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A 22-Amino-Acid Peptide Restores DNA-Binding Activity to Dimerization-Defective Mutants of the Estrogen Receptor

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We have identified residues within the estrogen receptor that are required for dimerization and high-affinity DNA binding. A 22-amino-acid peptide encompassing these residues was sufficient to restore DNA-binding activity to a mutant receptor lacking most of the hormone-binding domain. Point mutagenesis of the fusion protein confirmed that this sequence continued to mediate dimerization in a manner similar to that within the native receptor, although its position relative to the DNA-binding domain was appreciably altered.

Steroid hormone receptors are members of a class of ligand-inducible transcription factors (2, 4, 9) which bind to their response elements as dimers (12, 19). We have recently shown that the mouse estrogen receptor binds to an estrogen response element in a band shift assay with a K_d of approximately 10^{-9} M. This high-affinity DNA binding is dependent upon the presence of an intact dimerization domain, which is located in the C-terminus of the receptor (5).

Point mutagenesis of the native receptor has identified a number of residues between positions 507 and 518 that are required for receptor dimerization. Any partial C-terminal deletion of the ligand-binding domain beyond this point abolishes both dimerization and DNA-binding activity. However, complete removal of the C-terminal sequences to residue 339 (MOR121-339) reactivates DNA binding but with reduced affinity (K_d, approximately 10^{-8} M). Kumar and Chambon have shown that analogous deletion mutants of the human estrogen receptor function in a similar manner and have concluded that DNA-binding domain itself contains a weak dimerization activity which is masked in some way in the presence of additional C-terminal sequences (12).

We have investigated the extent of the major dimerization domain by analyzing fusion proteins in which the putative dimerization motif (DM) has been linked to mutant non-dimeric receptors which are unable to bind to DNA. A 22-amino-acid peptide, containing amino acids 501 to 522, was selected because it encompasses arginine 507, leucine 511, and isoleucine 518, residues previously shown to be critical for wild-type receptor dimerization (5).

Initially an oligonucleotide encoding DM was cloned into a unique *Eco*RI site at the 3′ end of pMOR121-384 (14). The encoded protein, MOR121-384DM, was then synthesized by in vitro transcription and translation and analyzed for DNA-binding activity in a band shift assay as described previously (14). The fusion protein MOR 121-384 DM was able to bind to the estrogen response element (Fig. 1) even though DM was positioned 117 amino acids closer to the DNA-binding domain. That the dimerization domain can function in a number of positions was supported by the observation that a 57-amino-acid deletion in the wild-type receptor (residues 444 to 500) also allowed DNA-binding activity (data not shown).

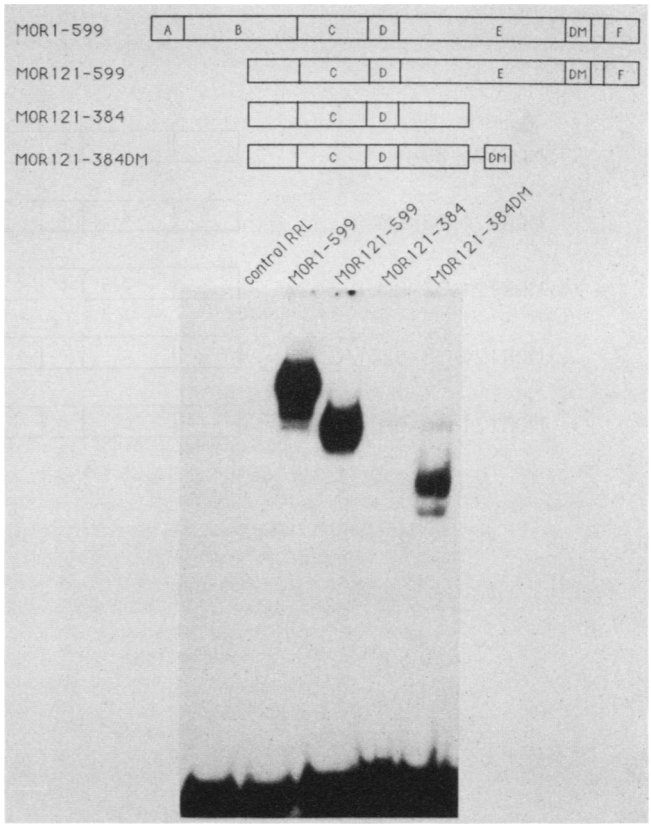

FIG. 1. DNA-binding activity of mouse estrogen receptor fusion proteins. The mutant receptors MOR1-599, MOR121-599, and MOR121-384 (14) are all named according to the residues which they retain. A double-stranded oligonucleotide encoding residues 501 to 522 (LQQQHRRLAQLLLILSHIRHMS) was cloned into pMOR121-384 at the unique *Eco*RI site located immediately after codon 384. The resulting receptor, MOR121-384DM, therefore contains a Glu-Phe linker (encoded by the *Eco*RI site) between the last receptor codon and the DM. A to F represent the regions of homology found within the steroid receptor superfamily (11). The receptor mutants were expressed from SP6-based vectors by in vitro transcription and translation and analyzed for DNA-binding activity in a gel shift assay (14) alongside control unprimed rabbit reticulocyte lysate (RRL).

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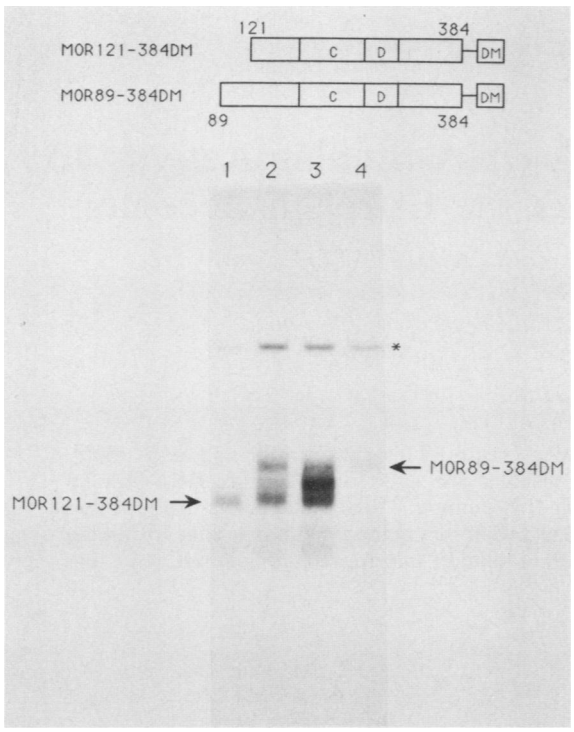
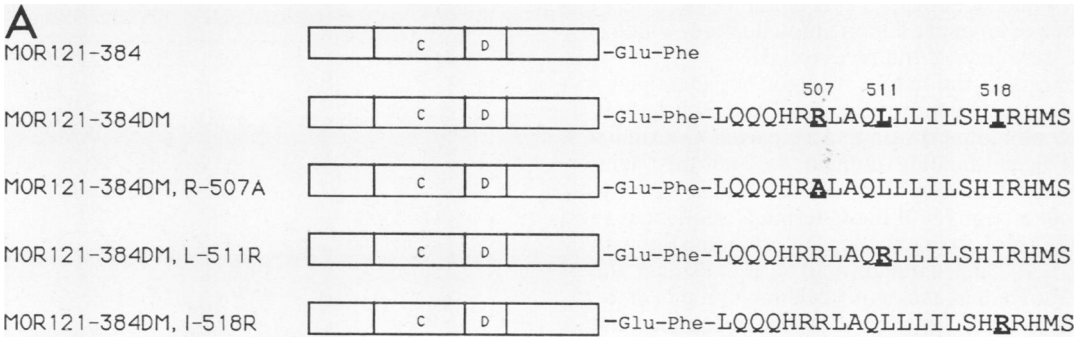


FIG. 2. Dimerization activity of receptor fusion proteins. MOR89-384DM was generated by transferring the N-terminal sequences of MOR89-599 (14) as a *Hind*III-*Xba*I fragment into MOR121-384DM. MOR121-384DM alone (lane 1), separate translations of MOR121-384DM and MOR89-384DM (lane 2), a cotranslation of MOR121-384DM and MOR89-384DM (lane 3), and MOR89-384DM alone (lane 4) were then incubated with 1 ng of the 35-base-pair, ^{32}P-labeled oligonucleotide probe and analyzed for DNA-binding activity in a band shift assay. The complex (*) corresponds to a lysate-specific binding protein.

The ability of the DM peptide 501-522 to promote dimerization was demonstrated by the analysis of heterodimer formation in the band shift assay. In order to conduct these experiments, we generated the mutant MOR89-384DM, in which the linear positioning of the dimerization and DNA-binding domains is identical to that in MOR121-384DM (Fig. 2). While MOR89-384 failed to bind in the band shift assay, MOR89-384DM bound to form a complex of reduced mobility relative to MOR121-384DM, consistent with its increased size (Fig. 2, compare lanes 1 and 4). When MOR89-384DM and MOR121-384DM were cotranslated, three complexes were observed, consistent with dimer formation (Fig. 2, lane 3). Few or no heterodimers were observed when these mutants were separately translated and mixed (Fig. 2, lane 2), demonstrating that they formed stable dimers in solution.



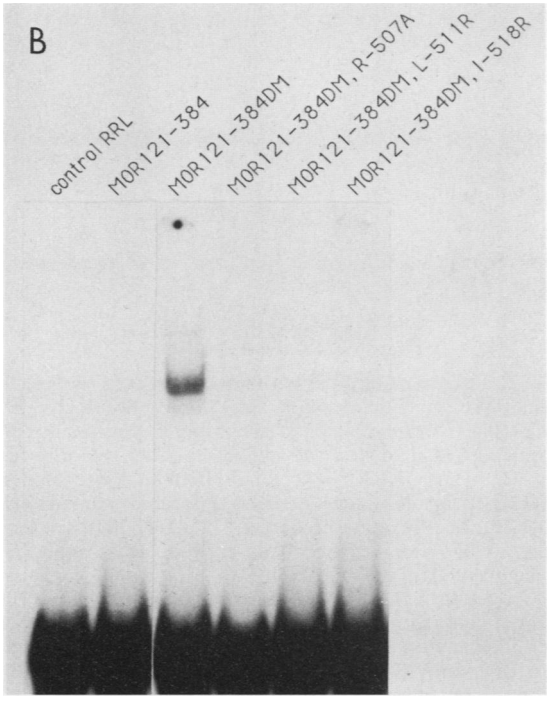


FIG. 3. Effects of point mutations within the DM of MOR121-384DM. (A) Double-stranded oligonucleotide cloning was used to introduce specific point mutations into the DM of MOR121-384DM. These changes were then verified by dideoxy sequencing. Resultant constructs were named according to the amino acid changed and its position in the wild-type receptor, followed by the substituting residue; e.g. MOR121-384DM R-507A contains an alanine at position 507 in place of arginine. The residues in boldface type in MOR121-384DM are critical for dimerization of the wild-type receptor, whereas those in boldface type in MOR121-384DM, R-507A; MOR121-384DM, L-511R; and MOR121-384DM, I-518R are the substituting residues. (B) These chimeric receptors were then translated in parallel in the presence and absence of [^{35}S]methionine, and the labeled reaction mixes were analyzed by electrophoresis to normalize the levels of unlabeled receptors. The DNA-binding activities of the point mutants were then compared with that of MOR121-384DM, alongside control unprimed rabbit reticulocyte lysate (RRL), in the band shift assay.

We previously identified three residues that are critical for dimerization by analyzing the effect of point mutations on the DNA-binding activity of the wild-type estrogen receptor (5). To confirm that the DM of MOR121-384DM functions in a manner similar to that within the native receptor, we tested the effect of these point mutations on the DNA-binding activity of the fusion protein. Substitution of either arginine 507 with alanine (MOR121-384DM R-507A), leucine 511 with arginine (MOR121-384DM L-511R), or isoleucine 518 with arginine (MOR121-384DM, I-518R) resulted in a marked reduction in DNA-binding activity (Fig. 3), similar to that previously observed in the native receptor (5). We therefore conclude that the function of the DM is retained when it is repositioned in the fusion protein.

Two structures have been implicated in the dimerization of DNA-binding proteins, the leucine zipper (7 10, 13, 18) and the helix-loop-helix motif (17). In those proteins characterized to date, these structures lie immediately adjacent to the DNA-binding domain. In contrast, this study demonstrates that the dimerization domain of the mouse estrogen receptor is located more than 250 amino acids from the DNA-binding domain of the native receptor. This motif is well conserved in both sequence and position throughout the steroid receptor family (5) and is contained within the region shown to be required for the heterodimerization of the thyroid hormone and retinoic acid receptors (6, 8).

From our previous work (5), we concluded that the steroid receptor dimerization domain does not involve a leucine zipper (7, 10, 13, 18) or coiled-coil structure (3), but despite the difference in positioning relative to the DNA-binding domain, we could not rule out the helix-loop-helix motif. Two potential amphipathic helices are located in this region of the mouse estrogen receptor, between residues 478 to 492 and 508 to 522. Since we have now demonstrated that residues 501 to 522 alone are sufficient to restore DNA binding, we conclude that a helix-loop-helix structure is unlikely to mediate receptor dimerization. The conserved motif within steroid receptors therefore appears to represent a novel dimerization domain for DNA-binding proteins.

Recently, we suggested that the structure of this motif may be similar to that of a major dimer interface within the progesterone-binding protein uteroglobin (1, 15, 16). This is generated by an antiparallel interaction of helix 3, β-turn 2, formed by residues 39 to 49 (1). A homologous sequence is present in all the steroid receptors, with highest similarity in the progesterone receptor (residues 871 to 881 in the human protein), and corresponds to a major portion of the dimerization motif described in this study. Since we have shown that this region mediates estrogen receptor dimerization, our data are entirely consistent with this model.

In summary, we have shown that receptor residues 501 to 522 are sufficient to induce DNA-binding activity and appear to encode a major portion of the dimer interface of the mouse estrogen receptor. This region is conserved throughout the steroid receptor superfamily but, in contrast to previously identified dimerization domains, is positioned more than 250 amino acids from the DNA-binding domain.

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