Analysis of Mechanisms That Determine Dominant Negative Estrogen Receptor Effectiveness*

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To analyze the mechanisms by which estrogen receptor (ER) activity is suppressed by dominant negative mutants, we examined the role of specific ER functions and domains in transcriptional repression. We previously described three transcriptionally inactive human ER mutants (the frameshift mutant S554fs, the point mutant L540Q, and the truncated receptor ER1–530), which act as effective dominant negative mutants, inhibiting the activity of wild type ER when they are co-expressed in mammalian cells. After additional mutational modifications, the ability of the ER mutants to suppress the activity of wild type ER was analyzed in cotransfection assays of the dominant negative mutants and wild type ER and an estrogen-responsive reporter gene (2ERE-TATA-CAT or 2ERE-p52-CAT). Eliminating the ability of the three dominant negative mutants to bind to estrogen response element (ERE) DNA (by introducing three point mutations in their DNA binding domains) dramatically reduced, but did not completely abolish, the dominant negative activity of the ER mutants. The mutation G521R, which rendered the three mutants incapable of binding estradiol, also reduced, but did not abolish, their dominant negative activity. Immunoprecipitation with monoclonal or flag antibodies followed by Western blotting demonstrated that each of the original dominant negative ER mutants formed heterodimers with wild type ER. Rendering the dominant negative mutants dimerization deficient by the mutation L507R strongly reduced, but did not eliminate, their dominant negative activity. Deletion of the N-terminal A/B domain resulted in the nearly complete loss of inhibitory activity of the three dominant negative mutants. However, these double mutants retained their ability to heterodimerize with wild type ER, suggesting that dominant negative interference also occurs at an additional step beyond dimerization. Our data indicate that competition for ERE binding, formation of inactive heterodimers, and specific transcriptional silencing can all contribute to the dominant negative phenotype and that these receptors suppress the activity of wild type ER by acting at multiple steps in the ER-response pathway.

The human estrogen receptor (ER) is a hormone-activated transcription factor that regulates the expression of specific genes in a variety of estrogen-responsive target cells (1–5). It is a member of a large superfamily of steroid/nuclear receptors, which shares a common domain structure identified on the basis of amino acid homology and functional similarity. The ER consists of six domains (A–F), which include at least two activation functions (present in the A/B and E domains), a DNA binding region (domain C), and ligand binding and dimerization regions present in domain E (1–7). Previous biochemical studies by us and others (8–12) have identified particular amino acids and regions of the receptor that are critically involved in some of these functions. Despite this considerable progress in mapping the ER, the interactions that lead to transcription activation in response to ligand binding still remain to be defined.

We have been interested in the development and analysis of dominant negative estrogen receptors, which suppress the activity of wild type ER when they are co-expressed in the same cells (13). These mutants should provide considerable insight into the mechanism of estrogen receptor action and how its various activities map onto its domain structure. In addition, these mutants have the potential to suppress estrogen receptor-stimulated responses and may ultimately be useful in regulating cell-specific gene expression and the proliferation of breast cancer and other estrogen-responsive cells.

Dominant negative receptor mutants are slightly altered proteins, which themselves are transcriptionally inactive but retain the ability to suppress the activity of the wild type receptor (14). Previously, we reported the generation and isolation of three dominant negative mutants of the human ER: a frameshift mutant (S554fs), a point mutant (L540Q), and a truncated receptor (ER1–530) missing the last 65 amino acids of the receptor (8, 13). These mutants, generated by random chemical mutagenesis of the E and F domains of the human ER, are altered such that the ligand-dependent transcription activation region in domain E of the ER is inactivated (S554fs and L540Q mutants) or missing (ER1–530 mutant) (13). However, as shown previously, the three mutants bind estradiol with good affinity (8, 13). When they are either co-expressed with wild type ER in cells (13) or introduced into cells containing endogenous ER (15), these dominant negative receptors were shown to effectively suppress the ability of wild type ER to activate transcription. Although naturally occurring dominant negative ERs have been reported in human breast tumors (16, 17) and breast cancer cell lines (18), these are exon deletion mutants that seem to be less potent suppressors of wild type
ER activity than our three dominant negative ERs. Little is known about the mechanisms of action of these exon-deleted trans-dominant negative receptor variants.

In this report, we examine the contributions of DNA binding, hormone binding, dimerization, and the activation functions of the receptor to dominant negative effective ERs. To determine the mechanisms by which dominant negative estrogen receptors are effective as inhibitors of wild type ER, we have introduced into our three original dominant negative estrogen receptors additional mutations that impair binding to the estrogen response element (ERE), that impair dimerization or hormone binding, or that eliminate activation function-1 activity. We find that any of several alterations that result in decreased ability of the mutant ERs to interact with the normal ER transactivation pathway diminish the potency of the dominant negative proteins.

EXPERIMENTAL PROCEDURES

Chemicals and Materials—Cell culture media and sera were purchased from Life Technologies, Inc. ER antibodies were obtained from Abbott Laboratories (Chicago). Flag M2 antibody was from Upstate Biotechnology, Inc. Anti-Flag M2 antibody was from Sigma. [3H]-Acetyl CoA and [3H]-protein A were obtained from DuPont NEN. The chemiluminescence-based ECL system was obtained from Amersham Corp.

Generation of ER Expression Vectors and Analysis of Mutant ER Expression Levels—Generation of pCMV5 ER wild type, ER1–530, L540Q, and L540S expression plasmids was described earlier (8, 13). Generation of A/B region-deleted ER expression vectors was performed by site-directed mutagenesis of the wild type pCMV5 ER as described (19, 20) followed by subcloning the HindIII fragment into each of the three dominant negative estrogen receptor vectors. These mutants initiate translation of the human ER cDNA from amino acid 176 (20). DNA binding domain mutations (E203G, G204S, A207V) (9). To destroy the ability to bind estradiol, a point mutation, G521R (6, 11), was introduced into each of the three dominant negative estrogen receptor vectors. The G521R mutant was introduced into each of the expression vectors by site-directed mutagenesis using the oligonucleotide 5′-CTGTACAGATGCTCCATGCGTTTGTTACTCAG-3′ (21). The construction of Flag-ER in pcDNA3 (Invitrogen), which encodes the Flag peptide (N-Asp-Tyr-Lys-Asp-Asp-Lys-C) at the N terminus of the ER is described elsewhere (21). Mutations were confirmed by sequencing. Equivalent expression levels were verified for the mutant ERs and wild type ER by Western blotting as described previously (13, 20), indicating wild type-like expression/stability of these mutants.

Other Plasmids—The reporter plasmids for transcriptional activation studies, pATCC (22) and ZERE-pS2-CAT (23), were described earlier. The plasmid pTZ19R used as carrier DNA was provided by Dr. Byron Kemper (University of Illinois). The β-galactosidase transfection efficiency internal control plasmids pCH110 and pCMVβ were obtained from Pharmacia Biotech Inc. and Clonetech.

Cell Culture and Transfection—Chinese hamster ovary (CHO) cells were cultured in culture and transfected as described (24, 25). For the transfection experiments, 1.8 × 10⁶ CHO cells were plated per 60-mm plate. These cells were maintained for 48 h in 5% CO2 at 37°C before changing the medium. The cells were then transfected using the calcium phosphate coprecipitation method as described (24, 25) using 0.4 ml of DNA precipitate containing 1.6 µg of pATCC or 1.0 µg of pERE-pS2-CAT, 0.3 µg of pCH110 or pCMVβ, 1.5–20 ng of pCMV5-ER expression vectors, and pTZ19R carrier DNA to 15 µg total DNA per 100-mm dish (25). Transfected cells were treated with estradiol or vehicle for 24 h before harvesting. Chloramphenicol acetyl transferase assays were performed as described previously (26).

COS-1 cells were used to express high levels of the ER for immunoprecipitation studies using a modification of the method of Reese and Katzenellenbogen (27) exactly as we have recently described (28). For study of in vitro heterodimer formation between the dominant negative ER mutant and wild type ER, COS-1 cells were transfected individually with the expression plasmid encoding a full-length dominant negative ER mutant and expression plasmid encoding a truncated ER (ER1–554). Cell extracts prepared from each transfection were then mixed together, incubated overnight on ice, and then analyzed by immunoprecipitation. For study of in vivo heterodimer formation between the dominant negative ER mutant and wild type ER, COS-1 cells were cotransfected with expression plasmids encoding a truncated ER (ER1–554 or ER1–530) and a full-length ER. Western blots (8, 13) were used to confirm that the proteins were expressed at equivalent levels in the cell.

Immuno-coprecipitation of Heterodimers between Dominant Negative ER Mutant and Wild Type ER—Cell extracts containing 100–200 µg of total protein were precipitated on ice for 30 min with 20 µg of rabbit anti-rat IgG in buffer containing 20 mM Hepes, pH 7.8, 50 mM KC1, 10% glycerol, 1 mM dithiothreitol, as well as protease inhibitors (50 µg/ml leupeptin, 5 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, 5 µg/ml aprotinin), 50 µl of a 10% slurry of zysorbin (Sigma) (zysorbin contains membrane-bound protein A, which binds most IgGs), which was prewashed three times just before use in Sarkosyl-TE (0.2% Sarkosyl, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.5% Nonidet P-40), and was then mixed and the mixture was incubated on ice for 30 min. Nonspecific protein complexes were sedimented by centrifugation at 12,000 × g in a microcentrifuge for 5 min at 4°C. The supernatant was then washed twice with 5 µl of anti-ER monoclonal antibody D75 on ice for 1 h, followed by incubation with 20 µg of rabbit-anti-rat IgG on ice for 30 min; the reaction mixture was then incubated with 50 µl of a 10% slurry of zysorbin (prewashed as described above). When monolocal flag antibody M2 was used to immunoprecipitate the Flag-HER, 20 µg of antibody M2 was used in the immunoprecipitation with the Flag-HER, and rabbit anti-mouse IgG was used as the second antibody. The immunoprecipitates were pelleted by centrifugation in a microcentrifuge at 12,000 × g for 5 min at 4°C, released into SDS sample buffer by boiling for 5 min, and the Zysorbin sedimented by centrifugation for 5 min at 40°C. The supernatants were then analyzed by SDS-polyacrylamide gel electrophoresis. Western blots were carried out using anti-ER monoclonal antibody DS47 as we have described (13).

GAL Mobility Shift Assays—GAL mobility shift assays using an ERE-containing oligonucleotide were carried out as we have recently described (13, 28).

RESULTS

Dominant Negative Estrogen Receptor Mutants Analyzed—Fig. 1 is a schematic of the wild type and mutant estrogen receptors that we analyzed in these studies. Shown are the three dominant negative mutants (S554fs, L540Q, and L540S) that we originally generated by random chemical mutagenesis of the E and F domains of the human ER. We previously demonstrated that these ER mutants are unable to activate transcription but retain the ability to bind estradiol and the ERE and are potent dominant negative mutants (13). To determine the mechanisms by which these dominant negative estrogen receptors are effective as inhibitors of wild type ER, we introduced additional mutations that eliminate various receptor functions or domains (Fig. 1). To block binding to the ERE, we made three amino acid changes in the DNA binding domain (E203G, G204S, A207V) (9). To destroy the ability to bind estradiol, a point mutation, G521R (6, 11), was introduced into each of the three dominant negative mutants; to impair receptor dimerization, amino acid 507 was changed from leucine to arginine (L507R) (6). To eliminate the N-terminal activation function (AF-1), the A/B domain of the three dominant negative receptors was deleted.

DNA Binding Ability Is Required for Efficient Dominant Negative Repression of Wild Type ER Activity—To evaluate the importance of competition for binding to the ERE in the effectiveness of the three dominant negative ER mutants, we introduced secondary mutations into the cDNAs encoding these
dominant negative mutants that change the specificity of DNA binding from being estrogen response element (ERE)-specific to progestin/glucocorticoid response element-specific (9). These secondary mutations (E203G, G204S, A207V) therefore eliminate the ability of the three dominant negative mutants to bind to and stimulate the transcription of reporter plasmids containing EREs (Ref. 9 and confirmed below).

In Figs. 2 and 3, we show the abilities of the three original dominant negative ERs alone (SSS45fs, L540Q, ER1–530) to suppress the transcriptional activity of the wild type ER, and we compare the potencies of these mutants with that of the same dominant negative ERs containing three additional point mutations in their DNA binding domain that eliminate ERE binding. The data in Figs. 2 and 3 are from transfections using two different estrogen-responsive reporter constructs, 2ERE-TATA-CAT, a simple synthetic promoter containing two consensus EREs and the Xenopus vitellogenin B1 TATA region, and 2ERE-pS2-CAT, containing two EREs inserted into the more complex promoter of the human pS2 gene. As expected, the three dominant negative receptors alone (SSS45fs, L540Q, and ER1–530) are transcriptionally inactive (open bars) and effectively suppress wild type transcriptional activity. The DNA binding mutant alone (denoted EGA→GSV) is also inactive. Cotransfections of a similar 3:1 and 10:1 excess of wild type (WT) ER or the DNA binding mutant (EGA→GSV) along with the WT-ER have no effect on wild type transcriptional activity so that measured CAT activity remains near 100%. On both promoter-reporter gene constructs, the frameshift (SSS45fs) and L540Q dominant negative receptors were more potent than ER1–530; however, at 1:10 molar ratios (Fig. 2), each of the dominant negative receptors achieved nearly complete suppression of the activity of the WT ER to the control (ethanol vehicle) level.

The ability of the dominant negative mutants to antagonize the activity of wild type ER was strongly suppressed when high affinity binding to the ERE was eliminated. At a low concentration ratio (1:3, wild type to mutant receptor), the three dominant negative receptors lost essentially all of their effectiveness as inhibitors of WT ER activity (Figs. 2 and 3). At higher molar concentrations (1:10, Fig. 2), the dominant negative receptors, although unable to bind to EREs and activate transcription from ERE-containing reporter plasmids, still gave substantial suppression of wild type activity. These data suggest additional sites of action, such as heterodimer formation or transcriptional suppression, in the inhibitory activity of these receptors.

L540Q Is Able to Form Heterodimers with a Shortened Wild Type ER in Vitro—To determine if the dominant negative receptors formed heterodimers with wild type ER, we developed an immunoprecipitation technique to analyze dimer formation in vivo and in vitro. To distinguish between the dominant negative ERs and wild type ER in immunoprecipitation and Western blotting, we used pairs of receptors that differed in size and in the ability to be recognized by monoclonal antibodies that specifically recognize unique epitopes. For example, by

**Fig. 1. Schematic diagram of the wild type and mutant estrogen receptors analyzed.** A schematic of the wild type human estrogen receptor (595 amino acids) is shown, including the relative locations of domains labeled A through F, activation functions 1 and 2 (AF-1 and AF-2), and the DNA binding region (domain C). The three original dominant negative mutants are shown in the next three lines (SSS45fs, L540Q, and ER1–530), and the location of their mutations is indicated. Each cDNA (wild type or dominant negative mutant) was further modified to include the additional mutations shown in the last four lines. These additional mutations include a triple point mutation in the DNA binding domain (E203G, G204S, A207V), which disrupts high affinity binding to EREs, L507R, which impairs receptor dimerization, G521R, which destroys estradiol binding, and deletion of the A/B domain.

**Fig. 2. Examination of the requirement for ERE DNA binding for the dominant negative effect.** CHO cells were cotransfected with reporter gene construct 2ERE-TATA-CAT, pCH110 β-galactosidase internal control plasmid, and a mixture of wild type and/or mutant ER expression vectors as indicated. Transfected cells were treated for 24 h with 10−9 M E2 or vehicle before preparing extracts and CAT activity analysis. Values are the means and S.E. of three or more independent experiments.
using monoclonal antibody D75, which recognizes an epitope near the C terminus of ER, full-length wild type ER or the full-length dominant negative mutant L540Q will be recognized, but not a truncated wild type ER (ER1–554), or the dominant negative mutants ER1–530 or S554fs. When a full-length ER and one of the truncated receptors not recognized by antibody D75 are co-expressed, and the extracts are immunoprecipitated with antibody D75, the truncated ER will not be present in the final immunoprecipitate unless it forms a heterodimer with the full-length receptor. Since the two members of the pair differ in size, they can be separated by SDS-polyacrylamide gel electrophoresis and quantitated by Western blotting with monoclonal antibody D547, which recognizes an epitope in the hinge region of ER (which is present in all of our ER mutants).

To test antibody specificities, COS cells were transfected individually with expression vectors for wild type ER or the ER mutants, and cell extracts containing each receptor were subjected to Western blotting with monoclonal antibody D547, which recognizes an epitope in the hinge region of ER (which is present in all of our ER mutants).

To investigate the ability of a dominant negative ER and wild type ER to form heterodimers in vivo, we co-transfected COS-1 cells with one of three pairs of estrogen receptors: wild type ER and ER1–554 (used as a control), L540Q and ER1–554, or ER1–530 and ER1–554. Western blotting was carried out with antibody D75 and quantitated by Western blot analysis with different antibodies (data not shown). The immunoprecipitation conditions we developed, which are described under “Experimental Procedures,” represent a balance between conditions that yield low levels of nonspecific protein binding and those that allow efficient retention of heterodimers.

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To investigate the ability of a dominant negative ER and wild type ER to form heterodimers in vitro, we transfected COS-1 cells separately with expression plasmids for the dominant negative mutant L540Q or a truncated ER (ER1–554). Cell extracts were prepared separately, mixed together, and incubated on ice overnight. Immunoprecipitation was then carried out on this mixture with the D75 monoclonal antibody. Since ER1–554 is not recognized by antibody D75, only homodimers of L540Q and heterodimers between L540Q and ER1–554 will be precipitated by this antibody. Precipitated proteins were analyzed by Western blotting with D547. The data presented in Fig. 4 demonstrates that ER1–554 is present in the final immunoprecipitate only after it is incubated with L540Q (Fig. 4, lane 3). Control experiments demonstrated that immunoprecipitation is not observed from an extract containing only ER1–554 (Fig. 4, lane 4) and that ER1–554 is not degraded in the extract since it is efficiently precipitated with monoclonal antibody H222 (Fig. 4, lane 6). This indicates that L540Q is able to form heterodimers with the wild type ER in vitro.

The Dominant Negative Mutants L540Q and ER1–530 Are Able to Form Heterodimers with Wild Type ER in Vivo—While the data presented in Fig. 4 demonstrated that prolonged incubation of a dominant negative ER and a truncated wild type ER resulted in heterodimer formation in cell-free extracts, they did not address the question of whether co-expression of the dominant negative receptors and wild type ER in the same cells results in in vivo heterodimer formation. To test this, we co-transfected COS-1 cells with one of three pairs of estrogen receptors: wild type ER and ER1–554 (used as a control),
L540Q and ER1–554, or ER1–530 and wild type ER. Cell extracts were prepared and immunoprecipitated with D75 monoclonal antibody, and precipitated proteins were analyzed by Western blot as described above. The data presented in Fig. 5A demonstrate that both dominant negative mutants L540Q and ER1–530 are able to form heterodimers with full-length wild type ER or ER1–554 about as efficiently as the wild type ER (Fig. 5, lanes 2, 5, and 9). In addition, the addition of ERE DNA to the extracts to be immunoprecipitated did not enhance heterodimer formation (Fig. 5, lanes 3, 6, and 10), suggesting that heterodimer formation probably does not occur after binding of ER monomers to the ERE.

An N-terminally Shortened Version of S554fs Is Able to Form Heterodimers with the Full-length Flag-ER—Because we could not distinguish between S554fs and wild type ER with the available antibodies, these studies did not address the question of whether the potent dominant negative mutant, S554fs, is able to form heterodimers with the wild type ER. To circumvent this problem, we made a Flag-ER, in which an 8-amino acid peptide containing the epitope for the commercially available monoclonal antibody M2 was engineered in frame on the N terminus of the wild type ER. In agreement with earlier studies indicating that deletion of N-terminal amino acids did not alter ER activity (29, 30), insertion of the 8-amino acid “flag” sequence does not alter the ability of the wild type ER to activate transcription (data not shown). To enable us to separate Flag ER and S554fs on a Western blot, we cotransfected COS-1 cells with expression plasmids encoding Flag-ER and a truncated form of S554fs (∆AB, S554fs), from which we deleted the N-terminal AB domain. Immunoprecipitation using Flag M2 monoclonal antibody was then carried out on the cell extract containing co-expressed Flag-ER and ∆AB, S554fs. The Western blot shown in Fig. 5B demonstrates that Flag M2 monoclonal antibody recognizes only Flag-ER but not ∆AB, S554fs (panel B, lane 2). The efficient immunoprecipitation of ∆AB, S554fs by the Flag M2 antibody (panel B) demonstrates that S554fs efficiently forms heterodimers with wild type ER.

Introducing a Single Point Mutation into the Dominant Negative Mutants to Impair Dimerization Substantially Reduced Their Dominant Negative Activity—Since the three dominant negative mutants are able to dimerize with the wild type ER, we wished to evaluate the importance of this heterodimer formation in dominant negative action. We introduced a single additional mutation in the hormone binding region of the original dominant negative mutants at amino acid 507, changing this leucine residue, which is highly conserved among estrogen receptors from different species, to arginine. This mutation is reported to severely impair the ability of mouse ER to dimerize in vitro (6). Introduction of this LS507R mutation into the WT ER reduced its transactivation activity by about 80% (Fig. 6). In addition, co-expression of L507R with WT ER did not suppress WT ER activity. When the L507R mutation was combined with the original dominant negative mutations, these double mutants showed a substantial reduction in their abilities to inhibit WT receptor activity, as compared to the original dominant negative mutants (Fig. 6). This implicates the formation of heterodimers between the dominant negative mutants and WT ER as at least part of the mechanism by which these mutants suppress the activity of WT ER.

Co-expression of S554fs and Wild Type ER and Co-expression of L540Q, EGA–GSV and Wild Type ER Inhibited Binding of the Wild Type to the ERE in Gel Mobility Shift Assays—While our immunoprecipitation studies provide direct evidence that the A/B-deleted S554fs mutant can form heterodimers with wild type ER, we wished to confirm that full-length S554fs heterodimerizes with wild type ER by an independent method based on one of the functions of ER. Although S554fs exhibits near wild type interaction with the ERE in in vivo promoter interference assays, it is unable to bind to the ERE in vitro gel mobility shift assays (13). Therefore, we wished to confirm that full-length S554fs heterodimerizes with wild type ER by an independent method based on one of the functions of ER. Although S554fs exhibits near wild type interaction with the ERE in in vivo promoter interference assays, it is unable to bind to the ERE in vitro gel mobility shift assays (13). To provide an independent confirmation of heterodimer formation between wild type ER and S554fs and to determine whether wild type ER–S554fs heterodimers are able to bind to the ERE, we prepared whole cell extracts from COS cells, which were cotransfected with a constant amount of expression vector for wild type ER, and increasing amounts of expression vector for S554fs. We then incubated these extracts with a radiolabeled DNA fragment containing an ERE and carried out gel mobility shift assays. The expected gel-shifted band was observed with wild type ER, while S554fs showed no binding to the ERE (Fig. 7). As the ratio of S554fs to wild type ER increased in the extracts, there was a progressive decline in binding to the ERE (Fig. 7, lanes 3–6). In a separate study at a 10:1 ratio of S554fs: wild type ER, binding to the ERE was completely abolished. These data provided a functional demonstration that full-length S554fs and wild type ER form functional heterodimers. Interestingly, the in vitro ERE binding properties of these heterodimers parallel those of the dominant negative mutant, not wild type ER.

Similar gel mobility shift assays were conducted using extracts from cells transfected with increasing ratios of the mutated L540Q containing the three point mutations in its DNA binding domain (E203G, G204S, A207V), which shift its specificity from ERE to glucocorticoid response element (see Fig. 1). The mutated L540Q did not bind to the ERE in gel shift assays.

**Fig. 5. In vivo heterodimer formation between the dominant negative mutants and wild type ER.** A, COS-1 cells were transfected wild type ER and ER1–554 (lanes 1–3), L540Q and ER1–554 (lanes 3–6), and wild type ER and ER1–530 (lanes 7, 9, and 10); to test that the two receptors in each pair were expressed at approximately equal levels, extracts from cells cotransfected with each pair of expression plasmids were prepared and analyzed by Western blotting (denoted by W) using antibody D547 (lanes 1, 4, 7) as described under “Experimental Procedures.” Immunoprecipitations using antibody D75 (denoted by I) were then carried out on each extract, and the precipitates were analyzed by Western blotting (lanes 2, 5, and 9; the damaged well in lane 8 was not used). Immunoprecipitation (with antibody D75) followed by Western blotting was also carried out on extracts to which 10 ng of a 51-base pair ERE-containing oligonucleotide was added (lanes 3, 6, 10). B, ∆AB, S554fs, and Flag-ER form heterodimers in vivo. Extracts were prepared from COS-1 cells cotransfected with expression plasmids for Flag-ER, and ∆AB, S554fs. Western blotting was carried out using antibody D547 (panel B, lane 1) or flag peptide-specific M2 antibody (panel B, lane 2). Cell extracts were immunoprecipitated with Flag M2 monoclonal antibody, and the immunoprecipitates were analyzed by Western blotting with antibody D547 as described under “Experimental Procedures” (panel B, right most blot).
However, there was a progressive decline in binding of wildtype ER to the ERE with increasing ratios of the mutant L540Q:wild type ER (Fig. 7, lanes 7–10); these data provide additional support that the L540Q mutant, like the mutant S554fs, forms functional heterodimers with wild type ER and is thereby able to inhibit binding of wild type ER to estrogen response element DNA.

Elimination of Hormone Binding Ability Weakens Dominant Negative Effectiveness—We previously reported that S554fs and L540Q bind estradiol with wild type affinity and that ER1–530 binds estradiol with an affinity about 7% that of wild type ER (1). To determine whether hormone binding affects the ability of the dominant negative mutants to repress wild type ER transcriptional activity, we introduced a single point mutation (G521R), which eliminates hormone binding by mouse ER (6, 11), into each of the dominant negative receptors. Since this is a single amino acid change, it seemed likely to disrupt estradiol binding without interfering with other functions of these receptors. Fig. 8 shows that the G521R mutants alone were transcriptionally inactive, suggesting that this mutation also renders the human ER unable to bind estradiol (and confirmed by us, data not presented). The G521R mutant did not affect the transcriptional activity of the wild type ER when they were co-expressed (Fig. 8, G521R + WT). Elimination of estradiol binding in the dominant negative ERs (Fig. 8, right-most bars) resulted in receptors with a greatly attenuated ability to repress wild type ER activity.

The A/B Domain of the Estrogen Receptor Influences Dominant Negative Activity—Since the ER mutants ER1–530, L540Q, and S554fs have an intact A/B domain that contains a hormone-independent constitutive activation function (AF-1), we postulated that elimination of the A/B domain and the AF-1 might result in a stronger repression by these dominant negative mutants. Since the AF-1 domain has not been precisely localized, we deleted essentially the entire A/B domain (residues 1–175) from each of the original dominant negative mutants. Cotransfection experiments utilizing these N-terminally truncated dominant negative mutants were conducted with both the 2ERE-TATA-CAT and 2ERE-pS2-CAT reporter gene constructs (Fig. 9, A and B).

As seen in Fig. 9, deleting the N-terminal A/B domain mark-
DISCUSSION

Our studies provide evidence that multiple mechanisms contribute to the ability of dominant negative estrogen receptors to act intracellularly as effective inhibitors of wild type ER activity. Our findings indicate that competition for ERE binding, formation of inactive heterodimers, and specific transcriptional silencing can all contribute to the dominant negative phenotype. In addition, our studies highlight that the presence of the A/B domain is required for strong dominant negative activity, even though it is not required for heterodimerization with wild type ER (Fig. 6), or for high affinity estradiol binding or DNA binding (data not presented). In addition, the ability to bind hormone enhances dominant negative effectiveness.

Overall, our studies indicate that the best dominant negative estrogen receptors will be proteins that are themselves transcriptionally inactive but retain the capacity to interact effectively at most steps in the hormone-receptor-response pathway in a manner that renders the wild type ER inactive. Three general mechanisms of dominant negative repression of wild type steroid/nuclear receptors by mutant receptors are commonly proposed: 1) There could be competition for binding to hormone response elements between active (wild type) receptors and inactive (mutant) receptors. 2) There may be formation of inactive heterodimers between wild type and mutant receptors. The formation of heterodimers would cause the depletion of wild type homodimers, thereby having two deleterious effects on transactivation by wild type receptor. The lower levels of wild type homodimers could lead to a concomitant reduction in transactivation by reducing the concentration of active dimers to sub-maximal levels, and the presence of inactive heterodimers could also compete with wild type homodimers for the hormone response elements. 3) Dominant negative steroid/nuclear superfamily receptors could cause a deleterious interaction with other nuclear factors or prevent the effective interaction of wild type ER with necessary adaptor or co-activator proteins. This could involve the sequestering of co-activator proteins or unproductive interactions with components of the basal transcription apparatus; or conversely, it could result in the stabilization of transcriptional repressor proteins on the regulatory regions of target genes.

In the case of the three dominant negative estrogen receptors discussed in this paper, we feel there is evidence for all three of these mechanisms. While it is appealing to be able to discriminate between mechanisms, this may not be possible since these three mechanisms are not mutually exclusive. To effect the strongest possible repression of the wild type ER, these transcriptionally inactive dominant negative mutants must interact with most of the wild type estrogen receptor transactivation pathway. In support of this position, we note that both DNA binding and dimerization are required for strong suppressive action of the mutants (Figs. 2, 3, and 7). While ER mutants that cannot dimerize effectively are also unlikely to bind effectively to the ERE, loss of ERE binding is unlikely to be the sole site at which a failure to dimerize impairs dominant negative function. Since the insertion of three point mutations that eliminate binding to the ERE reduced, but did not abolish, the ability of the double mutated proteins to act as dominant negative mutants, these mutants must be able to act at an additional site. Our observation that one of these mutants is able to form inactive heterodimers with wild type ER provides additional support for the view that both competition for ERE binding and formation of heterodimers are important components of the dominant negative phenotype.

Heterodimerization of these dominant negative mutants with wild type ER appears to be necessary, but probably not sufficient, to cause a strong dominant negative effect. In Figs.
4–6, we demonstrate that the dominant negative ERs have the ability to form homodimers, and we show that these ER mutants become greatly impaired in their ability to repress wild type transcription when they are mutated to contain L507R. However, wild L507R mutants still show some repressive activity with wild type ER, indicating that additional activities beyond heterodimerization are needed for optimal dominant negative activity. Likewise, the fact that A/B-deleted S554fs is able to heterodimerize with wild type ER and yet unable to effectively suppress wild type ER transcription shows that heterodimerization of an inactive ER mutant with wild type ER is necessary but not sufficient to explain the dominant negative effect of our mutants.

Further, we find that the dominant negative effect is augmented by the ability of the mutants to bind estradiol. Since the binding of the ER to the ERE is ligand dependent in the 231 cells used in the current work (25), the non-hormone binding forms of our dominant negative mutants may lose effectiveness through failure to heterodimerize and to bind to the ERE.

Perhaps most surprising is the observation that the three dominant negative mutants deleted of their A/B domains are much weaker dominant negatives than their unmodified progenitors. The wild type ER contains both a hormone-dependent transcription activation function (known as AF-2), which encompasses amino acids 534–548 in domain E of the human ER (7,8,12), and a ligand independent activation function in the A/B domain (31,32). Since our dominant negative ERs are altered in or missing AF-2, we reasoned that the intact AF-1 of these receptors might oppose the dominant negative effect exhibited by these mutants. Instead, this N-terminal truncation eliminated almost all of the trans dominant repression of the unmodified dominant negative receptors. One plausible explanation for these data is that a repressive cellular factor is no longer able to bind to these doubly mutated ER derivatives. This reasoning is bolstered by several observations. First, it has been shown that A/B-deleted wild type ER retains some capacity for estradiol-dependent transcription (20,31–34). Second, we show effective dimerization of the A/B-deleted S554fs ER with a wild type ER derivative (Flag-ER). This shows that the A/B-deleted mutants are still able to participate in some wild type interactions. It is also possible that AF-1 and AF-2 interact together with one or more proteins required for trans-activation by ER. By inactivating only AF-2, unproductive interaction with these proteins may be maintained, preventing their interaction with wild type ER. However, deletion of AF-1 and mutation of AF-2, by preventing interaction with these proteins, frees them to interact productively with wild type ER. Whether deletion of the A/B region contributes to the dominant negative phenotype by releasing a repressor or activator protein, our data strongly imply that additional interaction(s) or step(s) beyond formation of inactive heterodimers and competition for ERs are critical for the dominant negative effect.

Many naturally occurring mutations of the thyroid hormone receptor generate resistance to thyroid hormone and are dominantly inherited. It is notable that these receptor mutations cluster in two areas within the hormone binding domain outside putative dimerization regions and that recent studies have shown that the introduction of artificial mutations that impair heterodimerization abrogated dominant negative activity (35–37). Additionally, Palumbo et al. (38) provide evidence that dominant negative androgen receptors function through the formation of inactive heterodimers and also through competition for androgen response elements. There is still no clear consensus regarding the mechanisms underlying the action of dominant negative thyroid receptors, with some studies providing evidence for competition for thyroid response elements by dominant negative thyroid receptors (39) and the dominant negative viral oncogenic homolog of thyroid receptor, v-erbA (40), while others (41) have reported that their dominant negative thyroid hormone receptors did not require the ability to bind to the thyroid response elements they studied. Response element-dependent differences in the dominant negative potential of thyroid receptors raises the additional possibility that dominant negative receptors may function as well through interaction with other nuclear factors (42). While our findings provide strong evidence for the roles of receptor dimerization, DNA binding, estradiol binding, and the AF-1 containing N-terminal A/B domain in the effectiveness of our dominant negative estrogen receptors, additional studies examining the interaction of these receptors with receptor-associated proteins known to interact with the activation function regions of the receptor (43–45) should provide further insight regarding their mechanism of action.

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