Determinants of Subnuclear Organization of Mineralocorticoid Receptor Characterized through Analysis of Wild Type and Mutant Receptors*

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The mineralocorticoid receptor (MR) is a hormone-dependent regulator of gene transcription that in the absence of ligand resides both in the cytoplasm and the nucleus. Agonists but not antagonists increase the number of MRs residing in the nucleus and cause aggregation of MRs into distinct clusters. To identify the functional determinants of MR nuclear organization, we examined the localization pattern of wild type MR and a series of mutants in the presence and absence of ligands using fluorescent protein chimeras in living cells. Our data show that although MR DNA binding is not necessary to mediate nuclear localization, it is absolutely required for wild type cluster formation as is an intact N-terminal or C-terminal activation function. In contrast, destabilization of a dimerization motif within the DNA-binding domain has no effect on subnuclear receptor architecture. These data suggest that normal MR cluster formation is dependent on both DNA binding and intact transcriptional activation functions but not on DNA-dependent receptor dimerization. Because dimer mutants bind with high affinity to hormone response element DNA multimers but not to single palindromic DNA sites, we suggest that clusters represent MR aggregates bound to DNA response element multimers in the vicinity of regulated genes.

The nuclear receptors are hormone-activated regulators of gene transcription. Following the binding of a cognate agonist, these receptors undergo a change in conformation, enter the nucleus, and/or alter their subnuclear localization (1, 2), steps that are essential for the subsequent regulation of gene expression (3). The mineralocorticoid receptor (MR), a member of the glucocorticoid receptor (GR) subfamily of steroid receptors that also includes the progesterone and androgen receptors (4), provides a particularly striking example of context-dependent regulation. It can either activate or repress gene transcription in a highly context-dependent fashion (5, 6), and moreover, the receptor and its close relative, the GR, have indistinguishable activities in some contexts, whereas in others their activities are strikingly distinct (5, 7, 8).

The “context” of receptor function is determined by both non-DNA factors that modify receptor activity (e.g. coactivators, corepressors, and other transcription factors) and the DNA itself. Several different types of cis-acting DNA sequences, termed hormone response elements (HREs), have been identified, each of which has distinct structural features and patterns of receptor response. 1) “simple” HREs are composed of two palindromic half-sites separated by three nucleotides (9, 10). An intact DBD dimer interface is essential for binding and activation at simple HREs, and the receptors bind as homodimers or heterodimers to stimulate transcription of linked genes (11, 12). 2) Compound HREs are composed of multiple simple HREs of variable spacing. Their behavior is similar to simple HREs in many respects (e.g. protein-DNA interaction is required for function, and they invariably mediate stimulation and never repression of gene transcription), however, the mode of receptor binding and activation of transcription are quite distinct from simple HREs. Synergy is an important determinant of receptor activity at compound HREs, and the DBD dimer interface is dispensable for the binding and activation of transcription (7, 13). 3) Tethering sites mediate repression of transcription through protein-protein interaction. The receptors do not bind directly to these sites but rather are “tethered” through interaction with other DNA-bound transcription factors (6, 14). 4) Lastly, composite elements bind both the receptor and non-receptor transcription factors and mediate either stimulation or repression of transcription depending on the composition of other transcription factors bound nearby (15–17).

Much has been learned about the mechanisms of transcriptional regulation by the steroid receptors through the functional analysis of both naturally occurring (18, 19) and artificially constructed (7, 20) mutant receptors at these various types of HREs. In particular, some receptor functions can be reconstituted by isolated structural motifs that are transferable from the receptor to a heterologous context (21, 22). Other functions require multiple receptor domains and are disrupted by elimination of any significant amount of receptor sequence. For example, trans-repression at composite or tethering sites is lost if either of the receptor N- or C-terminal sequences is deleted (6, 7, 14). In contrast, MR or GR deleted of its C terminus containing the ligand-binding domain and activation function-2 motif strongly stimulates transcription of genes linked to simple or compound HREs even in the absence of
agonists (23, 24), whereas the deletion of the N-terminal activation function motif markedly diminishes activation at simple but not compound HREs (7, 8, 12).

Recently, derivatives of jellyfish green fluorescent protein (GFP) have been used effectively as tags to examine the subcellular distribution of a variety of proteins including steroid and nuclear receptors (1, 2, 25–29). Interestingly, in contrast to GR, MR is both nuclear and cytoplasmic in the absence of hormone in many cell types (1), although it is excluded from the nucleolus. Upon hormone addition, the remaining extranuclear MR accumulates in the nucleus, and a striking alteration in nuclear organization occurs. The receptors coalesce into 1500–4500 clusters, each containing ~10–50 MR molecules that remain excluded from the nucleolus (1). A similar pattern of subnuclear organization has been observed for GR in the presence of agonists, however, GR is almost entirely cytoplasmic in the absence of hormone (2, 30).

The role of subnuclear localization and organization in transcriptional regulatory behavior and the determinants of cluster formation remain poorly understood not only for MR but for other nuclear receptors as well. Previous studies have characterized steroid receptor nuclear localization signals (30, 31), however, the role of determinants other than that of the nuclear localization signal (NLS) on receptor accumulation and retention remains poorly understood. Furthermore, the functional determinants of clustering and, in particular, its relationship to DNA binding and activation of gene transcription are unknown. Therefore, we set out to characterize the determinants of subnuclear distribution of MR and to relate these to the determinants of receptor transcriptional activity by examining the nuclear distribution pattern and transcriptional activities of a series of MR mutants.

EXPERIMENTAL PROCEDURES

Plasmids—Construction of the GFP-MR plasmid (pEGFP-MR), containing sequences encoding fluorescence-enhanced jellyfish GFP (8) fused to full-length rat MR (11) in plasmid pEGFP-C1 (CLONTECH, Palo Alto, CA, see Ref. 26), has been described. In the resulting fusion protein, the C-terminus of EGFP is coupled to the N-terminus of full-length MR through a Ser-Gly-Leu-Arg-Ser sequence. Yellow fluorescent protein (YFP)-tagged and cyan fluorescent protein (CFP)-tagged MR derivatives were similarly generated using the vectors pYFP-C1 and pCFP-C1 (CLONTECH), respectively. In all cases, the autofluorescent proteins are N-terminal to the MR sequences. Truncated MR derivatives (shown in Fig. 1) were generated as follows. pEGFP-MR/BglII-BglII fragment in pEGFP-MR with BamHI-BglII fragment in pEGFP-MR was of expected sizes (data not shown), and although the transfected with 50 ng of TAT3-luciferase (TAT3-LUC) and 50 ng TAT1-CAT reporter and 500 ng of pEGFP-3RMR or appropriate mutant as described previously (1, 7). Activities of fluorescence-tagged proteins were tested the mutants to be used in the localization studies. In all cases, the behavior of the GFP-fusion derivative was qualitatively similar or point-mutants as specified below. Fluorescence images were captured on a PXL-cooled CCD camera (Photometrics) attached to a Olympus IMT2 microscope equipped with an epifluorescence attachment and standard fluorescein isothiocyanate, 4,6-diamidino-2-phenylindole, and Texas Red filter sets using a ×60 planapo objective (N.A. 1.4, Nikon). For fluorescence confocal microscopy, a Bio-Rad MRC-1024 laser-scanning confocal system was used.

RESULTS

We previously demonstrated that agonists increase MR nuclear predominance and cause a marked change in its subnuclear distribution. To determine the relationship between MR activity and its subnuclear distribution, we examined the transcriptional activity and localization of a series of MR deletion and point mutants (shown schematically in Fig. 1, A and B) fused to jellyfish autofluorescent proteins. We first examined the transcriptional activities of the fusion proteins to confirm that their functional characteristics reflected those of their untagged counterparts. As shown in Figs. 2, A and B, GFP-MR gave strong hormone-dependent activation of reporter genes driven by either one HRE (TAT1-CAT) or three HREs (TAT3-luciferase) as shown previously (1, 7). We next tested the mutants to be used in the localization studies. In all cases, the behavior of the GFP-fusion derivative was qualitatively similar to that of the untagged version (Ref. 1, and data not shown). Aldosterone-induced transcriptional activity was only seen at very high hormone levels when receptors were not cotransfected (≥10−8 M, data not shown) presumably because of the activation of small amounts of MR present in the cells (5). Finally, GFP-MR-immunoreactive proteins were characterized by immunoblotting using anti-GFP antibody as described under "Experimental Procedures." The fusion proteins were all made and were of expected sizes (data not shown), and although the truncated proteins were expressed at higher levels than the full-length proteins, all of the full-length proteins were expressed at comparable levels including the transcriptionally non-functional MR/C604S. As addressed under "Discussion," it seems improbable that differences in activity or subcellular localization were the result of differences in expression levels.

We next examined the cellular distribution of the fusion proteins in the presence and absence of hormone. We began transfections were carried out using LipofectAMINE with 20 ng of the GFP/YFP/CFP-MR plasmid/coverslip. Expression of GFP-fusion proteins in CV1 cells was confirmed by immunoblotting using standard methods as described previously (32). Immunoblots were probed using living cells monocular antibody JL-8 against GFP (CLONTECH) at 1:1000 dilution, and detected by enhanced chemiluminescence using sheep anti-mouse Ig horseradish peroxidase-linked whole antibody (Amersham Biosciences, Inc.).

Determination of Transcriptional Activity—CV1 cells were transfected with 50 ng of TAT3-luciferase (TAT3-LUC) and 50 ng TAT1-CAT reporters and 500 ng of pEGFP-3RMR or appropriate mutant as described previously (1, 7). Activities of fluorescence-tagged proteins were determined in the presence and absence of the MR proteins as described previously (1). 20 ng of Rous sarcoma virus-β-galactosidase plasmid was included as an internal control, and the BlueScript KS vector plasmid was used as a carrier to bring total amount of DNA to 1 μg. Fresh medium containing 5% stripped serum was added to cells 18 h before transfection, and the cells were transfected using 1 μl of Opti-MEM I-reduced serum medium containing LipofectAMINE-DNA complex and incubated at 37 °C. 5 h later, 1 μl of fresh Dulbecco’s modified Eagle’s medium containing 10% stripped fetal bovine serum was added without removing the transfection mixture, and the cells were kept at 30 °C. 16 h later, the medium containing 10−8 M aldosterone was added to one of two identical transfections. 24 h later, cells were harvested and assayed for luciferase and CAT activity as described previously (1, 7).

Fluorescence Microscopy—6–18 h after transfection with MR constructs, the medium was replaced with one containing no serum, and the coverslips were placed into a heated chamber under the fluorescence microscope. The cells were kept at 37 °C during microscopy. The chamber was perfused with culture medium (control period) followed by MR agonists or antagonists as specified below. Fluorescence images were captured on a PXL-cooled CCD camera (Photometrics) attached to a Olympus IMT2 microscope equipped with an epifluorescence attachment and standard fluorescein isothiocyanate, 4,6-diamidino-2-phenylindole, and Texas Red filter sets using a ×60 planapo objective (N.A. 1.4, Nikon). For fluorescence confocal microscopy, a Bio-Rad MRC-1024 laser-scanning confocal system was used.
with the MR N-terminal deletion derivative (MRΔN). The transcriptional behavior of this MR mutant is very similar to wild type MR in some regards but is strikingly different in others. In particular, as shown in Fig. 2, A and B, MRΔN stimulates transcription in a hormone-dependent fashion with activity comparable to that of wild type at genes driven by multimerized "simple" HREs, such as TAT3 (Fig. 2B), but activates transcription poorly from a single HRE, like TAT1 (Fig. 2A) (7). Moreover, in addition to the activation function-1 transcriptional activation function, the receptor N terminus is a key determinant of steroid receptor-mediated repression of transcription (5, 6). Hence, it was interesting to examine the distribution of this mutant and compare it with wild type MR to determine whether selective abrogation of MR activities associated with the N terminus would influence its nuclear distribution. CV1 cells were cotransfected with YFP-tagged MRΔN and CFP-tagged wild type MR and prepared for epifluorescence microscopy. As shown in Fig. 3A, in the absence of hormone, MRΔN was found in both the nucleus and cytoplasm in a pattern qualitatively similar to wild type. Aldosterone triggered a rapid increase in nuclear predominance and reconfiguration of the mutant receptor into clusters (Fig. 3B) also in a manner similar to wild type (data not shown, and Ref. 1). However, unlike wild type receptor, MRΔN showed a striking tendency to accumulate in a perinucleolar distribution (Fig. 3B). MRΔN enters the nucleoli and other structures from which the unliganded wild type MR is excluded. Importantly, the mutant receptor segregated into distinct clusters in the presence of the agonist but not of the antagonist (data not shown), although the clusters were less prominent than those observed with the wild type MR.

We next examined the distribution pattern of a C-terminal deletion mutant (MRΔC) fused to GFP. This derivative like the analogous derivative of the GR is constitutively active (i.e. stimulates transcription in the absence of hormone) in some contexts. In particular, it stimulates the transcription of genes linked to simple HREs (Fig. 2, and Refs. 7 and 8). However, unlike the wild type receptor, MRΔN does not repress gene transcription, consistent with the idea that determinants in both the N and C termini are required for the repression of gene transcription (6, 14). The nuclear localization and pattern of nuclear accumulation of GFP-MRΔC was examined by epifluorescence microscopy in the absence and presence of aldosterone. As shown in Fig. 4, MRΔC was found almost entirely in the nucleus in the absence of hormone, and as expected, its distribution was unaffected by hormone (data not shown). Importantly, in both the presence and absence of aldosterone, the receptor was found in subnuclear clusters indistinguishable from those seen with wild type receptor in the presence of aldosterone. Hence, the accumulation of MR into clusters is linked to receptor activation; it is not dependent on the ligand-binding domain and is not attributed to any direct effect of hormone per se.
MR showed prominent perinucleolar accumulation. MR formed clusters in response to hormone (Fig. 2, and B). Unlike wild type MR, BA

Confocal imaging revealed that wild type and N-terminally deleted MR formed clusters in response to hormone (Fig. 2, B). Unlike wild type MR, MRΔN showed prominent perinucleolar accumulation.

MRΔC is exclusively nuclear and forms clusters in the absence of hormone. CV1 cells were transfected as shown in Fig. 2 with GFP-tagged MRΔC. Shown is a confocal image of a cell in the absence of aldosterone. A pattern in the presence of aldosterone was indistinguishable (data not shown).

It is clear from the above observations that MR, deleted of either its N- or C-terminal activation function, still translates to the nucleus and forms clusters that are morphologically similar to those formed by wild type, although additionally, MRΔN but not MRΔC accumulates in a perinucleolar pattern. Two major reasons were considered for the similarity between wild type and mutant clusters. 1) The key determinants of cluster formation and nuclear localization are contained within the DBD, and both N- and C-terminal regions are dispensable. 2) N- and C-terminal regions are redundant, and one but not both is required. To determine whether the DBD and NLS alone are sufficient to direct nuclear localization and cluster formation, we next examined the distribution of the GFP-tagged MR DBD fragment (Fig. 1). As with all of the steroid receptors, this derivative of MR can bind to DNA but has very low transcriptional activity (Fig. 2, A and B). As shown in Fig. 5, GFP-MR/DBD, like GFP-MRΔC, is entirely nuclear in the absence of hormone, consistent with observations with other steroid receptors that a nuclear localization signal is found within the DBD (30, 31, 33). Note also that a canonical NLS is present at the C-terminal end of the MR DBD (Fig. 1B). However, the pattern of nuclear distribution of the DBD alone is quite different from that of wild type or MRAN (in the presence of hormone) or of MRΔC (in the absence of presence of hormone). First, it displays prominent nuclear localization. Second, although its distribution within the nucleus is not completely homogenous, clusters reminiscent of those formed by the wild type MR are conspicuously absent. Furthermore, the nuclear distribution of the GFP-MR/DBD consistently parallels chromatin density as revealed by Hoechst staining (data not shown). This is in sharp contrast to the clusters formed by the wild type MR, which are excluded from regions of condensed chromatin, rather showing a predilection for the regions of transcriptionally active euchromatin (1). The lack of cluster formation is reminiscent of the wild type MR in the presence of the antagonist. However, antagonist-bound wild type MR is still excluded from the nucleolus, in striking contrast to MR/DBD. Further supporting the idea that cluster formation is linked to receptor competence to stimulate gene transcription is the observation that an MR mutation, which renders it capable of activating but not repressing transcription (20), forms wild type clusters (data not shown).

We next wanted to examine the role of DNA binding on nuclear localization and cluster formation. MR and GR, together with the progesterone and androgen receptors, form a subgroup within the nuclear receptor superfamily that binds to overlapping palindromic DNA response elements and has homologous DNA-binding domains. Within this region are determinants of both protein-DNA interaction (within the first zinc finger, ZF1) and dimerization (within the second zinc finger, ZF2) as shown schematically in Fig. 1B. The ZF2 dimer interface stabilizes receptor DNA binding to some but not all palindromic sites (7, 18), whereas an α-helix within ZF1 intercalates into the major groove of the HRE and is essential for DNA binding (34, 35). The above observations suggested that the receptor DBD and associated NLS are sufficient to mediate nuclear localization but not cluster formation. To examine whether DNA binding is necessary for stable nuclear localization and cluster formation, we next examined the effect of mutations that disrupt the DBD dimer interface on receptor distribution in response to aldosterone. As shown in Fig. 6, the behavior of the dimer mutant, GFP-MR/R643D is very similar to that of the wild type MR, despite its strikingly different behavior at two different types of HRE (Fig. 2, A and B). Notably, the clusters formed by MR/R643D in the presence of aldosterone demonstrated the same dynamic characteristics as

![Image](https://via.placeholder.com/150)

**Fig. 3.** MR deleted of its N terminus demonstrates enhanced perinucleolar localization compared with wild type. CV1 cells were cotransfected with CFP-tagged wild type MR (CFP-MR) and YFP-tagged MRΔN (YFP-MRΔN) and exposed to 1 nM aldosterone (aldo) or vehicle as shown. Standard epifluorescence images were then obtained (A). Confocal imaging revealed that wild type and N-terminally deleted MR formed clusters in response to hormone (B). Unlike wild type MR, MRΔN showed prominent perinucleolar accumulation.

**Fig. 4.** GFP-MR/DBD accumulates in the nucleus but does not form clusters. CV1 cells were transfected again as in Figs. 2 and 3 with GFP-tagged MR/DBD. Shown is a nucleus in the absence of hormone. Cells exposed to 1 nM aldosterone were indistinguishable. MR accumulation is entirely nuclear, but clusters are not formed and the DBD peptide is not excluded from the nucleolus.

**Fig. 5.** MR/DBD accumulates in the nucleus but does not form clusters. CV1 cells were transfected as shown in Figs. 2 and 3 with GFP-tagged MR/DBD. Shown is a nucleus in the absence of hormone. Cells exposed to 1 nM aldosterone were indistinguishable. MR accumulation is entirely nuclear, but clusters are not formed and the DBD peptide is not excluded from the nucleolus.
cells were transfected as in Figs. 2–4 with GFP-tagged MR/R643D and imaged under control conditions (left hand panel), exposed to 1 nM aldosterone for 30 min (middle panel), and then exposed to 100 nM of the MR antagonist ZK91587 for 60 min (right hand panel).

Those formed by wild type. An addition of an excess of the MR antagonist ZK91587 caused rapid transformation of MR from a clustered to a diffuse pattern (Fig. 6) (data not shown, and Ref. 1). The distribution of another salt bridge mutant GFP-MR/D645R was also indistinguishable from that of wild type MR (data not shown).

We conclude from these results that nuclear localization and cluster formation do not require the DBD dimer interface and may not require specific DNA binding. However, it is important to note that the disruption of the DBD dimer interface disrupts MR and GR DNA binding and activity at only a subset of HREs. Indeed, dimer mutants of either MR or GR bind to compound elements (composed of multiple HREs in tandem) with affinity similar to that of wild type, and interestingly, their activity at such sites is greater than that of wild type MR (7) (Fig. 2B). Moreover, the dimer interface mutations do not interfere with receptor-mediated repression, and the replacement of the wild type GR gene with a GR dimer mutant is non-lethal in mice in contrast to GR deletion, which is uniformly lethal (36, 37).

Hence, the disruption of the DBD dimer interface interaction surface imparts a complex phenotype, and the dimer mutants do not reflect the simple abrogation of DNA binding (7, 18).

With the above observations in mind, we next wanted to examine the effect on nuclear localization and organization of completely destabilizing the receptor-DNA interaction. Therefore, we examined the cellular distribution of MR mutants that were unable to bind DNA because of disruption of the zinc coordination motif of the first zinc finger within the DBD (Fig. 1B). This mutant fails to stimulate transcription at either a single (TAT1) or compound (TAT3) HRE (Fig. 2). As shown in Fig. 7, distribution in cytoplasm and nucleus of the mutant MR/C604S in the absence of hormone is similar to that of wild type MR. However, although aldosterone drives the remaining receptor into the nucleus with a similar time course to that of wild type, MR/C604S forms clusters in the presence of aldosterone that are markedly different from those formed by wild type MR, both in their morphology and in their fewer numbers. Another DNA-binding-deficient MR, GFP-MR/C607S, behaved very similarly to GFP-MR/C604S (data not shown).

**DISCUSSION**

The wild type MR undergoes not only a change in cellular distribution in response to agonists (26) but forms distinct subnuclear clusters that are correlated with the activated state of the receptor (1). Importantly, although both agonists and antagonists induce nuclear localization of the receptor, only agonists induce clustering, implying an association between cluster formation and the active conformation of the receptor. By characterizing a series of mutants designed to selectively disrupt various receptor functions, our present study offers insight into the functional determinants of MR subnuclear organization and the relationship between nuclear distribution and activity. One of the most striking findings presented here is that the C-terminally deleted MR is not only localized predominantly to the nucleus in the absence of hormone, but that it forms clusters indistinguishable from those formed by wild type MR in the presence of agonists but not antagonists. We have shown previously that the C-terminally deleted MR does not repress transcription under conditions where the full-length does. However, like its close relative, the GR, this MR mutant does bind to DNA and stimulates a reporter gene driven by simple HREs (7). Indeed, the receptor C-terminal domain appears to provide a general inactivation function that is released by hormone and that can be transferred to other transcription factors and indeed other unrelated proteins (38).

However, as witnessed by the failure of receptor derivatives lacking this function to repress transcription, it is clear that this is not the sole function of the ligand-binding domain. Indeed, other functions have been clearly localized to the steroid receptor ligand-binding domain including an activation function (most often referred to as “AF2”), nuclear localization signal, and coregulator interaction domains (22, 30, 39).

Interestingly, although the DBD fragment including the NLS was strongly localized to the nucleus, it did not form clusters. These data suggest that although the DBD is sufficient to mediate nuclear localization, cluster formation requires either N- or C-terminal sequences but not both to be present. Importantly, the DNA-binding-deficient mutant MR/C604S does aggregate into clusters (Fig. 7), although they are abnormal in morphology and number, consistent with the idea that wild type clusters require DNA binding for appropriate targeting and interreceptor interactions through either N- or C-terminal sequences for aggregation. Aggregation can occur in the absence of DNA binding, but aggregates are abnormal presumably because DNA targeting is absent or abnormal. In contrast, the normal morphology of the clusters formed by dimerization-deficient mutants (Fig. 6) (1) indicates that the interaction through this motif is not required for aggregation, nor is binding to single palindromic HREs. MR dimer mutants bind to compound HREs (i.e. HREs consisting of multiple palindromic receptor binding sites) with an affinity similar to that of wild type (7), and their nonspecific DNA binding is indistinguishable from wild type,2 however, their binding to single palindromic HREs is poor. It is also possible, but seems less likely, that the C604S mutation disrupts a function of the DBD unrelated to DNA binding that is required for cluster formation (e.g. interaction with the nuclear matrix).

One striking difference between the subnuclear localization of MRbN and either wild type MR or MRAC is the prominent perinucleolar signal that this mutant demonstrates, consistent

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2 D. Pearce, unpublished results.
with localization to the perinuclear compartment. Although this difference is not seen in all cell types (for example, it was found in CV1 cells but not in RCCT cells, data not shown), it suggests that the N terminus may exclude the receptor from the perinuclear compartment. It should also be noted that the perinuclear accumulation of MR\(\Delta N\) was not induced by the MR antagonist ZK91587, indicating that this accumulation like cluster formation is a function of the activated receptor. The perinuclear compartment is a site of active transcription and RNA metabolism, although the enrichment of MR\(\Delta N\) at these sites is of uncertain significance.

It seems unlikely that any of the differences in receptor behavior are because of differences in expression levels among the various mutants, although this possibility must be considered since the truncated derivatives were expressed at higher levels than the full-length receptors (data not shown). Importantly, the truncated derivatives were all expressed comparably to each other as were the full-length receptors to each other, and none of the differences in activity or cellular localization, such as perinuclear accumulation of MR\(\Delta N\) or a lack of cluster formation of MR/DBD and MR/CD604S, segregated according to receptor size. Furthermore, in a series of transfections of MR/DBD in which the expression level varied over more than a 20-fold range, there was no change in the underlying pattern, only in the signal intensity (data not shown). Hence, it seems most likely that the differences in receptor behavior are because of inherent differences in their function, not expression levels.

Taken together, the above observations suggest that the clusters represent higher order receptor complexes organized on HRE multimers. Particularly relevant to this conclusion is the indistinguishable appearance of clusters formed by wild type and dimer mutant MRs in live cells (Figs. 4 and 6) (1) and the ability of both to form higher order complexes at HRE type and dimer mutant MRs in live cells (Figs. 4 and 6) (1) and cause of inherent differences in their function, not expression.

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