinnadurai, G. (1998) *Oncogene* **16**, 3105-3114.
68. Ueda, T., Bruchovsky, N., and Sadar, M. D. (2002) *J Biol Chem* **277**, 7076-7085.
69. Almeida, O. F., Conde, G. L., Crochemore, C., Demeneix, B. A., Fischer, D., Hassan, A. H., Meyer, M., Holsboer, F., and Michaelidis, T. M. (2000) *Faseb J* **14**, 779-790.
The NTD of MR inhibits glucocorticoid-induced apoptosis

**Abbreviations**

AR, androgen receptor; Cyt c, cytochrome c; DBD, DNA binding domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC(s), glucocorticoid(s); GR, glucocorticoid receptor; GRE, glucocorticoid response element; HRE, hormone response element; IAP, inhibitor of apoptosis protein; LBD, ligand binding domain; mAb, monoclonal antibody; MR, mineralocorticoid receptor; NFM, nonfat milk; NGS, normal goat serum; NSS, nonspecific serum; NTD, N-terminal domain; NTD-MR, N-terminal domain of MR; pAb, polyclonal antibody; PARP, poly (ADP)-ribose polymerase; PBS, phosphate buffered saline; TA, triamcinolone acetonide; TNF, tumor necrosis factor.
Acknowledgements

We are grateful to R. M. Evans for providing us with the expression plasmids for the human MR and the human GR. We also thank Dr. Jim Zangrilli and Dr. Annette Hastie for their critical review of the manuscript and members of the Litwack lab for their helpful discussions.
The NTD of MR inhibits glucocorticoid-induced apoptosis

Figure Legends

Figure 1. (A and C) Detection of recombinant NTD-MR and NTD-GR in stably transfected 697 cells. Samples containing whole cell extracts prepared from 1 x 10⁶ cells transfected with vector alone (pCMV), NTD-MR/c-myc or with NTD-GR/c-myc were subjected to SDS-PAGE followed by immunoblotting with a c-myc mAb. Molecular mass markers are indicated (kDa).

(B and D) Kinetics of 697 cell survival following GC treatment. At time 0, 697 cells stably transfected with either vector alone, NTD-GR (clone D6), or NTD-MR (clones H7 and A2) were cultured in the presence or absence of 1µM TA, and survival was analyzed over time by trypan blue exclusion. Data shown are means and standard deviations for three experiments in which each was performed in triplicate.

Figure 2. Stable expression of NTD-MR inhibits GC-induced apoptosis in 697 cells. 697 cells with vector or NTD-MR (MR-H7) were incubated in the presence or absence of 1µM TA. At the 24-hour time point, aliquots of cell suspensions were processed using the TUNEL assay (green) and counterstained with DAPI (blue) to detect changes in nuclear morphology.

Figure 3. Subcellular localization and expression levels of endogenous GR in MR-H7 cells treated with GC. (A) 697 cells with vector or NTD-MR were treated with 1µM TA over a 48-hour time course, harvested at designated time points, and subjected to SDS-PAGE followed by immunoblotting with a GR pAb. Accuracy of protein loading and transfer was confirmed by striping and reprobing with a β-actin pAb (Santa Cruz). (B) Untreated MR-H7 cells (T₀) or cells treated with 1µM TA for 1 hour (T₁) were processed for immunofluorescence using a GR
The NTD of MR inhibits glucocorticoid-induced apoptosis

pAb and confocal microscopy.

**Figure 4. NTD-MR physically associates with endogenous GR.** EMSA was performed using a GR/MR specific response element as a probe and nuclear extracts from 697 cells with vector or NTD-MR (MR-H7) treated with 1µM TA for 1 hour. (A) A GR pAb (αGR) or c-myc mAb (αmyc) was incubated with nuclear extracts 10 min prior to the addition of probe (lanes 2 and 4, respectively). Lane 1 contained a buffer control and lane 3 a preimmune nonspecific serum (NSS). The asterisk denotes GR-specific binding and the arrow indicates the antibody-mediated shifts on DNA-protein complexes. (B) A αMR (lane 4) or αhMRsN (lane 6) pAb was incubated with nuclear extracts 10 min prior to the addition of probe. Similarly, a c-myc mAb (αmyc) from Invitrogen or from CN Biosciences was incubated with samples in lanes 3 and 5, respectively. Lane 1 contained a buffer control, lane 2 αGR, and lane 7 a preimmune NSS. (C) Schematic representation of NTD-MR derivatives 103, 203, 303, 403, MR-H7, and 603 cloned into the pCMV/myc/nuc vector. (D) 697 cells with vector, NTD-MR, or stably expressed NTD-MR derivatives 103, 203, 303, 403 and 603 were treated with 1µM TA and harvested after 3 hours. Cellular lysates were subjected to immunoprecipitation with c-myc mAb and analyzed by Western Blot using a GR pAb (top) or a c-myc mAb (bottom) as a control for the amount of immunoprecipitated protein. In lane 3, lysate from cells transfected with full-length GR served as a positive control.

**Figure 5. Effect of TA on the expression of c-myc mRNA in 697 and MR-H7 cells.** (A) Gel electrophoresis of semi-quantitative RT-PCR using 1µg total cellular RNA from 697 cells with
vector or NTD-MR (MR-H7) harvested at indicated time points throughout a 48-hour incubation with 1 µM TA. GAPDH was included as an internal control. Fold induction (FI) of c-myc mRNA expression is indicated. (B) Total cellular RNA was isolated from 697 cells with vector (Lanes 1-5) or NTD-MR (MR-H7) (Lanes 6-10) at 0-48 hours after TA (1 µM) treatment. Equal amounts of RNA (15 µg/lane) were fractionated on an agarose-formaldehyde gel (top) and transferred by blotting onto Hybond-N nylon membranes as described in “Materials and Methods.” The c-myc mRNA was identified by hybridization using a 1 kilobase 32P-labeled human c-myc probe (bottom). The locations of the 28S and 18S rRNAs are indicated. The periods of TA treatment (h) are indicated at the top of each lane.

Figure 6. Stable expression of NTD-MR inhibits caspase activation and PARP cleavage during GC-induced apoptosis. Western blot analysis of caspase-8, caspase-9, caspase-3 and PARP status after treatment with TA. (A) 697 cells with vector or NTD-MR (MR-H7) were treated with 1 µM TA for a 48-hour time course. Samples containing whole cell extracts from 1 x 10^6 cells were subjected to SDS-PAGE followed by immunoblotting with a mAb to caspase-8 and pAbs to caspase-9 and active caspase-3. (B) For PARP analysis, samples (50 µg) were electrophoresed on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and probed with anti-PARP mAb. Molecular mass markers are indicated (kDa).

Figure 7. Effect of GC on Bcl-2 and Bfl-1 mRNA expression and protein levels in 697 and MR-H7 cells. (A) Gel electrophoresis of semi-quantitative RT-PCR using 1 µg total cellular RNA from 697 cells with vector or (NTD-MR) MR-H7 harvested at indicated time points...
throughout a 48-hour incubation with 1µM TA. RT-PCR was performed as described in Experimental Procedures using the following primer pairs: (Bcl-2-forward 5'-ATGGCGCACGCTGGGAGAACGGGGTACGAC-3', Bcl-2-reverse 5'-TCACTTGTGGCTCATGATAGGCACCCAGGGT-3'; Bfl-1-forward 5'-ATGACAGACTGTGAATTTGGATTATTTAC-3', Bfl-1-reverse 5'-TCAACAGTATTGCTTCAGGAGAGATAGCAT-3'). GAPDH was included as an internal control. Fold induction (FI) of Bcl-2 and Bfl-1 mRNA expression is indicated. (B) Mitochondrial and cytosolic extracts from 697 cells with vector or NTD-MR (MR-H7) treated with 1µM TA for a 48-hour time course were subjected to SDS-PAGE followed by immunoblotting with a mAb to Bcl-2 (BD Biosciences) and a pAb to Bfl-1 (Santa Cruz). To ensure that the same content of mitochondrial and cytosolic protein was loaded in each case, the membrane was stripped and successively reprobed with a VDAC pAb (Santa Cruz) as a marker for the mitochondrial fraction and a β-actin pAb as a marker for the cytosolic fraction.

Figure 8. (A) Localization of Bid, Bax, Cyt c and Smac in GC-treated 697 and MR-H7 cells.

Mitochondrial and cytosolic extracts from 697 cells with vector or NTD-MR (MR-H7) treated with 1µM TA for a 48-hour time course were subjected to SDS-PAGE followed by immunoblotting with a pAb to Bid (Biosource), a pAb to Bax (Santa Cruz), a mAb to Cyt c (BD Biosciences) and a pAb to Smac/DIABLO (Upstate Biotechnology). To ensure that the same content of mitochondrial and cytosolic protein was loaded in each case, the membrane was stripped and successively reprobed with a VDAC pAb as a marker for the mitochondrial fraction.
The NTD of MR inhibits glucocorticoid-induced apoptosis

and a β-actin pAb as a marker for the cytosolic fraction. (B) **NTD-MR inhibits degradation of XIAP after treatment with TA.** Cytosolic extracts from 697 cells with vector or NTD-MR (MR-H7) treated with 1μM TA for a 48-hour time course were subjected to SDS-PAGE followed by immunoblotting with a mAb to XIAP (Stressgen). Accuracy of protein loading and transfer was confirmed by striping and reprobing with a β-actin pAb.
Figure 1
Figure 3
Figure 4

A

697 + TA

No Ab  aGR  NSS  c-myc

GR/DNA *

1  2  3  4

B

MR-H7 + TA

No Ab  aGR  c-myc  aMR  c-myc  aMR&cN  NSS

GR/DNA *

1  2  3  4  5  6  7

C

NTD-MR Derivatives

103  c-myc

203  c-myc

303  c-myc

403  c-myc

MR-H7 (1-516)  c-myc

603  c-myc

D

No lysate  697 control  Recombinant GR  NTD-MR-103  NTD-MR-203  NTD-MR-303  NTD-MR-403  NTD-MR-H7  NTD-MR-603  + TA

1  2  3  4  5  6  7  8  9

αGR

αmyc
Figure 5
Figure 6
Figure 7
Figure 8
Inhibition of glucocorticoid-induced apoptosis in 697 Pre-B lymphocytes by the mineralocorticoid receptor N-terminal domain
Sonia L. Planey, Assia Derfoul, Andrzej Steplewski, Noreen M. Robertson and Gerald Litwack

J. Biol. Chem. published online August 22, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205085200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts