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Steroid receptor heterodimerization demonstrated *in vitro* and *in vivo*

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ABSTRACT **The mineralocorticoid and glucocorticoid receptors (MR and GR, respectively) are members of the intracellular receptor superfamily that bind as homodimers to the same hormone response elements (HREs). Physiological evidence suggests that MR and GR interact with each other in cells that express both receptors, implying that they might directly interact in the regulation of transcription initiation. Indeed, we have found that coexpressed MR and GR interact functionally at the transcriptional level and furthermore that they interact physically through heterodimer formation at a shared HRE *in vitro* and *in vivo*. We suggest from these findings that heterodimerization may play an important role in steroid receptor transcriptional regulation.**

The effects of the corticosteroid hormones are mediated by two closely related intracellular receptors, the mineralocorticoid and glucocorticoid receptors (MR and GR, respectively) that bind with apparently equal affinities to common hormone response elements (HREs) (1–3). Many of the HREs are imperfect inverted hexanucleotide repeats, separated by 3 nucleotides, to which the receptors bind as "head-to-head" homodimers *in vitro* (4, 5). Molecular biological studies have demonstrated that in cells expressing only one of the receptors, receptor-mediated transcriptional regulation from these HREs is mediated by homodimers (6, 7). However, physiological studies in various systems suggest that MR and GR also functionally interact with one another. In isolated hippocampal neurons, for example, selective activation of GR inhibits MR-induced neuronal excitability (8), while whole animal studies suggest that the receptors interact in the regulation of circadian rhythm, feeding, and blood pressure (9, 10).

We were interested in determining whether or not interaction between MR and GR at the level of transcriptional regulation might underlie the interactions observed at the physiological level. We therefore performed cotransfection and gel-shift experiments aimed at identifying functional and physical interactions between the receptors. We furthermore used complementary salt bridge mutations to examine genetically whether MR and GR heterodimerize in cultured cells.

MATERIALS AND METHODS

Cell Culture and Transfection. Sixty to eighty percent confluent monolayers of monkey kidney CV-1b cells (Cell Culture Facility, University of California, San Francisco) were grown in 5% CO_2/95% air (relative humidity, 90%) at 37°C in Dulbecco's Modified Eagle's medium H-16 (Cell Culture Facility, University of California, San Francisco) supplemented with 5% fetal calf serum (GIBCO/BRL). Cells were transfected by the calcium phosphate method as described (11), with 2 μg of MR expression plasmid (11), 2 μg of GR expression plasmid (12), or 2 μg of each expression plasmid together, along with 0.2 μg of the reporter plasmid TAT3-TATA and 1 μg of BlueScript KS$^-$ vector plasmid as carrier DNA. The reporter TAT3-TATA contains a trimerized HRE with unit sequence TGTACAGGATGTTCT fused to the *Drosophila* alcohol dehydrogenase minimal promoter (−33/+4) driving luciferase expression (a generous gift of W. Matsui and K. R. Yamamoto, University of California). Fresh medium containing 5% stripped serum (charcoal treated to remove endogenous steroids) and penicillin G at 100 units/ml and streptomycin sulfate at 100 μg/ml was added 4 hr before transfection. Calcium phosphate DNA precipitates were prepared using 125 mM $CaCl_2$ and HEBS (pH 6.93) (25 mM Hepes/0.75 mM Na_2HPO_4/140 mM NaCl). Cells were incubated 12 hr in medium containing precipitate, washed two times in 37°C phosphate-buffered saline, and refed with fresh medium. In all transfection experiments, corticosterone (10 nM) was added to one of two identical transfections; 24 hr later, cells were harvested and extracts were prepared as described (11). The extracts were assayed for luciferase activity. The luciferase values presented were all normalized to protein concentration. In some experiments, luciferase activities were normalized to β-galactosidase activity (expression driven by the Rous Sarcoma Virus promoter) without any qualitative difference.

***In Vitro* Measurement of DNA Binding.** For *in vitro* measurements, receptors were expressed in bacteria as DNA-binding zinc finger regions (ZFRs) lacking the ligand-binding domains and N termini. (ZFR and DNA-binding domain are both used to denote the region, common to all intracellular receptors, that is necessary and sufficient for specific DNA binding.) The rat MR gene was cloned into the plasmid pET-14b (Novagen) and expressed in *Escherichia coli* strain BL21 (DE3) pLysS as a recombinant protein with the sequence MGSP(MR residues 604–684) RL (87 residues; 9.5 kDa). Purification was as described (13), except that the MR was precipitated by addition of 50% ammonium sulfate and then redissolved in HEGDZ50 [20 mM Hepes, pH 7.5/0.5 mM EDTA/10% (vol/vol) glycerol/5 mM dithiothreitol (DTT)/50 μM $ZnSO_4$/50 mM NaCl]. This material was then applied to a CM-Sepharose fast flow column, and MR was eluted with a salt gradient at ≈310 mM NaCl. MR was then applied to a DNA-cellulose column (1 mg of salmon sperm DNA per ml) in HEGDZ50 buffer and eluted with a salt gradient at ≈250 mM NaCl, yielding >95% pure material. Rat GR construct T7X556 (13) with the sequence MASMTGGQQMGRGSP(GR residues 407–556)MGELEFPGLEDPST (179 residues; 19.0 kDa) was expressed and purified as described above, except that the DNA cellulose step was omitted.

A double-stranded oligonucleotide was constructed by annealing overlapping single-stranded oligonucleotides containing an inverted repeat HRE sequence: TTGGAACCCGGGAGAACATCATGTTCTGAATTC (the 15-nucleotide pal-

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Abbreviations: MR, mineralocorticoid receptor; GR, glucocorticoid receptor; HRE, hormone response element; ZFR, zinc finger region.

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indromic HRE is underlined). The oligonucleotide was end-labeled with T4 DNA kinase (Boehringer Mannheim) and [γ-^{32}P]ATP (Amersham) and subjected to gel purification.

Binding was quantified by an electrophoretic mobility-shift assay. GR or MR DNA-binding domains (1–10 ng) were mixed with 8000 cpm of 5′ ^{32}P-end-labeled HRE in reaction buffer (50 mM NaCl/20 mM Tris·HCl, pH 7.9/1 mM EDTA/10% glycerol/0.1% Nonidet P-40/1 mM DTT/200 ng of poly(dIdC)/100 ng of bovine serum albumin) in a total vol of 16 μl. An anti-GR monoclonal antibody, BuGR (14), directed against amino acids 407–420, was included in the reaction mixtures where indicated (see Fig. 2). After incubation for 15 min at room temperature, reaction mixtures were applied to a 7.8% nondenaturating polyacrylamide gel (37.5:1, acrylamide/bisacrylamide; National Diagnostics) buffered with 0.5× TBE (44.5 mM Tris·HCl/44.5 mM boric acid/1 mM EDTA, pH 8), and resolved by electrophoresis (250 V) at room temperature, followed by autoradiography.

Mutagenesis. Point mutations were introduced into N-terminal deletion derivatives of MR and GR using PCR as described (11). Oligonucleotides were synthesized using an Applied Biosystems DNA/RNA synthesizer (model 394). Silent restriction sites were incorporated into the oligonucleotides in addition to the desired point mutations in amino acid sequence. PCR on a wild-type MR or GR template was accomplished using the desired mutagenic primer paired with a primer either downstream or upstream (depending on the location of the mutation) of a convenient restriction site. The resulting fragments (with the point mutation and desired ends) were then introduced into the vector 6RMR (11) or 6RGR (12) using appropriate restriction sites. PCR-amplified regions of all constructs were sequenced by the dideoxynucleotide chain-termination method (Sequenase; United States Biochemical) to confirm the desired mutation and absence of additional mutations.

RESULTS

We first transfected MR or GR with TAT3-TATA, a reporter construct with three tandem HREs driving a minimal promoter, into CV1b cells. These cells lack endogenous MR and GR and therefore provide a null background for expression of transfected wild-type or mutant receptors (11). We found that MR maximal activity was ≈20% that of GR, as shown in Fig. 1. When we coexpressed the two receptors with TAT3-TATA (Fig. 1, MR + GR), activity was comparable to that achieved by MR alone, indicating that MR has an inhibitory effect on the transcriptional activity of GR in this context. Interestingly, this observation is in contrast to a recent report that identified synergy between MR and GR in a different regulatory context (3). We considered four possible mechanisms for this inhibition: (*i*) occlusion, in which case MR homodimers could block access of GR homodimers to response elements (without direct interaction between the receptors); (*ii*) MR titration of a coactivator (squelching); (*iii*) receptor heterodimerization with formation of a MR–GR heterodimer that is weakly active at TAT3; (*iv*) disruption of GR homodimer activity by an MR homodimer bound at an adjacent site.

In a series of reporter and expression vector titration experiments (data not shown), we found that MR inhibited GR at both high reporter/receptor ratios and at low absolute levels of MR and GR, suggesting that MR inhibition of GR was not due to occlusion or squelching (mechanisms *i* and *ii* above) and consistent with inhibition through direct interaction between the receptors through either heterodimer formation or interaction of receptor homodimers at adjacent sites. We were therefore interested in examining directly whether this inhibitory interaction was mediated by physical contact between the two proteins and, in particular, whether they could heterodimerize. We therefore performed both *in vitro* and *in vivo*

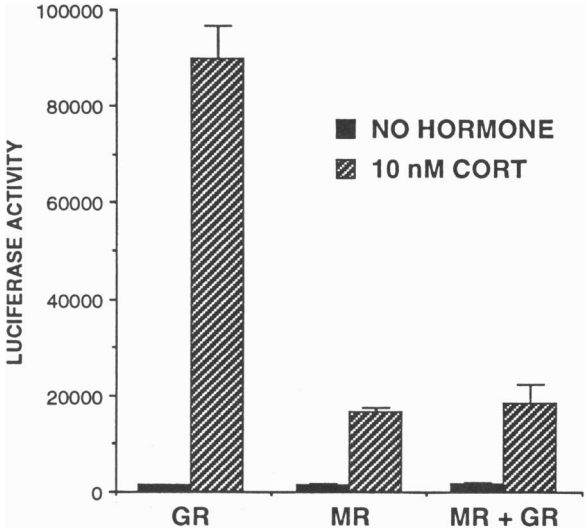

FIG. 1. MR inhibition of GR activity in transfected cells. Receptor-deficient CV-1b cells were transiently transfected with the HRE-containing reporter plasmid TAT3-TATA possessing three near-consensus HREs upstream of a minimal promoter driving expression of the luciferase gene (see text). Expression vectors for the rat GR, rat MR, or both were cotransfected. Cells were incubated for 24 hr with 10 nM corticosterone (cort) as indicated, harvested, and assayed for luciferase activity. Shown is the average of six experiments (±SEM) from four independent transfections.

experiments designed to determine whether MR and GR could form heterodimers.

Previous genetic, biochemical, and structural evidence indicated that GR (5–7, 15–17) and perhaps MR (1, 2) form homodimers through a dimer interface within their ZFRs. MR and GR share complete sequence identity within this ZFR dimer interface, thus suggesting that this region might mediate heterodimerization as well. With these considerations in mind, we first examined whether the ZFR could mediate heterodimerization *in vitro*.

We performed gel-shift experiments using truncated MR and GR derivatives encompassing the ZFR expressed in *E. coli* and purified to near homogeneity. As shown in Fig. 2*A*, incubation of the HRE-containing oligonucleotide with MR or GR derivatives of different sizes resulted in shifted bands with readily distinguishable electrophoretic mobilities, representing receptor homodimers (compare lanes 1 and 11), as demonstrated (2, 17). In lanes 2–10, MR and GR were mixed in various ratios, keeping the total amount of protein constant. A band of intermediate size appears in reaction mixtures containing both receptors, strongly supporting heterodimer formation (18). This intermediate band was disrupted by specific unlabeled DNA competitor but not by nonspecific competitor (data not shown). Furthermore, in experiments in which an anti-GR antibody was included, both the upper band (GR homodimer) and the intermediate band (MR–GR heterodimer) were supershifted, but the lower band (MR homodimer) was not (Fig. 2*B*). An antibody directed at the MR ZFR is not available at this time; however, in preliminary results with an influenza hemagluttinin (HA)/epitope-tagged derivative of MR ZFR (HA/MR DNA-binding domain), we have found a similar supershift of both the MR homodimer and MR–GR heterodimer band by anti-HA antibody. A similar heterodimer band was observed in experiments with a TAT HRE-containing oligonucleotide identical in sequence to the HRE in the TAT3-TATA reporter (data not shown). Thus, we conclude that MR and GR heterodimerize *in vitro*, consistent with the recent observations of Trapp *et al.* (3).

We next sought to evaluate whether or not the receptors heterodimerize at TAT3-TATA *in vivo*. The observation that

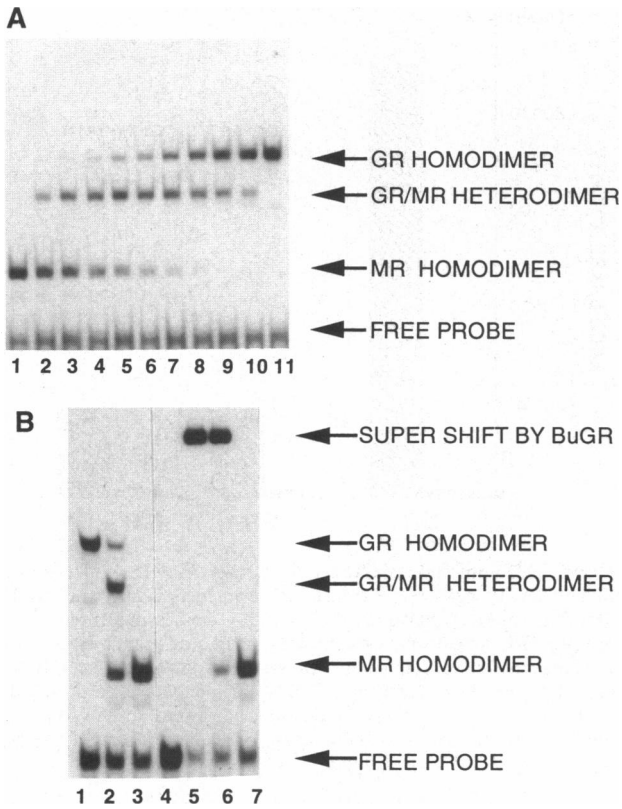

FIG. 2. Heterodimer formation by MR and GR ZFRs *in vitro*. (*A*) Purified ZFR protein fragments incubated with a radiolabeled HRE. Lanes: 1, 10 ng of MR, no GR; 2–10, increasing amounts of GR and decreasing amounts of MR were added, keeping the total protein constant at 10 ng; 11, 10 ng of GR, no MR. (*B*) Anti-GR antibody was included in the reaction mixture (lanes 4–7). Note that the intermediate band (MR–GR) and the upper band (GR) are supershifted by the antibody but the lower band (MR) is not. Lanes: 3 and 7, 10 ng of MR; 2 and 6, 5 ng of MR plus 5 ng of GR; 1 and 5, 10 ng of GR; 4, antibody and probe alone.

the ZFR alone is capable of mediating MR–GR heterodimerization *in vitro* suggested an approach to detecting heterodimer formation in transfected cultured cells; crystallographic (5) and biochemical (17) evidence demonstrated that the dimer interface in the GR ZFR was stabilized by a pair of symmetry-related Asp-Arg salt bridges, which could potentially be used for introduction of compensatory mutations (19–21). If equivalent residues in MR and GR participated in formation of the putative GR–MR heterodimer then genetic complementation might be exploited to demonstrate heterodimer formation (Fig. 3 *A* and *Bi*). Thus, we predicted that an Arg-to-Asp mutation at residue 643 in MR (MR/R643D), for example, would weaken the homodimer interface, thereby decreasing receptor activity (Fig. 3*Bii*), and, similarly, that an Asp-to-Arg mutation at residue 481 in GR (GR/D481R) would reduce its activity (Fig. 3*Biii*). If MR and GR heterodimerized using the same salt bridge, then coexpression of complementary mutants might reestablish the ionic interaction and restore heterodimer formation and, consequently, transcriptional activity (Fig. 3*Biv*).

To avoid the confounding effects of full-length MR's lower activity and inhibitory effect on full-length GR (Fig. 1), we used N-terminal deletion derivatives of MR and GR (MRΔN and GRΔN, respectively) for introduction of point mutations. These derivatives contain receptor sequences necessary for homodimerization but, importantly, they display similar activities at TAT3-TATA (Fig. 4*A*) and other reporters (22) and do not significantly affect each other's activities. Therefore, using these derivatives, heterodimer formation can be probed by

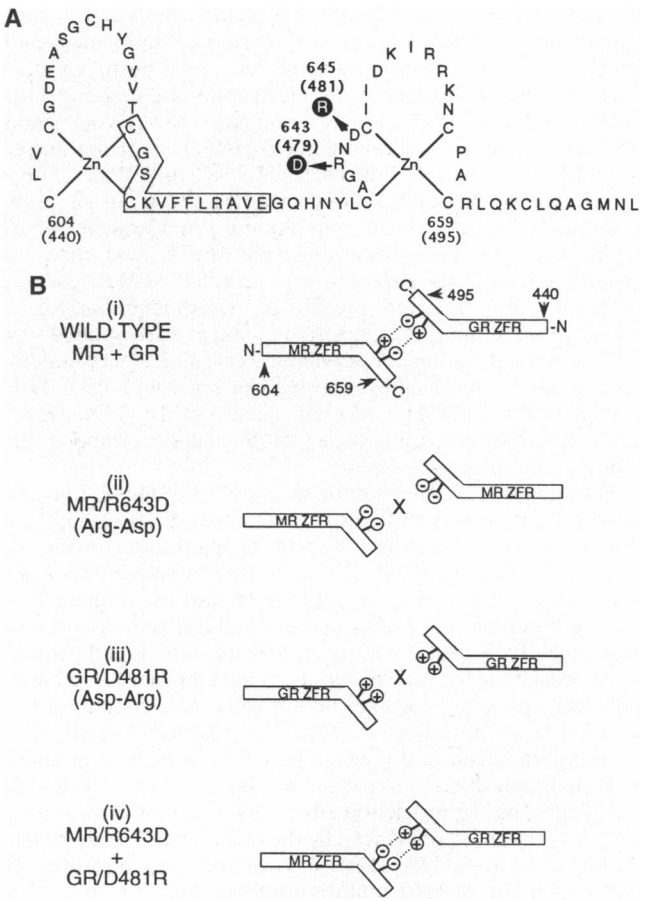

FIG. 3. Schematic diagrams of receptor ZFRs showing amino acid sequence and putative salt bridges. (*A*) Amino acid sequence of MR ZFR is shown with residues that were mutated to create salt bridge mutants illustrated by solid circles with white letters. Boxed region is DNA recognition helix. Numbering corresponds to MR amino acid positions with GR numbers below in parentheses. Note that MR and GR sequences are identical in the DNA recognition helix and the salt bridge region. (*B*) Schematic representation of hypothesized wild-type and mutant ZFR salt bridge interactions. (*i*) Heterodimer interface showing putative Arg (+)–Asp (−) salt bridges (see *A* for amino acid numbers). Dotted line represents ionic interaction. (*ii* and *iii*) Disrupted MR and GR homodimers that are predicted to result from mutations MR/R643D (Arg-to-Asp) and GR/D481R (Asp-to-Arg), respectively. X, Disrupted salt bridge. (*iv*) Hypothesized restoration of dimerization by coexpression of the complementary MR and GR salt bridge mutants. In these schematic views, the DNA is pictured as running horizontally in the plane of the paper and the dimer interface is pictured as coming toward the reader out of the plane of the paper.

comparing the activities of the salt bridge mutants expressed alone and together.

We introduced single point mutations into MRΔN and GRΔN, as shown in Fig. 3, and transfected each with the reporter TAT3-TATA into CV1b cells as in Fig. 1. Consistent with the idea that the Arg-to-Asp and the Asp-to-Arg mutations disrupt receptor homodimerization, the activity of each mutant is markedly decreased relative to the corresponding wild-type receptor (Fig. 4*B*, MRΔN/R643D, MRΔN/D645R, and GRΔN/D481R). We then cotransfected the complementary mutants, MRΔN/R643D and GRΔN/D481R, keeping the total amount of receptor expression vector constant. Coexpression of the complementary mutants restored transcriptional activity to a level approaching that of wild type, strongly suggesting that the receptors heterodimerize at TAT3-TATA *in vivo* (Fig. 4*B*, compare MRΔN/R643D + GRΔN/D481R with MRΔN + GRΔN).

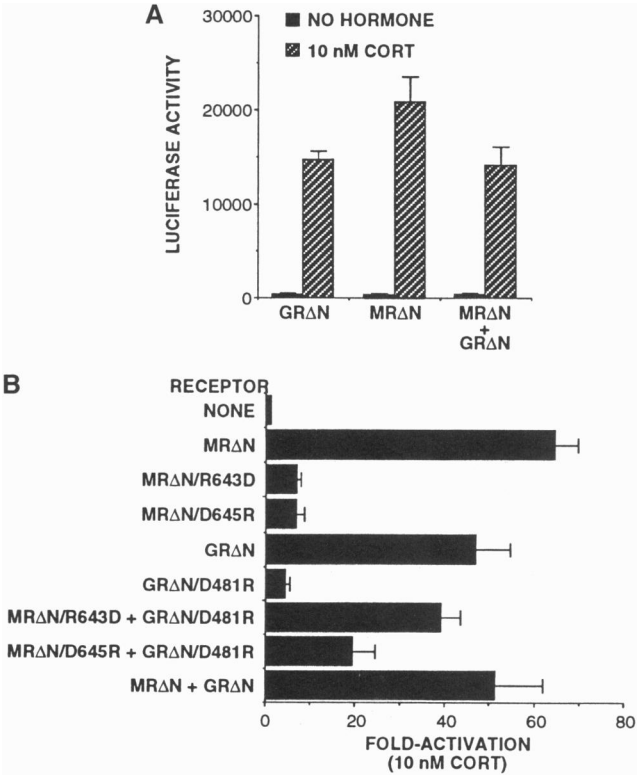

FIG. 4. (*A*) Comparable activities of MR and GR N-terminal deletion derivatives on the reporter TAT3-TATA. TAT3-TATA was cotransfected with MRΔN [also termed MR-596C to indicate that it begins at amino acid 596 and ends with the last amino acid of the C terminus (11)], GRΔN [a similar N-terminal deletion mutant of GR also termed GR-407C (12)], or both, by calcium phosphate precipitation as in Fig. 1. Shown is the average of six experiments (±SEM), representing four different transfections. (*B*) Transcriptional activities of coexpressed complementary and noncomplementary salt bridge mutants in CV1B cells. TAT3-TATA was cotransfected into CV1B cells as in *A* with wild-type receptors or salt bridge mutants as shown; cells were incubated with 10 nM corticosterone. Total expression vector was held constant at 1 μg. Thus, in experiments with MR/R643D and GR/D481R or MR/D645R and GR/D481R there is 0.5 μg of each vector. We also performed these experiments with 1 μg of each vector and obtained approximately twice the level of activity obtained with 0.5 μg of each (not shown). Fold activation is based on no receptor = 1. Shown are means ± SEM (n = 6).

The activity of coexpressed noncomplementary mutants MRΔN/D645R and GRΔN/D481R (Fig. 4*B*) supported the importance of the ZFR salt bridge but also produced a surprising result: transcriptional activity was partially restored (although not to the same level as when the complementary mutants were coexpressed). On the one hand, this result confirms the importance of the salt bridges; however, it also suggests that additional sequences, perhaps in the ligand-binding domain, contribute to receptor heterodimerization. Moreover, while these sequences may be involved in homodimerization as well, they appear to preferentially mediate heterodimerization. Consistent with this idea, homodimer and heterodimer interfaces have been identified in the ligand-binding domains of other intracellular receptors (23–25). The presence of heterodimer-preferring sequences outside the ZFR is also suggested by comparison of our gel-shift data with that of Trapp *et al.* (3), who recently reported that full-length MR and GR heterodimerize preferentially, while we found ZFR heterodimers to form in an ≈1:2:1 ratio (GR homodimer/MR–GR heterodimer/MR homodimer), consistent with random assortment (Fig. 2). Further studies will be needed to localize and characterize these heterodimer-preferring sequences.

DISCUSSION

Taken together, our data suggest that MR inhibition of GR transcriptional activity at TAT3-TATA proceeds through a mechanism involving heterodimer formation. In view of previous reports that GR is capable of synergizing at multiple HREs while MR (at least in some cases) is not, it is plausible that the inhibition we observe results from formation of an MR–GR heterodimer that is incapable of synergy in this context. In particular, we propose that a region within the MR N terminus disrupts GR self-synergy when it is brought into contact with GR through heterodimer formation (mediated by receptor sequences in their respective ZFRs and ligand-binding domains). This interpretation is consistent with observations that the GR N terminus mediates self-synergy (26) and is particularly appealing in light of recent findings suggesting that MR N-terminal sequences prevent MR from self-synergizing (22). Moreover, in preliminary data with a reporter with a single TAT HRE (TAT1-TATA), we find that MR does not inhibit GR activity, also consistent with the idea that inhibition occurs through disruption of synergy. The regulatory role of MR N-terminal sequences with respect to synergy and inhibition will require further examination.

Surprisingly, in contrast to our observations, Trapp *et al.* (3) recently reported that MR and GR activate transcription synergistically through heterodimer formation. The explanation for this opposite effect and the role of heterodimer formation are unclear—the transfection conditions, cell types, and reporters were different. However, it is unlikely that differences in MR/GR ratio play a role in this discrepancy. Trapp *et al.* found synergy at several MR/GR ratios, while we find inhibition at ratios ranging from 1:4 to 4:1 (data not shown). It is appealing to speculate that the disparate results are due to a context-dependent regulatory switch; perhaps nonreceptor regulatory factors and/or DNA target sequence shift the MR–GR heterodimer from inhibitory to synergistic in a manner reminiscent of the influence of nonreceptor factors on receptor behavior at composite response elements (27–29). Alternatively, receptor behavior might be altered by posttranslational modification such as phosphorylation.

We have shown both *in vitro* and *in vivo* that MR and GR can form functionally significant heterodimers and have identified specific residues involved in heterodimerization through the construction of complementary salt bridge mutants. Together with the recent demonstration of heterodimer formation *in vitro* by Trapp *et al.*, these findings challenge the commonly held view that steroid receptors form only homodimers (30, 31). In view of physiological studies suggesting receptor interaction, it seems likely that MR–GR heterodimers play an essential role in mediating responses to corticosteroids in some tissues. In particular, receptor heterodimers may contribute to the biphasic excitatory response of hippocampal neurons to corticosterone (8) as well as the inhibitory effect of corticosterone in the brain on blood pressure (10).

It is interesting to note that the androgen and progesterone receptors (AR and PR, respectively) share a high degree of sequence homology with MR and GR in their ZFRs including Asp and Arg in the homologous positions of the dimer interface, suggesting the potential for heterodimer formation with other members of the subfamily. Heterodimerization between GR or MR and AR would be consistent with physiological observations of interaction between the corticosteroid and androgen pathways (32–34). Thus, heterodimerization may be a general feature of the steroid receptor family, as has been found with the nuclear receptors (35). It seems likely that the integrated regulatory effects of MR and GR (and perhaps AR and PR) are influenced by receptor dimerization state as well as nonreceptor regulatory factors (11, 31, 36) at diverse DNA targets. The resulting regulatory flexibility would allow a few simple components to produce complex cell

type-specific patterns of gene expression in response to hormonal signals.

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