17α-(Haloacetamidoalkyl)estradiols Alkylate the Human Estrogen Receptor at Cysteine Residues 417 and 530†

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ABSTRACT: Results obtained in a previous study suggested that cysteine residues in the estrogen receptor were covalent attachment sites for four 17α-(haloacetamidoalkyl)estradiols (halo, bromo or iodo; alkyl, methyl, ethyl, or propyl). To identify the putative concerned cysteines, we expressed wild-type and various cysteine → alanine mutants of the human estrogen receptor in COS cells and determined their ability to be alkylated by the four electrophiles. The quadruple mutant, in which all the cysteines (residues 381, 417, 447, and 530) of the hormone-binding site were changed to alanines, showed very little electrophile labeling, whereas the four single mutants (C381A, C417A, C447A, and C530A) were alkylated as efficiently as the wild-type receptor. These results (i) demonstrate that cysteine residues were covalent attachment sites of electrophiles and (ii) indicate that more than one cysteine residue could be alkylated. Analysis of three double mutants (C381A/C530A, C417A/C530A, and C447A/C530A) provided strong evidence that only C417 and C530 were sites for electrophile covalent attachment. Since C530 was also alkylated by tamoxifen aziridine, a nonsteroidal affinity-labeling agent, we propose a selective mode of superimposition of tamoxifen-class antiestrogens with estradiol, which could account for the relative positioning of the two types of ligands in the receptor hormone-binding pocket. According to the structure of the hormone-binding pocket of nuclear receptors, as inferred from crystallographic studies and general sequence alignment of hormone-binding domains, C417 and C530 appear to be (1) located at the extreme border or in structural elements involved in delineation of the hormone-binding pocket, (2) spatially in close proximity to each other, and (3) in positions highly homologous to those of glucocorticoid receptor sites alkylated by affinity- and photoaffinity-labeling agents, respectively.

The nuclear receptor superfamily (Evans, 1988; Mangelsdorf et al., 1995b) presently includes more than 150 related intracellular proteins which act as transcriptional regulators and are involved in development, differentiation, and homeostasis. This superfamily is made up of a group of established receptors, which are ligand-modulated transcriptional factors that include receptors for steroid and thyroid hormones, vitamins D, retinoids, and related compounds, and of a group of orphan receptors, including proteins without ligands and possibly proteins whose cognate ligands are still unknown (Mangelsdorf & Evans, 1995a). Nuclear receptors are modular proteins with evolutionarily conserved DNA- and ligand-binding domains (Krust et al., 1986). In the steroid hormone receptor subgroup, hormone binding, through a receptor conformation change, induces receptor dimerization, interaction of the dimer with target DNA sequences, usually located in the vicinity of hormone-regulated genes, and interaction with transcription factors, thus allowing modulation of transcription of these genes (Tsai et al., 1994).

The DNA-binding domain of nuclear receptors includes about 70 amino acids; its structure, with two perpendicular helices, was established in the early 1990s, through NMR (Hard et al., 1990; Schwabe et al., 1990) and crystallographic studies (Luisi et al., 1991). The nuclear receptor hormone-binding domain includes about 250 amino acids; in addition to the hormone-binding function, this domain is involved in binding of 90 kDa heat-shock proteins (Pratt, 1993), the receptor dimerization process (Glass, 1994), and activation of transcription (Tora et al., 1989). The overall structure of this domain was unknown until recent reports involving crystallographic studies of the hormone-binding domain from unliganded human retinoic X receptor α (RXRα)[1] (Bourguet et al., 1995), liganded retinoic acid receptor γ (RARγ) (Renaud et al., 1995), and rat thyroid hormone receptor α_1 ($TR\alpha_1$) (Wagner et al., 1995). The hormone-binding domain of these receptors appears to comprise 11 (or 12) α-helices and 2 (or 4) β-strands. The helices are organized in a three-layer structure with 5 (or 4) internal helices sandwiched between the most external helices. The ligand-binding pocket is predominantly formed by hydrophobic residues located in 8 (or 10) structural elements (Renaud et al., 1995; Wagner et al., 1995), some identical and others differing with respect to RARγ and $TR\alpha_1$.

On the basis of a comparison of structures determined for unfilled and hormone-filled receptors, Wurtz et al. (1996) proposed a mechanism whereby a ligand-induced confor-

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[1] Abbreviations: RXRα, retinoic X receptor α; RARγ, retinoic acid receptor γ; $TR\alpha_1$, thyroid hormone receptor α_1; DME medium, Dulbecco's modified Eagle's tissue culture medium; PBS, phosphate-buffered saline; T_{20}, 20 mM Tris-HCl buffer at pH 8.5.

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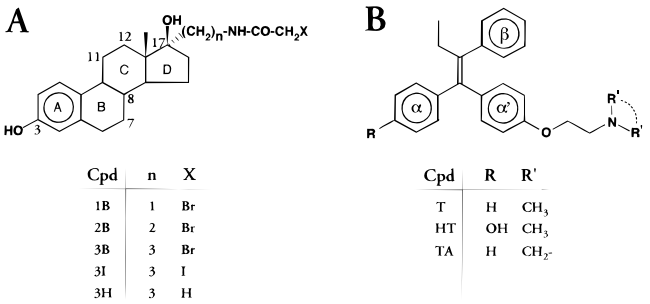

FIGURE 1: Structures of steroidal affinity labels of the estrogen receptor. (A) The four steroidal affinity labels of the estrogen receptor used in the study are 17α-(bromoacetamidomethyl)estradiol (1B), 17α-(bromoacetamidoethyl)estradiol (2B), 17α-(bromoacetamidopropyl)estradiol (3B), and 17α-(iodoacetamidopropyl)estradiol (3I); 17α-(acetamidopropyl)estradiol (3H), a chemically inert analog of these electrophiles, was used as a blank in receptor affinity labeling experiments. (B) Structure of nonsteroidal antiestrogens, tamoxifen (T) and 4-hydroxytamoxifen (HT), which are conventional ligands of the estrogen receptor, and tamoxifen aziridine (TA) which is an affinity label of the receptor.

mational transition could transform the receptor into a transcriptionally active species. Moreover, using information obtained on the basis of the crystal structure of RARγ and through the general sequence alignment of ligand-binding domains of nuclear receptors, the above-cited authors inferred the common ligand-binding pocket of RXRα and the glucocorticoid receptor. These authors suggested that the hormone-binding domain fold, the ligand-binding pocket, and the ligand activation mechanism are similar for members of the nuclear receptor superfamily.

Affinity labeling of receptors enables direct identification of amino acid residues of the hormone-binding site which are in contact with or in close proximity to electrophilic ligands. This approach was first used for the estrogen receptor by the groups of B. S. Katzenellenbogen and J. A. Katzenellenbogen, which developed several series of efficient affinity- or photoaffinity-labeling agents. The studies showed that two nonsteroidal electrophilic ligands, tamoxifen aziridine (an antiestrogen) (Katzenellenbogen et al., 1983) and ketononestrol aziridine (an estrogen) (Zablocki et al., 1987), only react with cysteine 530 of the human estrogen receptor (Harlow et al., 1989). Moreover, the receptor was still alkylated by tamoxifen aziridine when cysteine 530 was mutated to alanine (C530A mutant) (Reese & Katzenellenbogen, 1991). Using double cysteine → alanine mutants, it was determined that C381 was the alternative attachment site (Reese et al., 1992).

Recently, we reported affinity labeling of the lamb estrogen receptor by 17α-(bromoacetoxyalkyl/alkynyl)estradiols (El Garrouj et al., 1993) and 17α-(haloacetamidoalkyl)-estradiols (El Garrouj et al., 1995) (Figure 1). These compounds, although displaying low affinity, were efficient affinity labels of the receptor. Prevention of receptor alkylation by methyl methanethiosulfonate, a thiol specific reagent, suggested that target amino acid residues of these electrophiles are SH groups of cysteines. Since the hormone-binding domain of estrogen receptors includes only four cysteines (C381, C417, C447, and C530 in the human receptor), perfectly conserved from amphibians (Weiler et al., 1987) to mammals (Green et al., 1986; Koike et al., 1987), results obtained in affinity labeling studies with the lamb estrogen receptor should also be obtained with the human receptor. In the present study, we use expression vectors coding for the wild-type and single, double, and quadruple cysteine → alanine mutants of the human estrogen receptor (Reese & Katzenellenbogen, 1991; Reese et al., 1992), demonstrate that amino acids alkylated by electrophilic estradiol 17α-derivatives are indeed cysteines, and then identify these cysteines. We then use these identified target cysteines to determine the positioning of triphenylethylene compounds relative to that of steroidal estrogens in the hormone-binding pocket. Finally, the identified electrophile covalent attachment sites are localized in the structure of the nuclear receptor hormone-binding domains, as defined through crystallographic studies and a general sequence alignment method, to determine whether the alkylation sites are actually located on the structural elements which have been described to delineate the hormone-binding pocket.

EXPERIMENTAL PROCEDURES

Chemicals and Materials. The electrophilic ligands of the estrogen receptor used in the study, 17α-(bromoacetamidoalkyl)estradiols (alkyl, methyl, ethyl, or propyl), 17α-(iodoacetamidopropyl)estradiol, and the chemically inert ligand 17α-(acetamidopropyl)estradiol, were synthesized as described previously (El Garrouj et al., 1995). [6,7-^{3}H]-Estradiol (specific activity of 1.96 PBq/mol, radiochemical purity of 98%) was purchased from Amersham International (Amersham, England). Estradiol and 17α-derivatives of estradiol used for affinity labeling were solubilized in absolute ethanol. Solutions were stored at −20 °C. The purity of solubilized compounds was checked before use by thin-layer chromatography.

Cell Culture Conditions and Transfections. COS cells were passaged in phenol red-free Dulbecco's modified Eagle's tissue culture medium (DME medium) supplemented with 10% charcoal/dextran-treated fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). COS cells transfected for use in receptor affinity labeling studies were plated in T150 tissue culture flasks in DME medium plus 10% charcoal/dextran-treated calf serum at a density of ∼10^6 cells in a humidified 5% CO_2 atmosphere. Four hours later, media were changed and cells were transfected with 30 μg of estrogen receptor expression plasmid (pRER) coding for wild type, or one of the various (single, double, or quadruple) cysteine → alanine mutants of the human estrogen receptor.

DNA Constructs. The preparations for plasmids coding for the wild-type receptor and the four single cysteine → alanine mutants (C381A, C417A, C447A, and C530A), the three double cysteine → alanine mutants (C381A/C530A, C417A/C530A, and C447A/C530A), and the quadruple cysteine → alanine mutant (C381A/C417A/C447A/C530A) of the human estrogen receptor have been described previously (Reese & Katzenellenbogen, 1991; Reese et al., 1992).

Cytosolic Estrogen Receptors. COS cells transfected with estrogen receptor expression vectors were harvested in phosphate-buffered saline (PBS) containing 1 mM EDTA by scraping with a rubber policeman. Cells collected by centrifugation were washed in ice-cold PBS. COS cells were resuspended in 0.25 mL/plate chilled 20 mM Tris-HCl buffer at pH 8.5 (T_{20}) containing 1 mM EDTA, 5% glycerol and a cocktail of protease inhibitors [8 μM pepstatin, 10 μM leupeptin, and 8 mM 4-(2-aminoethyl)benzenesulfonyl fluoride]. Cells were homogenized by ∼40 strokes in a glass dounce homogenizer (B pestle) and incubated on ice for 30

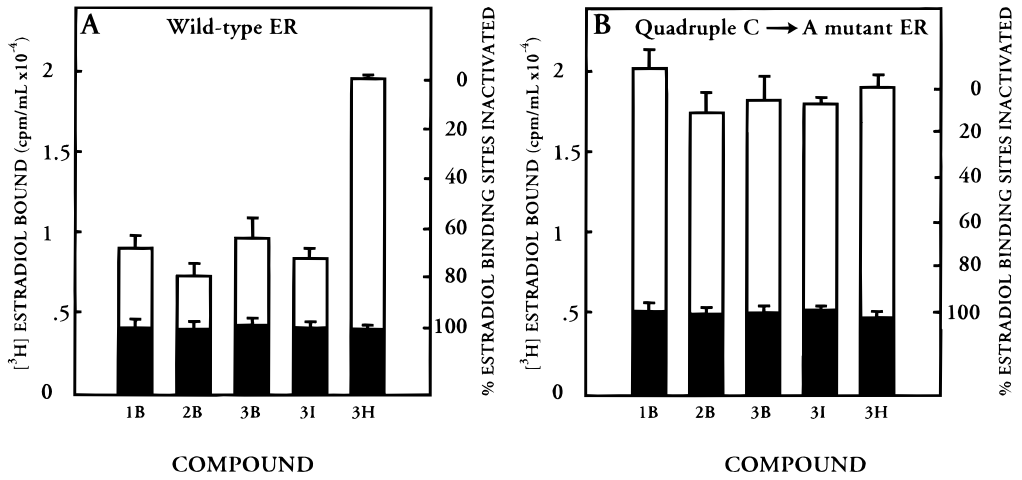

FIGURE 2: Estradiol binding to wild-type and quadruple cysteine → alanine mutant estrogen receptors after exposure to estradiol 17α-derivatives. Cytosols, from COS cells transfected with pRER containing (A) wild-type or (B) quadruple cysteine → alanine mutant estrogen receptor cDNA, were incubated for 15 h at 0 °C with 3 μM 1B, 2B, 3B, or 3I or with the chemically inert compound 3H. Unbound steroids were removed by charcoal treatment, and then aliquots were incubated under exchange conditions (4 h at 20 °C) with [^{3}H]estradiol (10 nM) in the absence or presence of unlabeled estradiol (5 μM) to determine total and nonspecific [^{3}H]estradiol binding, respectively, as described in Experimental Procedures. Concentrations of total (solid bars + open bars) and nonspecific (solid bars) bound [^{3}H]estradiol are represented according to the steroid incubated with the cytosolic extracts. Values are means of duplicate determinations; error bars indicate standard deviations. The tops of open bars also indicate the percent of specific estradiol binding sites inactivated by electrophiles (right scale).

min. The homogenate was then centrifuged at 180000*g* at 4 °C for 30 min to yield the cytosol. The cytosol protein concentration (1−2 mg/mL) was determined according to the method of Bradford (1976).

Irreversible Binding Assay. Since the electrophiles used are non-radiolabeled ligands of the estrogen receptor, alkylation of the estrogen receptor hormone-binding site by electrophiles was determined indirectly, on the basis of the fact that the difference between the concentrations of specific binding sites for [^{3}H]estradiol in control cytosol and electrophile-exposed cytosol reflects the extent of affinity labeling of the receptor (El Garrouj et al., 1993, 1995). The receptor was alkylated with 17α-(haloacetamidoalkyl)estradiols by incubation of cytosol aliquots with 3 μM compounds for 15 h at 0 °C; as controls, other cytosol aliquots were incubated with 17α-(acetamidopropyl)estradiol or without steroid. To remove unbound steroids, samples were treated with an equal volume of charcoal suspension (1% charcoal and 0.1% dextran T70 in T_{20} at pH 7.4) for 30 min at 0 °C and then centrifuged in order to pellet the charcoal. The concentration of specific estradiol-binding sites in supernatants was determined by incubation of aliquots with 10 nM [^{3}H]estradiol, under exchange conditions (4 h at 20 °C) in the absence and presence of 5 μM radioinert estradiol, respectively; total and nonspecific [^{3}H]estradiol binding were measured by charcoal assay.

Molecular Mechanics Studies. Molecular structures were determined using the MAD 2.20 software package (1993, Oxford Molecular Ltd., Oxford, England), and their lowest-energy conformations were jointly optimized by molecular mechanics (MM_2 force field coupled to steepest descent and Newton−Rephson mimizers). Graphical visualization and molecular superimposition were monitored using the Insight 2.2.0 software package (1993, Biosym Technologies, San Diego, CA).

RESULTS

Covalent Labeling of the Estrogen Receptor by 17α-(Haloacetamidoalkyl)estradiols Occurs at Cysteine Residues. The fact that irreversible inactivation of the hormone-binding site of the lamb estrogen receptor by 17α-(bromoacetamidoalkyl)estradiols (alkyl, methyl, ethyl, or propyl) and 17α-(iodoacetamidopropyl)estradiol (Figure 1) (i) markedly increased from pH 7.0 to 8.5 and (ii) was prevented by pretreatment of the estrogen receptor with methyl methanethiosulfonate (El Garrouj et al., 1995) suggested that cysteines were the electrophile covalent attachment sites. However, other possibilities cannot be excluded; the prevention of receptor affinity labeling by methyl methanethiosulfonate could have been due, for instance, to steric hindrance at the covalent attachment site, resulting from modification of a neighbor cysteine.

To obtain evidence that cysteines are indeed the covalent attachment sites of 17α-(haloacetamidoalkyl)estradiols (compounds 1B, 2B, 3B, and 3I) to the receptor, we used expression vectors coding for the wild type and for a mutant of the human estrogen receptor obtained by replacing all four cysteines in the hormone-binding domain with alanines (Reese et al., 1992). This quadruple cysteine → alanine mutant and the wild-type human estrogen receptors were expressed in COS cells, and their ability to be affinity labeled by 17α-(haloacetamidoalkyl)estradiols was evaluated in terms of the decrease in their capacity to bind estradiol after electrophile exposure. The results of a representative experiment are shown in Figure 2. Exposure of cytosol containing wild-type receptor to 3 μM 1B, 2B, 3B, or 3I for 15 h at 0 °C and pH 8.5 resulted in a specific binding of estradiol ∼3.5-fold lower than that measured in cytosol exposed to the chemically inert analog 3H (Figure 2A). Contrary to results obtained with the lamb estrogen receptor, there was no marked difference in the efficiencies of the four compounds in alkylating the human estrogen receptor. With cytosol containing the quadruple cysteine → alanine mutant receptor, the effect of the four electrophiles, if any, was much less pronounced, since the mean decrease in estradiol binding was under 10% (Figure 2B). This established the fact that covalent labeling of the receptor by electrophilic estradiol

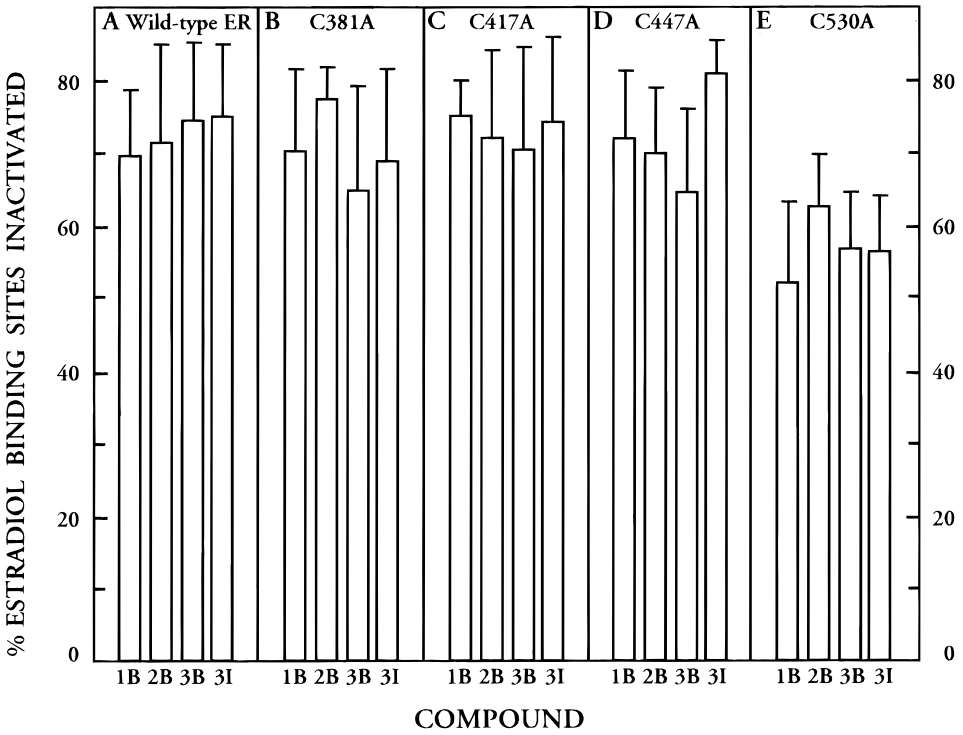

FIGURE 3: Inactivation of estrogen binding sites in wild-type and single cysteine → alanine mutants of the estrogen receptor by electrophilic estradiol 17α-derivatives. Cytosols from COS cells transfected with the expression vector coding for (A) wild-type, (B) C381A, (C) C417A, (D) C447A, or (E) C530A mutant estrogen receptor were incubated for 15 h at 0 °C with 3 μM 1B, 2B, 3B, or 3I or chemically inert compound 3H. Total and nonspecific binding of [^{3}H]estradiol in cytosols incubated with 1B, 2B, 3B, 3I, or 3H were determined as described in Figure 2 and Experimental Procedures. The percentage of estradiol specific binding sites inactivated by electrophiles (calculated from the difference in estradiol-binding site concentrations between cytosols incubated with compound 3H and cytosols incubated with the various electrophiles) is represented. Data were obtained from three to five separate experiments; error bars indicate standard deviations.

17α-derivatives occurred almost exclusively at cysteine residues.

Cysteines 417 and 530 Are the Covalent Attachment Sites of 17α-(Haloacetamidoalkyl)estradiols. With the aim of determining how many and which of the four cysteines in the hormone-binding site were alkylated by 17α-(haloacetamidoalkyl)estradiols, we exposed the four single cysteine → alanine mutants (C381A, C417A, C447A, and C530A) (Reese & Katzenellenbogen, 1991) to electrophiles. As observed with the wild-type receptor, with each of the four single mutants, proportions of inactivated hormone-binding sites were similar for all the electrophiles tested (Figure 3). Moreover, with C381A, C417A, and C447A mutants, these proportions were pratically identical to those obtained with wild-type receptor (~70%); with the C530A mutant, the mean proportion of inactivated hormone-binding sites was slighty lower (~60%), but this difference was not statistically significant. This suggested that, in the hormone-binding pocket, two or more cysteine residues could react alternatively with the estradiol derivatives.

If only two cysteines are alkylated, the use of the various double cysteine → alanine mutants should enable identification of the two cysteines. We therefore tested the three double cysteine → alanine mutants, C381A/C530A, C417A/C530A, and C447A/C530A, in which cysteine 530 was mutated along with one of the other three cysteines in the hormone-binding domain (Reese et al., 1992). With the two C381A/C530A and C447A/C530A double mutants, the percentages of hormone-binding sites inactivated by the four electrophiles (between 50 and 65%) were similar to levels obtained with the C530A single mutant (Figure 4), whereas the percentages of hormone-binding sites inactivated with the C417A/C530A double mutant (between 7 and 12%) were as low as those obtained with the quadruple cysteine → alanine mutant. This indicates that cysteines 417 and 530 are the major covalent attachment sites of 17α-(haloacetamidoalkyl)estradiols in the hormone-binding pocket of the human estrogen receptor.

DISCUSSION

The present results establish that C417 and C530 are covalent attachment sites of 17α-(haloacetamidoalkyl)estradiols in the hormone-binding pocket of the human estrogen receptor. This conclusion is similar to that drawn for affinity labeling of the receptor by tamoxifen aziridine (Reese et al., 1992). With this nonsteroidal affinity-labeling agent, two cysteines were also found to be the receptor covalent attachment sites (C381 and C530). However, due to the different approaches used, in the earlier study, we were able to distinguish between the primary (C530) and secondary (C381) labeling sites using tritiated affinity-labeling ligands. In the present study, however, the method used did not reveal whether C417 or C530 was the potential primary labeling site.

Relative Positioning of Cysteines 381, 417, and 530 in the Hormone-Binding Pocket. From the steroid nucleus, the electrophilic carbon of estradiol 17α-derivatives displays a low degree of freedom, especially in the case of 17α-(bromoacetamidomethyl)estradiol, whose haloacetamido-bearing alkyl chain includes only one carbon. The fact that such a low-flexibility compound can alkylate both C417 and C530 suggests that C530, although separated from C417 by 112 amino acids in the receptor linear sequence, is probably

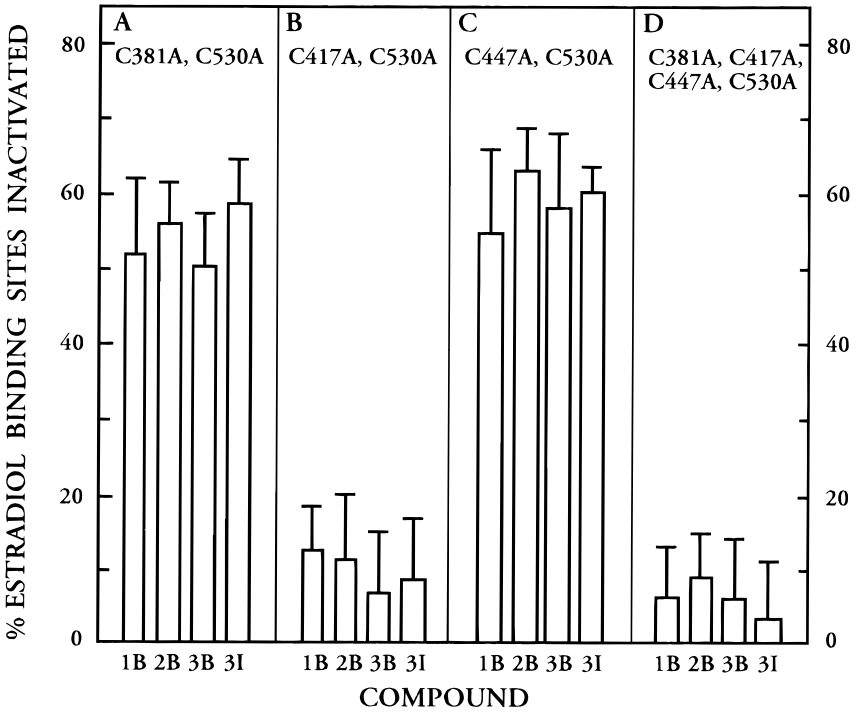

FIGURE 4: Inactivation of estrogen-binding sites in double and quadruple cysteine → alanine mutants of the estrogen receptor by electrophilic estradiol 17α-derivatives. Cytosols from COS cells transfected with the expression vector coding for (A) C381A/C530A, (B) C417A/C530A, (C) C447A/C530A, or (D) quadruple cysteine → alanine mutant estrogen receptor were incubated with electrophiles 1B, 2B, 3B, or 3I or with compound 3H. Total and nonspecific binding of [^{3}H]estradiol in cytosols incubated with 1B, 2B, 3B, 3I, or 3H were determined as described in Figure 2 and Experimental Procedures. The percentage of estradiol specific binding sites inactivated by the electrophiles (calculated from the difference in estradiol-binding site concentrations between cytosols incubated with compound 3H and cytosols incubated with the various electrophiles) is represented. Data were obtained from two or three separate experiments; error bars indicate standard deviations.

a neighbor of C417 when the estradiol 17α-derivative is bound to the receptor. C530 is a receptor covalent attachment site, common to tamoxifen aziridine and 17α-(haloacetamidoalkyl)estradiols, whereas C381 and C417 are covalent attachment sites specific to the first and second type of electrophiles, respectively. This suggests that the electrophilic carbons of the two types of affinity labels (one of the two aziridinyl carbons and the terminal halogen-bearing carbon, respectively), although located in close proximity to C530, are not in the same environment when compounds are in the ligand-binding pocket.

The different patterns of residue labeling with the two types of affinity labels could reflect (i) inherent differences in the reactivity of a haloacetamide function vs an aziridine, (ii) different orientations that these ligands may have when bound within the receptor, and (iii) different conformations that the receptor may adopt around agonists vs antagonists [tamoxifen and its derivatives behave mainly as antagonists, whereas 17α-(haloacetoamidoalkyl)estradiols are partial estrogen agonists with weak antagonist activity (El Garrouj et al., 1995)]. At this point, it is not yet possible to definitively determine which of these three hypotheses is correct.

Superimposition of Tamoxifen-Class Antiestrogens with Steroidal Estrogens. The previous (Harlow et al., 1989; Reese et al., 1992) and the present results highlight the type of superimposition of triphenylethylene derivatives and steroidal estrogens that must be selected to explain the binding properties and hormonal activity of these ligands. Crystallographic and energy determination data have shown that triphenylethylenic compounds occur in a conformation such that the molecule looks like a propeller (Figure 5) with a shaft in the middle of the double bond perpendicular to the plane of the double bond, with values between 40 and 65° for the three dihedral angles defined by each of the phenyl rings and by the plane of the ethylenic bond (Précigoux et al., 1979; Gilbert et al., 1983; Pons et al., 1984).

Structure/affinity relationships for triphenylethylene compounds, including tamoxifen, *cis*-tamoxifen, 4-hydroxytamoxifen, and triphenylacrylonitrile compounds, suggest that the transtilbene element formed by the ethylenic bond and the α- and β-rings, as for the potent estrogen diethylstilbestrol, mimics the whole structure of steroidal estrogens. With 4-hydroxytamoxifen, the α-ring, not the β-ring, is thought to play the role of the estradiol A-ring (Durani et al., 1979; Duax et al., 1981; Raynaud et al., 1985) so that the two phenolic hydroxyls coincide. However, with tamoxifen (and tamoxifen aziridine), which lacks the phenolic function, the alternative superimposition (β-ring of tamoxifen with the A-ring of estradiol) might be equally favorable. However, even considering the α-ring/A-ring orientation, there are two possible superimpositions of tamoxifen with estradiol: one in which the substituted aromatic α′-ring is located above the median plane of the steroid at the level of carbons 11 and 12 and the other (obtained from the former by a 180° rotation of the molecule around the bond linking the α-ring to the central ethylenic bond) in which the α′-ring is located under the median plane at the level of carbons 7 and 8 (Figure 5).

If we assume that in the hormone-binding pocket the relative positioning of tamoxifen aziridine and 17α-(bromoacetamidomethyl)estradiol corresponds to the above-mentioned optimal superimposition of tamoxifen and estradiol, then the fact that 17α-(bromoacetamidomethyl)estradiol

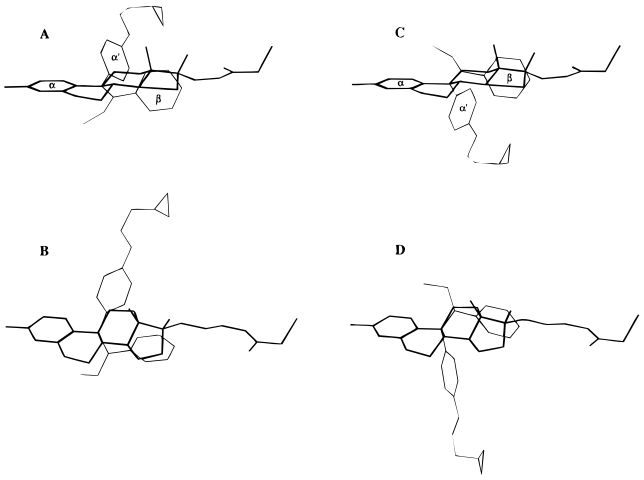

FIGURE 5: Molecular superimposition showing potential binding orientations of tamoxifen aziridine relative to 17α-(haloacetamidoalkyl)estradiols. Structures of 17α-(bromoacetamidomethyl)-estradiol (A and C) and 17α-(bromoacetamidopropyl)estradiol (B and D) were determined by molecular mechanics. The structure of tamoxifen aziridine (A−C) is derived from the tamoxifen conformation established by X-ray crystallographic studies (Précigoux et al., 1979); values for the dihedral angles between the plane of the ethylenic bond and the α-, α′-, or *β*-ring are 51.1, 51.05, and 49.76°, respectively (Pons et al., 1984). In these conformations, energies of the molecules were 79.44, 57.68, and 93.72 kcal/mol for 17α-(bromoacetamidomethyl)estradiol, 17α-(bromoacetamidopropyl)-estradiol, and tamoxifen aziridine, respectively. The α-ring of tamoxifen aziridine (thin lines) is superimposed on the A-ring of the steroidal derivatives (thick lines) so C-4 of tamoxifen aziridine coincides with C-3 of the steroids. The two possible superimpositions of the triphenylethylene derivative with each of two steroids are shown. The α′-ring of tamoxifen aziridine is oriented either (i) above the median plane of 17α-(bromoacetamidomethyl)estradiol (A) and 17α-(bromoacetamidopropyl)estradiol (B) or (ii) under the median plane of 17α-(bromoacetamidomethyl)estradiol (C) and 17α-(bromoacetamidopropyl)estradiol (D).

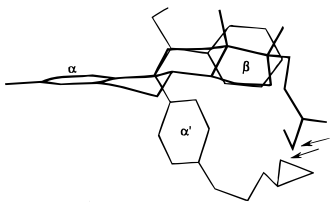

FIGURE 6: Spatial proximity of electrophilic carbons in superimposed tamoxifen aziridine and 17α-(bromoacetamidomethyl)estradiol. Tamoxifen aziridine (thin lines) was superimposed on 17α-(bromoacetamidomethyl)estradiol (thick lines) as described in Figure 5C. Mobilities of the 17α-substituent of the steroid (two degrees of freedom) and the aromatic substituent of the triphenylethylene derivative (four degrees of freedom) were then used to bring closer the bromide-bearing carbon of the steroid and one of the aziridinyl carbons of the triphenylethylene derivative. In the conformations shown, the distance between the above-mentioned electrophilic carbons (arrows) is 1.88 Å.

(whose electrophilic carbon displays mobility only within a short conical space under the median plane of the steroid) and tamoxifen aziridine both alkylate C530 indicates that the α′-ring (bearing the aziridine function), like the 17α-substituent, should be under the median plane of the steroid. From this superimposition, the mobilities of the bromoacetamidomethyl substituent in the steroid and *N*-aziridinylethyloxy group in tamoxifen aziridine could bring the respective electrophilic carbons of the two molecules into close proximity (Figure 6). This could account for the common alkylation of C530 by the two compounds. Due to a longer 17α-substituent, bringing the electrophilic carbons into close proximity would be a much easier process using a bromoacetamidoethyl or bromo- or iodoacetamidopropyl derivative rather than 17α-(bromoacetamidomethyl)estradiol. Hence, to explain the binding characteristics of triphenylethylene derivatives related to those of estradiol, the right type of superimposition is probably that with the α′-ring located under the median plane of the steroid (Figure 5C,D).

Locations of the Covalent Attachment Sites on the Structural Elements Which Putatively Delineate the Hormone-Binding Pocket. The structure of the ligand-binding pocket of nuclear receptors, as defined from crystallographic studies and sequence alignment of hormone-binding domains of nuclear receptors, can be used to predict whether the covalent attachment sites of steroidal and triphenylethylenic affinity labels are actually located on structural elements delineating the hormone-binding pocket. In the case of RAR*γ*, these elements would be helices 1, 3, and 5, a *β*-turn, loop 6−7, helix 11, loop 11−12, and helix 12 (Renaud et al., 1995), whereas for TRα_1, these elements would be helices 2, 3, 5, and 6, a *β*-turn, helix 7, loop 7−8, and helices 8, 11, and 12 (Wagner et al., 1995) (the RAR*γ* helix numbering will be used).

Considering the locations of the three cysteines alkylated by tamoxifen aziridine and 17α-(haloacetamidoalkyl)estradiols in the human estrogen receptor, C381 is at the junction of helices 4 and 5, C417 is the last C-terminal amino acid of helix 6, just ahead of loop 6−7, and C530 is the penultimate C-terminal amino acid of helix 11. The fact that C381, C417, and C530 are located on, or in very close proximity to, structural elements thought to constitute the hormone-binding pocket, especially with respect to RAR*γ*, agrees with proposals related to the structural organization of the hormone-binding domain of nuclear receptors, as determined from crystallographic studies. Moreover, in the model proposed for the hormone-filled RAR*γ*, the fact that the C-terminal ends of helix 6 and helix 11 are closely positioned (i) agrees with our results which suggest that C417 and C530 are in close proximity when the estrogen receptor is binding steroidal estrogenic ligands and (ii) indicates that this model could also account for the ligand-activated estrogen receptor.

Conversely, the fact that in the liganded RAR*γ* model the C-terminal ends of helix 4 and helix 11 are relatively far apart suggests that the RAR*γ* model cannot account for the structure of the estrogen receptor binding an antiestrogen. This may be a logical supposition since the model of liganded RAR*γ* corresponds to an activated receptor, whereas the tamoxifen-liganded estrogen receptor is usually a nonactivated receptor. Tamoxifen binding could elicit a receptor conformation characterized by close proximity of the C-terminal ends of helix 4 and helix 11, together with impaired exposure of helix 12, which encompasses the core of the ligand-dependent transactivation function.

The above argument assumes that, in labeling both C381 and C530, tamoxifen aziridine is bound in a single orientation (the α-ring of tamoxifen aziridine superimposed with the A-ring of estradiol, with the α′-ring projecting downward toward the 7−8 position; see above). While this orientation is a reasonable assumption for hydroxytamoxifen, it is less certain that tamoxifen derivatives that lack a phenol, such as tamoxifen aziridine, will be bound in only one orientation. Thus, the labeling by tamoxifen aziridine of both C381 and

C530, which are separated by some distance in the RARγ model based on bound agonists, may arise not from a different conformation of the receptor when bound to an antagonist but rather from two alternate binding modes of this affinity label; the α-ring/A-ring binding mode, which may label C530, and the β-ring/A-ring mode (perhaps with the α'-ring projecting in the 11−12 direction), which may label C381.

Homologies between Locations of Covalent Attachment Sites in Glucocorticoid and Estrogen Receptors. There are striking similarities when the locations of covalent attachment sites for affinity-labeling agents on the human estrogen and glucocorticoid receptors are considered. Dexamethasone mesylate exclusively alkylates C638 (C656 in the rat glucocorticoid receptor) (Simons et al., 1987), which according to the general sequence alignment is located in loop 6−7, only two or three amino acids away from position C417 of the estrogen receptor. Triamcinolone acetonide, a photoaffinity-labeling agent, alkylates M604 and C736 (M622 and C754, respectively, in rat) (Carlstedt-Duke et al., 1988). M604 is located in helix 5 and is six amino acids away from position C381 of the human estrogen receptor. C736 is in helix 11 and is five amino acids away from position C530 of the human estrogen receptor.

In conclusion, affinity labeling of the estrogen receptor appears to be a reliable technique for identifying nucleophilic amino acid residues of the hormone-binding pocket, which are located in the neighborhood of bound ligands. Our findings with this method could be useful for ligand-directed mapping of the hormone-binding pocket, to evince the differential positioning of estrogens and antiestrogens responsible for the antihormonal action of the latter and also to specify the structure of hormone- and antihormone-filled receptor. Finally, it could be used to validate the structural organization of the ligand-binding pocket, as inferred from crystallographic determinations and sequence alignement of hormone-binding domains of nuclear receptors.

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REFERENCES

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., & Moras, D. (1995) *Nature 375*, 377−382.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Carlstedt-Duke, J., Stromstedt, P.-E., Persson, B., Cederlund, E., Gustafsson, J.-A., & Jornvall, H. (1988) *J. Biol. Chem. 263*, 6842−6848.
Duax, W. L., Griffin, J. F., Rohrer, D. C., Swenson, D. C., & Weeks, C. M. (1981) *J. Steroid Biochem. 15*, 41−47.
Durani, S., Agarwal, A. K., Saxena, R., Setty, B. S., Gupta, R. C., Kole, P. L., Ray, S., & Anand, N. (1979) *J. Steroid Biochem. 11*, 67−77.
El Garrouj, D., Aumelas, A., & Borgna, J.-L. (1993) *J. Med. Chem. 36*, 2973−2983.
El Garrouj, D., Aliau, S., Aumelas, A., & Borgna, J.-L. (1995) *J. Med. Chem. 38*, 2339−2348.
Evans, R. M. (1988) *Science 240*, 889−895.
Gilbert, J., Miquel, J.-F., Précigoux, G., Hospital, M., Raynaud, J.-P., Michel, F., & Crastes de Paulet, A. (1983) *J. Med. Chem. 26*, 693−699.
Glass, C. K. (1994) *Endocr. Rev. 15*, 391−407.
Green, S., Walter, P., Kumar, V., Krust, A., Bornet, J.-M., Argos, P., & Chambon, P. (1986) *Nature 320*, 134−139.
Hard, T., Kellenbach, E., Boelens, R., Maler, B. A., Dahlman, K., Freedman, L. P., Carlstedt-Duke, J., Yamamoto, K. R., & Gustafsson, J.-A. (1990) *Science 249*, 157−160.
Harlow, K. W., Smith, D. N., Katzenellenbogen, J. A., Greene, G. L., & Katzenellenbogen, B. S. (1989) *J. Biol. Chem. 264*, 17476−17485.
Katzenellenbogen, J. A., Carlson, K. E., Heiman, D. F., Robertson, D. W., Wei, L. L., & Katzenellenbogen, B. S. (1983) *J. Biol. Chem. 258*, 3487−3495.
Koike, S., Sakai, M., & Muramatsu, M. (1987) *Nucleic Acids Res. 15*, 2499−2513.
Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornet, J.-M., & Chambon, P. (1986) *EMBO J. 5*, 891−897.
Luisi, B. F., Xu, W. X., Otwinowski, Z., Freeman, L. A., Yamamoto, K. R., & Sigler, B. P. (1991) *Nature 352*, 497−505.
Mangelsdorf, D. J., & Evans, R. M. (1995a) *Cell 83*, 841−850.
Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., & Evans, R. M. (1995b) *Cell 83*, 835−839.
Pons, M., Michel, F., Bignon, E., Crastes de Paulet, A., Gilbert, J., Miquel, J.-F., Précigoux, G., Hospital, M., Ojasoo, T., & Raynaud, J.-P. (1984) in *Progress in Cancer Research and Therapy* (Bresciani, F., et al., Eds.) Vol. 31, pp 27−36, Raven Press, New York.
Pratt, W. B. (1993) *J. Biol. Chem. 268*, 21455−21458.
Précigoux, G., Courseille, C., Geoffre, S., & Hospital, M. (1979) *Acta Crystallogr. B 38*, 312−315.
Raynaud, J.-P., Ojasoo, T., Bouton, M.-M., Bignon, E., Pons, M., & Crastes de Paulet, A. (1985) in *Estrogens in the Environment* (McLachlan, J. A., Ed.) pp 24−42, Elsevier, Amsterdam.
Reese, J. C., & Katzenellenbogen, B. S. (1991) *J. Biol. Chem. 266*, 10880−10887.
Reese, J. C., Wooge, C. H., & Katzenellenbogen, B. S. (1992) *Mol. Endocrinol. 6*, 2160−2166.
Renaud, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., & Moras, D. (1995) *Nature 378*, 681−689.
Schwabe, J. W. R., Neuhaus, D., & Rhodes, D. (1990) *Nature 348*, 458−461.
Simons, S. S., Pumphrey, J. G., Rudikoff, S., & Eisen, H. J. (1987) *J. Biol. Chem. 262*, 9676−9680.
Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., & Chambon, P. (1989) *Cell 59*, 477−487.
Tsai, M. J., & O'Malley, B. W. (1994) *Annu. Rev. Biochem. 63*, 451−486.
Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., & Fletterick, R. J. (1995) *Nature 378*, 690−697.
Weiler, I. J., Lew, D., & Shapiro, D. J. (1987) *Mol. Endocrinol. 1*, 355−362.
Wurtz, J.-M., Bourguet, W., Renaud, J.-P., Vivat, V., Chambon, P., Moras, D., & Gronemeyer, H. (1996) *Nat. Struct. Biol. 3*, 87−94.
Zablocki, J. A., Katzenellenbogen, J. A., Carlson, K. E., Norman, M. J., & Katzenellenbogen, B. S. (1987) *J. Med. Chem. 30*, 829−838.

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