Pergamon

0960-0760(94)00090-5

J. Steroid Biochem. Molec. Biol. Vol. 50, No. 5/6, pp. 241–252, 1994

0960-0760/94 $7.00 + 0.00

Oligomeric Structures of Cytosoluble Estrogen-Receptor Complexes as Studied by Anti-Estrogen Receptor Antibodies and Chemical Crosslinking of Intact Cells

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The structure of estrogen-receptor complexes recovered in cytosolic extracts of MCF-7 cells treated with hormone at 2°C was probed by chemical crosslinking of intact cells and sample analysis with four monoclonal anti-estrogen receptor antibodies. When MCF-7 cells were treated with either glutaraldehyde or dithiobis(succinimidyl propionate), cytosoluble estrogen-receptor complexes consisted of two major forms sedimenting as 4 S monomers and 8–9 S salt-resistant oligomers. By high salt sucrose density gradient centrifugation, we could observe that the four monoclonal anti-estrogen receptor antibodies bound different forms of receptor complexes from crosslinked cells. While H222 and H226 antibodies could interact with any form we detected, the D75 and D547 monoclonals could only recognize those showing sedimentation coefficients lower than 7 S. When cytosolic extracts from [^{35}S]-methionine-labeled cells were subjected to immunoprecipitation with H222 and D75 anti-estrogen receptor antibodies, electrophoretic analysis of material extracted from immunoprecipitates revealed the presence of 65 kDa estrogen receptors. If extracts were prepared from crosslinked cells, instead, two more components with estimated molecular masses of 220 and 100 kDa were specifically immunoprecipitated by the H222 antibody, whereas only the 100 kDa component and the estrogen receptor were found in immunoprecipitates obtained with the D75 monoclonal. When estrogen-receptor complexes were immunopurified from extracts prepared after cells had been crosslinked with dithiobis(succinimidyl propionate), and the oligomers were dissociated by treatment with β-mercaptoethanol, electrophoretic analysis of our samples showed that only the 65 kDa estrogen receptor and a 50 kDa protein were selectively immunoprecipitated by anti-estrogen receptor antibodies. We concluded that the structures of cytosoluble estrogen-receptor complexes in MCF-7 cells treated with hormone at 2°C, include oligomeric forms which contain a 50 kDa non-steroid binding protein.

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INTRODUCTION

The characterization of steroid hormone receptor structures represents a major issue in elucidation of the molecular mechanism of action of these ligand-dependent transcription factors [1]. While cloning of receptor genes and construction of deletion mutants, have provided the primary structures of these proteins and the definition of several functional properties of their domains [1, 2], less detailed information is available regarding the supramolecular arrangements of steroid receptors in intact cells.

The original models of quaternary structures of steroid receptors and their changes in different functional states [3, 4] have been refined in the last few years, leading to a better understanding of their structure–function relationships at this supramolecular level. The emerging concept is that the hormone-free receptors and steroid-receptor complexes in the state with low affinity for DNA acceptor sites, are composed of a single receptor subunit associated with heat shock proteins, including hsp90 [5–21] and hsp56 [20–25], whereas the

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Received 20 Dec. 1993; accepted 20 Apr. 1994.
Abbreviations: hsp90, hsp70, hsp56, heat shock proteins of Mr 90,000, 70,000, 56,000; DSP, dithiobis (succinimidyl propionate); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

interaction with hsp70 [23, 26, 27] has remained controversial [18, 20, 21]. The transformation of steroid–receptor complexes to the states with high affinity for DNA, in turn, would involve dissociation of receptor complex heterooligomers [8, 28–30].

The proposed structure of untransformed steroid-receptor complexes has mainly been based on data obtained with glucocorticoid [6, 8–10, 13, 16, 18, 19, 22, 24, 27] and progesterone [5, 7, 11, 17, 21–24, 26] receptors in cell-free systems. In those studies, the interactions between receptors and associated components have been stabilized by the inclusion of molybdate ions in buffers employed in preparation and analysis of steroid receptors [3–17, 22–27], and/or by sample treatment with chemical crosslinkers [11, 14–16, 18–21, 24]. The usefulness of the latter procedure is based on the capability of certain compounds to induce the formation of covalent bonds between proteins (or proteins and nucleic acids) in direct contact [31–33], and has been employed to study androgen [34, 35], estrogen [24, 36, 37], glucocorticoid [14–16, 18–20, 38], and progesterone [11, 21, 24] receptors. Chemical crosslinking has been also performed using intact cells [14, 15, 19, 20, 37, 39–42], in order to characterize the structures of steroid receptors *in vivo*. Using this approach, indications of the association of hsp90 and hsp56 with glucocorticoid receptors in intact cells have been obtained [15, 19, 20], but similar evaluations of proteins interacting with estrogen receptors *in vivo* have not been performed yet.

In this study we have employed chemical crosslinking of intact MCF-7 cells to investigate the structures of soluble estrogen-receptor complexes. By sample analysis using four monoclonal anti-estrogen receptor antibodies, we could show that cytosoluble estrogen-receptor complexes from crosslinked cells include oligomeric forms, involving the receptor and a non-steroid binding protein of about 50 kDa.

EXPERIMENTAL

Materials

[2, 4, 6, 7-^{3}H(N)]17β-estradiol (104.4 Ci/mmol) was purchased from New England Nuclear. L-[^{35}S] methionine (*in vivo* labeling grade, specific activity greater than 1,000 Ci/mmol), [^{125}I]-labeled anti-rat and anti-mouse immunoglobulin (from sheep), Hybond-C Extra, Hyperfilm-^{3}H and Hyperfilm-MP were purchased from Amersham. Dithiobis(succinimidyl propionate), catalase, bovine serum albumin, goat anti-rat IgG-agarose and prestained molecular mass markers for sodium dodecyl sulfate–polyacrylamide gel electrophoresis were obtained from Sigma. All other reagents were of analytical grade.

Antibodies

Rat anti-human estrogen receptor antibodies H222, H226, D75, D547 [43, 44] were a generous gift of Geoffrey L. Greene (The University of Chicago, Chicago, IL, U.S.A.). Mouse anti-hsp90 antibody AC88 [45], was a kind gift of David O. Toft (Mayo Clinic, Rochester, MN, U.S.A.). Serum obtained from normal rats or mice was the source of nonspecific antibodies used in the experiments involving anti-estrogen receptor and anti-hsp90 antibodies, respectively.

Buffers

Buffer A was composed of 20 mM phosphate buffer, pH 7.4 and 0.15 M NaCl. Buffer B was composed of 20 mM Tris-HCl, pH 7.5 at 2°C, 1.5 mM $MgCl_2$ and 10 mM NaCl. Buffer C was composed of 20 mM Tris–HCl, pH 7.5 at 2°C, 1.5 mM EDTA, 5% (v/v) glycerol and 0.3 M NaCl. Buffer D was composed of 20 mM Tris–HCl, pH 7.5 at 20°C, and 0.2% (v/v) Tween 20. Immunoblotting buffer was composed of 50 mM Tris–HCl, pH 7.5 at 25°C, 0.15 M NaCl, containing either 0.2 or 0.6 % (v/v) Tween 20, depending on whether the buffer was used for immunoblotting of estrogen receptor or hsp90, respectively.

Cell culture conditions

MCF-7 cells were grown in 5% carbon dioxide in air at 37°C, in Petri dishes, with a culture medium composed of Dulbecco's Modified Eagle Medium containing antibiotics (100 units/ml penicillin, and 100 μg/ml streptomycin) 1% nonessential amino acids, 6 ng/ml insulin and 10% fetal calf serum. Four days before the experiment, cells were seeded in the culture medium lacking phenol red and containing 10% charcoal-stripped [46] fetal calf serum. On the day of the experiment, cells were harvested by treatment for 5 min at room temperature with 0.25% trypsin, 5 mM EDTA in buffer A.

Whole cell labeling of estrogen receptor protein

In these experiments we used cells at semi-confluency and maintained for 4 days in phenol red-free medium. The culture medium was changed with Dulbecco's Modified Eagle Medium lacking phenol red and L-methionine and containing 10% charcoal-stripped and dialyzed fetal calf serum. After cells were preincubated for 1 h at 37°C, the medium was removed, and cells were incubated for 2 h at 37°C in fresh medium containing 125 μCi L-[^{35}S]methionine/ml. Cells were then harvested, incubated with nonradioactive 17β-estradiol, and subjected (or not) to crosslinking, before being processed to prepare cytosolic extracts, as described below.

Formation of estrogen-receptor complexes in intact cells

Harvested cells were brought to 2°C, recovered by centrifugation for 8 min at 600 *g*, and washed once by suspension in buffer A and low speed centrifugation. Cells were then dispersed in buffer A and were treated with either radioactive or nonradioactive 17β-estradiol. When estrogen receptor was labeled with radioactive hormone, cells were incubated for 2 h at 2°C with 5 nM tritiated 17β-estradiol and in the presence, or in the absence, of a 200-fold molar excess of nonradioactive 17β-estradiol. Nonradioactive estrogen-receptor complexes were obtained by cell incubation for 2 h at 2°C with 5 nM unlabeled 17β-estradiol.

Crosslinking of intact cells

Cell suspensions were crosslinked with either glutaraldehyde or dithiobis(succinimidyl propionate) (DSP). In the first case cell suspensions in buffer A were treated for 90 min at 2°C with 0.0125% (w/v) glutaraldehyde, as already described [41]. Crosslinking with DSP was performed by the procedure of Rexin *et al.* [15]. Briefly, cell suspensions in PBS were adjusted to 5% dimethyl sulfoxide and were incubated for 1 h at 2°C with 2 mg/ml DSP. At the end of the incubation, crosslinking was stopped by the addition of lysine at a final concentration of 0.12 M and cell incubation for 30 min at 2°C. Crosslinked cells were washed once with buffer A before being used for preparation of cell extracts.

Preparation of cytosolic extracts

Cells were disrupted by suspension in buffer B containing 3% (w/v) digitonin and incubation for 30 min at 2°C with occasional vortexing [40]. The homogenate was then centrifuged for 1 h at 105,000 *g* to obtain the cytosolic extract.

Sucrose density gradient centrifugation

Sedimentation properties of estrogen-receptor complexes were evaluated by linear 5–20% (w/v) sucrose gradients in buffer C. Cytosolic extracts were treated with a dextran-coated charcoal pellet [47], before being loaded onto the gradients, and centrifugations were performed with a Beckman SW-60 rotor in a Beckman L8-70 ultracentrifuge for 18 h at 45,000 rpm. At the end of the centrifugation, gradients were fractionated into 38 fractions.

Immunopurification of estrogen-receptor complexes

Cytosolic extracts were subjected to an immunoprecipitation procedure, in order to immunopurify estrogen-receptor complexes. The extracts were adjusted to 1.5 mM EDTA, 0.3 M NaCl and 0.2% Tween 20, and aliquots (0.3 ml) were incubated for 1 h at 2°C with either 1 μl of normal rat serum, or 5 μg of anti-estrogen receptor antibodies, as specified in the text. Samples were then transferred to a mixture containing anti-rat IgG-agarose, nonfat dry milk, NaCl and Tween 20, in order to continue the incubation for 1 h at 2°C in buffer D containing 2.5% (v/v) affinity matrix, 0.5 M NaCl and 4% (w/v) nonfat dry milk. At the end of the incubation, the affinity matrix was recovered by centrifugation for 5 min at 16,000 *g*, and was washed four times by resuspension in 0.6 ml of buffer D containing 0.5 M NaCl, and centrifugation for 10 min at 16,000 *g* through a 0.6 ml cushion of 10% (w/v) sucrose in buffer D containing 0.5 M NaCl. The affinity matrix was finally washed once by resuspension in 0.6 ml of buffer D and centrifugation for 10 min at 16,000 *g* through a 0.6 ml cushion of 10% sucrose in buffer D. The material bound to the affinity matrix was extracted by incubation for 10 min at 100°C in the presence of 10 mM Tris–HCl, pH 7.0 at 2°C, 2% SDS, 1.5 mM EDTA and 5% β-mercaptoethanol. The mixtures were then centrifuged for 10 min at 16,000 *g*, and the supernatant was collected and subjected to SDS–PAGE.

Fractionation of proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Cytosolic extracts were adjusted to 1.5 mM EDTA, 2% SDS and 5% β-mercaptoethanol, and were incubated for 5 min at 100°C. Cytosolic extracts, or the material obtained from immunoprecipitates, were fractionated by SDS–PAGE according to Laemmli [48], using 6, 7 or 10% separating gels, as specified in the text, and a 3% stacking gel. Molecular mass markers were: α_2-macroglobulin subunit, 180 kDa; β-galactosidase subunit, 116 kDa; fructose-6-phosphate kinase subunit, 84 kDa; pyruvate kinase subunit, 56 kDa; fumarase subunit, 48.5 kDa; lactic dehydrogenase subunit, 36.5 kDa; triosephosphate isomerase subunit, 26.6 kDa.

Detection of [^{35}S]methionine-labeled proteins was performed by fluorography [49] of dried gels, using Hyperfilm-MP.

Immunoblotting

Estrogen receptor and hsp90 were immunoblotted following the procedures of Greene *et al.* [50] and of Riehl *et al.* [45], respectively, with minor modifications. Proteins fractionated by SDS–PAGE were electrophoretically transferred to Hybond-C Extra and binding sites remaining on the membranes were blocked by incubation of blots for 1 h at room temperature with immunoblotting buffer containing 3% nonfat dry milk. Blots were then incubated for 1 h at room temperature with either normal serum or monoclonal antibodies in immunoblotting buffer containing 1% nonfat dry milk. When immunoblotting of estrogen receptor was performed, membranes were incubated with either 0.4 μl of rat serum/ml, or 4 μg of anti-estrogen receptor antibody/ml, as specified in the text. If immunoblotting of hsp90 was carried out, membranes were incubated with either 1 μl of mouse serum/ml, or

10 μg of AC88 antibody/ml. Membranes were then washed four times with immunoblotting buffer containing 1% nonfat dry milk for 5 min, followed by a fifth wash for 10 min at room temperature. Membranes were next incubated for 1 h at room temperature with immunoblotting buffer containing 1% nonfat dry milk and 0.3 μCi [^{125}I]-labeled secondary antibody/ml, consisting of either anti-rat (estrogen receptor immunoblotting) or anti-mouse (hsp90 immunoblotting) immunoglobulin. Membranes were then washed as described above, followed by a final overnight incubation at room temperature with immunoblotting buffer containing 1% nonfat dry milk. Radioactive bands were next visualized by direct autoradiography of dried membranes using Hyperfilm-^{3}H.

RESULTS

Sedimentation properties of estrogen-receptor complexes in cytosolic extracts from crosslinked MCF-7 cells

The formation of covalent bonds between steroid-receptor complexes and associated components induced by crosslinking of intact cells can be easily monitored by the appearance of salt resistant oligomers [15, 39, 41]. In preliminary experiments we then evaluated the effect of our crosslinking procedures on sedimentation properties of estrogen-receptor complexes (Fig. 1). When MCF-7 cells were not subjected to crosslinking, sucrose density gradient centrifugation of cytosolic extracts in the presence of 0.3 M NaCl, resulted in the detection of estrogen-receptor complexes sedimenting as an homogeneous peak at

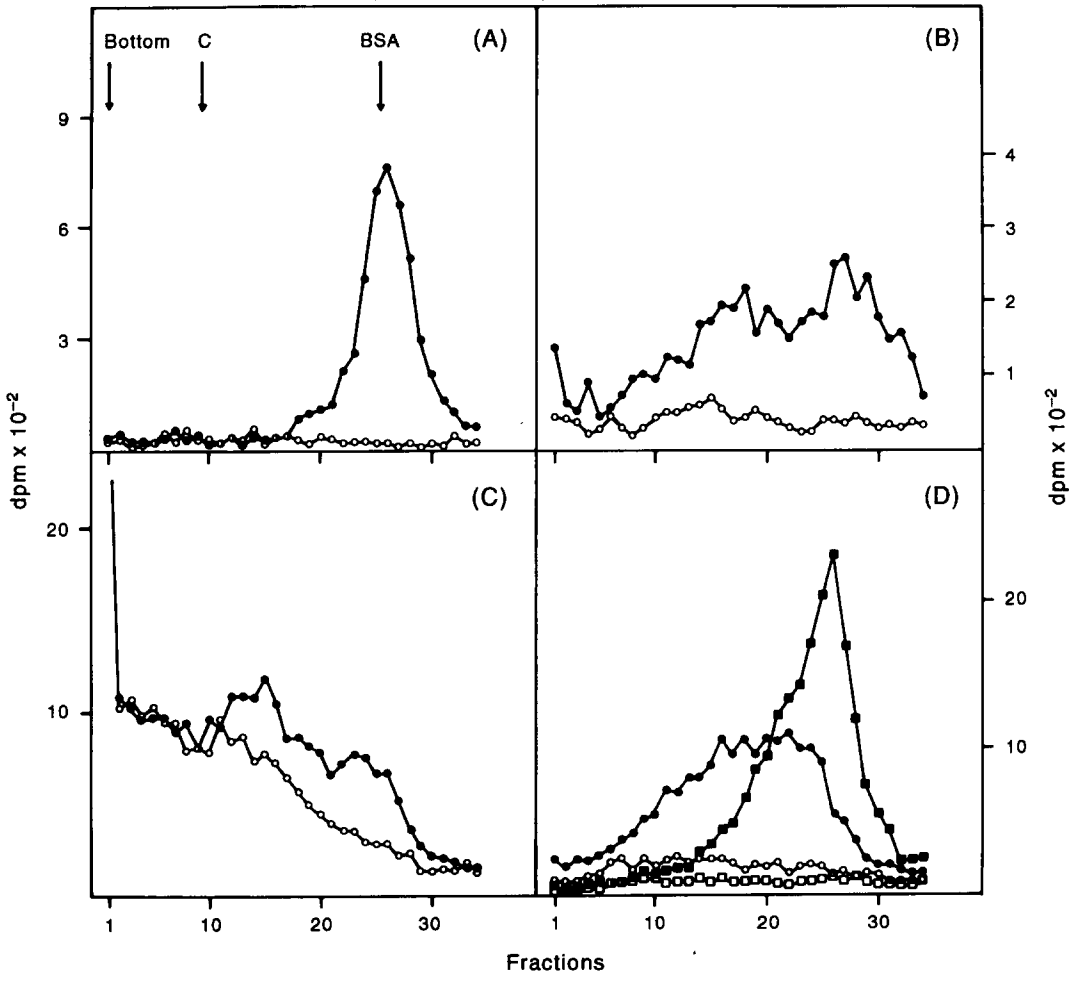

Fig. 1. Effect of crosslinking of MCF-7 cells on sedimentation properties of cytosoluble estrogen-receptor complexes. MCF-7 cells were incubated for 2 h at 2°C with 5 nM tritiated 17β-estradiol and in the presence (open symbols) or in the absence (closed symbols) of a 200-fold molar excess of nonradioactive competitor. At the end of the incubation, cells were either left untreated (panel A), or were subjected to crosslinking with glutaraldehyde (panel B) or DSP (panels C and D), as described under Experimental. Cells were then processed to prepare cytosolic extracts, which were treated (□,■), or not (○,●), with 5% β-mercaptoethanol, before being subjected to sucrose density gradient centrifugation in the presence of 0.3 M NaCl. The data of panel D were obtained in a separate experiment, carried out 1 week after that of panel C. The sedimentation positions of catalase (C, 11.3 S) and bovine serum albumin (BSA, 4.4 S) are denoted by arrows.

4.2 ± 0.2 S ($n = 3$) (Fig. 1, panel A). If cytosoluble estrogen-receptor complexes were prepared after crosslinking of MCF-7 cells with glutaraldehyde, salt-resistant oligomers were detected in addition to 4 S monomers (Fig. 1, panel B). As is apparent from the data shown in Fig. 1, specific estrogen binding sedimented between 10 and 4 S and this heterogeneous pattern most likely reflected the presence of receptor complex forms sedimenting at about 6 S (see below). This type of sedimentation pattern of receptor complexes is often observed when intact cells are subjected to mild crosslinking conditions [41]. In 8 out of 11 separate experiments performed, however, estrogen-receptor complexes from glutaraldehyde-treated cells could be detected as two broad peaks consisting of the 4 S monomer and 8.3 ± 0.4 S oligomers (see for instance Fig. 2, panel A).

The sedimentation properties of cytosoluble estrogen-receptor complexes from MCF-7 cells crosslinked with DSP were similar to those found after glutaraldehyde crosslinking (Fig. 1, panels C and D). In agreement with the chemical properties of the disulfide bridge present in DSP [15, 51], cytosol treatment with β-mercaptoethanol caused the dissociation of estrogen-receptor complex oligomers and the consequent accumulation of monomers (Fig. 1, panel D). The data shown in Fig. 1 (panels C and D) also document a phenomenon we have evaluated in preliminary experiments. Treatment of MCF-7 cells with DSP resulted in detection of rapidly sedimenting, uncompetable 17β-estradiol binding. The amount of this nonspecific binding was particularly high when freshly prepared DSP solutions were used to crosslink MCF-7 cells, so that it could mask detection of cytosoluble estrogen-receptor complexes. The capacity of DSP to induce nonspecific 17β-estradiol binding, however, progressively declined upon ageing of stock DSP solutions (Fig. 1, panel C) and it was negligible when cells were crosslinked employing solutions prepared 2 weeks before use (Fig. 1, panel D). In subsequent experiments, therefore, crosslinking of MCF-7 cells with DSP was carried out exclusively with stock solutions prepared 10–40 days before use.

Interaction of anti-estrogen receptor antibodies with cytosoluble estrogen-receptor complexes from crosslinked MCF-7 cells

The evaluation of the structures of crosslinked estrogen-receptor complexes was performed next. In these experiments we used H222, H226, D75 and D547 monoclonal antibodies, which recognize epitopes located in different portions of the estrogen receptor protein [44, 50, 52].

When cytosolic extracts from MCF-7 cells crosslinked with glutaraldehyde were incubated with nonspecific antibodies, the sedimentation pattern of estrogen-receptor complexes in the presence of 0.3 M NaCl was not changed (Fig. 2, panel A). If samples were incubated with D75 (Fig. 2, panel B) or D547 (not

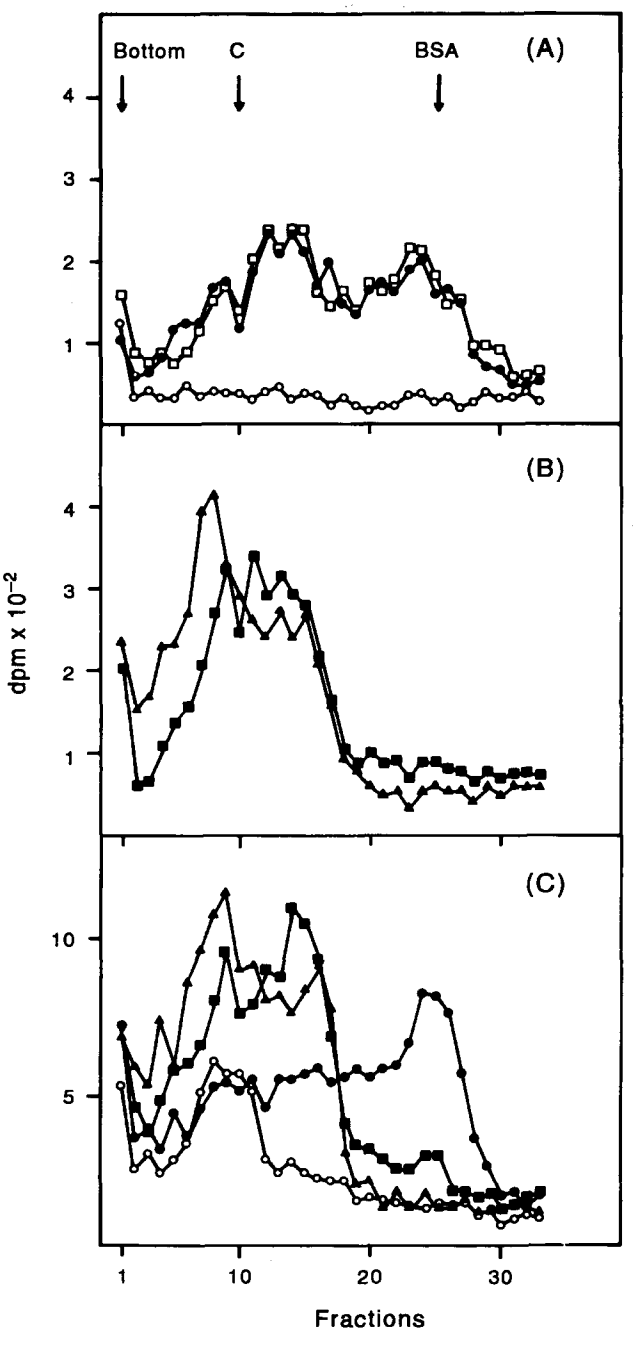

Fig. 2. Effect of anti-estrogen receptor antibodies on sedimentation properties of cytosoluble estrogen-receptor complexes from crosslinked MCF-7 cells. MCF-7 cells were incubated for 2 h at 2°C with 5 nM tritiated 17β-estradiol and in the presence (○), or in the absence (□,●,▲,■) of a 200-fold molar excess of nonradioactive competitor. At the end of the incubation, cells were crosslinked with either glutaraldehyde (panels A and B) or DSP (panel C), before being processed to prepare cytosolic extracts. Aliquots (0.2 ml) of cytosols were then brought to a 0.15 M NaCl concentration and were incubated for 1 h at 2°C with 5 μg of H222 (▲) or D75 (■) anti-estrogen receptor antibodies, 1 μl of normal rat serum (□), or without any addition (○,●), before being subjected to sucrose density gradient centrifugation in the presence of 0.3 M NaCl. Sedimentation positions of catalase (C, 11.3 S), and bovine serum albumin (BSA, 4.4 S) are denoted by arrows.

shown) anti-estrogen receptor antibodies, specific estrogen binding sedimenting at 4 S disappeared, with its concomitant accumulation at 8–10 S. When the H222 (Fig. 2, panel B) or the H226 (not shown) monoclonals were used, instead, the disappearance of 4 S estrogen-receptor complexes and their shift to 8–10 S was also accompanied by detection of a second peak of specific binding at 12–13 S. When these experiments were repeated using cytosolic extracts prepared from cells crosslinked with DSP, the data we obtained after subtraction of nonspecific binding confirmed that both D and H anti-estrogen receptor antibodies shifted 4 S receptor complexes to 8–10 S and that a second 12–13 S peak was consistently observed only after sample incubation with the H monoclonals (Fig. 2, panel C). These data showed that all of the monoclonals we have used could interact with monomeric receptor complexes, and indicated that only the H antibodies could efficiently bind the 8–9 S forms of estrogen-receptor complexes.

In an attempt to gain a preliminary insight into the estrogen-receptor forms bound by the D monoclonals, we first subjected cytosolic extracts from glutaraldehyde-crosslinked cells to sucrose density gradient centrifugation, in order to separate receptor complexes into three fractions of 11–8 S, 7–5 S and 4 S species, which were then incubated with D monoclonals and analyzed by sucrose density gradient centrifugation. The results we obtained confirmed those reported in Fig. 2 and showed that interaction of D antibodies with estrogen-receptor complexes with a sedimentation coefficient higher than 7 S was negligible (not shown).

We could then conclude that H and D antibodies can discriminate between the 8–9 S estrogen-receptor complex oligomers and smaller forms and that the epitopes recognized by D monoclonals are unaccessible to these antibodies when estrogen receptor proteins are part of 8–9 S oligomers.

Analysis of crosslinked estrogen-receptor complexes by immunoprecipitation and SDS–PAGE

A more precise estimate of the size of estrogen-receptor complexes from crosslinked cells was next performed by *in vivo* labeling of receptors with [^{35}S]-methionine and analysis of immunopurified receptor complexes by SDS–PAGE. In preliminary experiments, we established that the incorporation of the radioactive amino acid supplemented to MCF-7 cells in methionine-free medium was linear for up to a 2–3 h incubation at 37°C. We then incubated MCF-7 cells with [^{35}S]methionine for 2 h at 37°C and, after harvesting, cells were brought to 2°C, incubated with nonradioactive 17β-estradiol, subjected, or not, to crosslinking with glutaraldehyde and cytosolic extracts were prepared. Samples were then treated with nonspecific and monoclonal anti-estrogen receptor antibodies, immunoprecipitated and the material recovered from immunoprecipitates was analyzed by SDS–PAGE.

The electropherogram we obtained by fluorography (Fig. 3), revealed that immunoprecipitates of extracts from cells which had not been previously subjected to crosslinking (lanes 2 and 3), contained only monomeric estrogen receptors with an estimated molecular mass of 65 ± 2 kDa ($n = 5$). Monomeric estrogen receptors were also detected in immunoprecipitates of cytosolic extracts from glutaraldehyde-treated cells (Fig. 3, lanes 5 and 6). In addition to this form, immunoprecipitates obtained with the H222 (lane 5), but not with the D75 (lane 6), antibody contained a component with an estimated molecular mass of 220 ± 10 kDa ($n = 3$). A third component with a molecular mass of 100 ± 6 kDa was also specifically immunoprecipitated by H monoclonals and, at a more limited extent, by D75 antibody (Fig. 3, lanes 5 and 6). This estrogen-receptor form was a minor species in immunoprecipitates and its low levels varied in the three separate experiments we have performed.

When these experiments were repeated using cytosolic extracts prepared after MCF-7 cell treatment with DSP, we confirmed the detection of 220 and 100 kDa estrogen-receptor complex oligomers in immunopre-

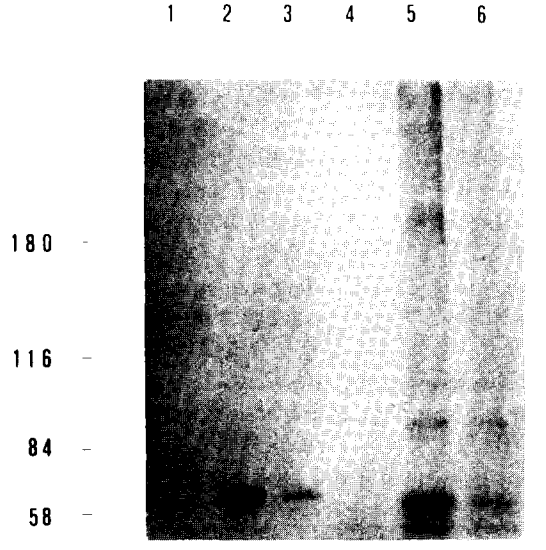

Fig. 3. SDS–PAGE analysis of [^{35}S]methionine-labeled estrogen-receptor complexes after immunopurification from cytosolic extracts of MCF-7 cells crosslinked with glutaraldehyde. MCF-7 cells were labeled with L-[^{35}S]methionine and were incubated with 5 nM nonradioactive 17β-estradiol, as described under Experimental. Cell suspensions were then treated or not with glutaraldehyde, before being processed to prepare cytosolic extracts. Samples were subjected to immunoprecipitation after they were treated with either 1 μl of normal rat serum (lanes 1 and 4), or 5 μg of H222 (lanes 2 and 5) or D75 (lanes 3 and 6) anti-estrogen receptor antibodies, as described under Experimental. The material recovered from immunoprecipitates of cytosols from control (lanes 1–3) and crosslinked (lanes 4–6) cells was then analyzed by SDS–PAGE with a 6% separating gel and radioactive proteins were detected by fluorography. Molecular mass markers were as described under Experimental.

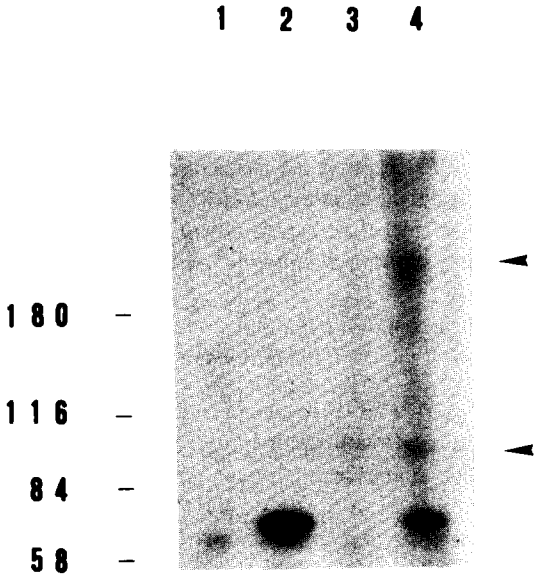

Fig. 4. SDS–PAGE analysis of [^{35}S]methionine-labeled estrogen-receptor complexes after immunopurification from cytosolic extracts of MCF-7 cells crosslinked with DSP. Experimental conditions were as described in the legend to Fig. 3, except that MCF-7 cells were crosslinked with DSP. Samples were subjected to immunoprecipitation after they were treated with either 1 μl of normal rat serum (lanes 1 and 3), or 5 μg of H222 (lanes 2 and 4) anti-estrogen receptor antibody, as described under Experimental. The material recovered from immunoprecipitates of cytosols from control (lanes 1 and 2) and crosslinked (lanes 3 and 4) cells was then analyzed by SDS–PAGE under non-reducing conditions with a 6% separating gel and radioactive proteins were detected by fluorography. Molecular mass markers were as described under Experimental.

cipitates, provided that non-reducing conditions were employed in extraction of material and in its analysis by SDS–PAGE (Fig. 4).

In order to ascertain whether estrogen-receptor complexes were actually present in the three radioactive components immunopurified by anti-estrogen receptor antibodies, we performed immunoblot analysis of cytosolic extracts (Fig. 5). The H222 monoclonal antibody allowed detection of monomeric receptor in samples from both control and crosslinked cells. In agreement with results shown in Figs 3 and 4, samples from crosslinked cells also contained the 220 kDa and low levels of the 100 kDa species. The components we have purified, therefore, represented different forms of estrogen-receptor complexes.

Analysis of protein components of crosslinked estrogen-receptor complexes

The use of a cleavable crosslinker, such as DSP, allowed a preliminary evaluation of the components of estrogen-receptor complex oligomers. To this end, [^{35}S]methionine labeled cells were crosslinked with DSP and the immunoprecipitates obtained from cytosolic extracts were treated with β-mercaptoethanol, in order to release the components of oligomeric receptor complexes. The material recovered from immunoprecipitates was then analyzed by SDS–PAGE (Fig. 6). Immunoprecipitates obtained with the H222 anti-estrogen receptor antibody contained the 65 kDa estrogen receptor and a second component with an estimated molecular mass of 50 ± 2 kDa ($n = 3$), which was barely detectable in control immunoprecipitates (compare lanes 1 and 2). In agreement with the inability of the D75 monoclonal to efficiently bind and immunoprecipitate 220 kDa receptor complex oligomers, the levels of estrogen receptor in immunoprecipitates obtained with this antibody were lower than those observed with its H counterpart and low levels of the 50 kDa component were also detected (compare lanes 2 and 3). In order to evaluate whether the levels of the 50 kDa component and of oligomeric receptor complexes in immunoprecipitates might show some quantitative relation, we used densitometric scanning of the autoradiography and calculated the ratios between the two entities after background subtraction and based on the content of oligomeric receptor complexes in paired samples analyzed by SDS–PAGE under non-reducing conditions. Our three determinations showed that the p50:oligomeric estrogen receptor (220 ± 100 kDa forms) ratios did not significantly differ in samples obtained with H222 and D75 anti-estrogen receptor antibodies, being 0.73 ± 0.09 and 0.64 ± 0.05, respectively. Thus, the levels of the 50 kDa component selectively immunoprecipitated by anti-estrogen receptor antibodies correlated with those of oligomeric receptor complexes,

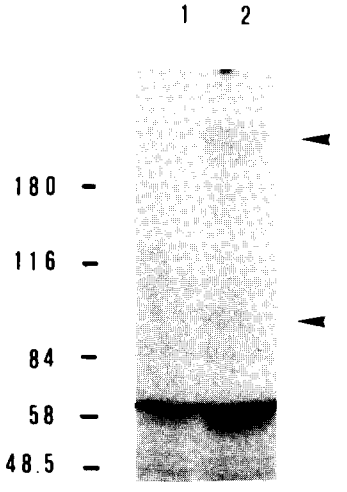

Fig. 5. Immunoblotting of estrogen-receptor complexes from cytosolic extracts of crosslinked MCF-7 cells. MCF-7 cells were incubated for 2 h at 2°C with 5 nM nonradioactive 17β-estradiol, and were then treated (lane 2) or not (lane 1) with glutaraldehyde. After crosslinking, cells were processed to prepare cytosolic extracts, which were fractionated by SDS–PAGE with a 7% separating gel and were subjected to immunoblotting with the H222 anti-estrogen receptor antibody. Molecular mass markers were as described under Experimental.

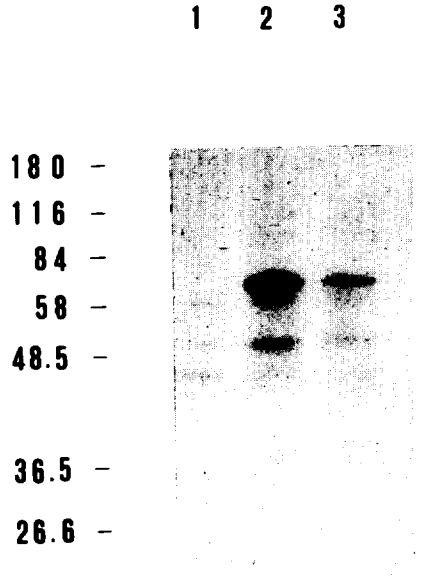

Fig. 6. Protein components of immunopurified estrogen-receptor complexes from cytosolic extracts of crosslinked MCF-7 cells. MCF-7 cells were labeled with L-[^{35}S]methionine and were incubated with 5 nM nonradioactive 17β-estradiol, as described under Experimental. The cell suspension was treated with DSP, before being processed to prepare cytosolic extracts, and samples were then subjected to immunoprecipitation using either normal rat serum (lane 1), H222 (lane 2) or D75 (lane 3) anti-estrogen receptor antibodies, as described in the legend to Fig. 3. The material recovered from immunoprecipitates was then analyzed by SDS–PAGE with a 10% separating gel and radioactive proteins were detected by fluorography. Molecular mass markers were as described under Experimental.

implying that the stoichiometry of estrogen receptor and p50 is the same in both 220 and 100 kDa oligomers.

The methodological approach we have used in these experiments has already been employed to study glucocorticoid-receptor complex oligomers, leading to the conclusion that hsp90 is a major component of these entities in intact cells [15, 19, 20]. The lack of a clear detection of 90 kDa proteins in our immunoprecipitates (Fig. 6), therefore, was of great interest and we considered it was important to directly evaluate the presence of hsp90 in these samples. We then analyzed the material recovered from immunoprecipitates obtained with nonspecific and anti-estrogen receptor antibodies and, after treatment with β-mercaptoethanol, by immunobloting using an anti-hsp90 antibody [45]. The results reported in Fig. 7 showed that similar levels of hsp90 could be detected in immunoprecipitates obtained with both nonspecific (lanes 1 and 4) and anti-estrogen receptor (lanes 2, 3, 5, 6) antibodies and regardless of whether cytosolic samples were prepared from control (lanes 1–3) or DSP-crosslinked (lanes 4–6) cells. Furthermore, the autoradiography shown in Fig. 7 has been obtained with a 6-week exposure, which suggests that the presence of hsp90 in our immunoprecipitates was due to a residual contamination. The same results were also obtained when these experiments were repeated using cytosolic extracts from cells which had not received 17β-estradiol (not shown).

As we could not detect a specific immunoprecipitation of hsp90 by anti-estrogen receptor antibodies in the three separate experiments we have performed, our results indicated that hsp90 is not a component of the oligomeric estrogen receptors we have analyzed.

DISCUSSION

The experimental protocol we have used in this study included hormonal treatment and cell handling at 2°C. Under these conditions, estrogen-receptor complexes remain in a state with low affinity for DNA acceptor sites in intact cells, which results in nuclear leakage of receptor complexes and their recovery in cytosolic extracts upon cell rupture [53–55]. In preliminary experiments we confirmed the observations made by Wrenn and Katzenellenbogen [37] on immobilization of estrogen receptors at the nuclear level upon chemical crosslinking of MCF-7 cells and we found that the amounts of estrogen-receptor complexes in cytosols prepared from crosslinked cells were about 50% of those measured in the extracts from control, non-crosslinked, cells. The estrogen-receptor complex forms we have studied in this investigation, therefore, represent a portion of the total cellular pool, and, being of nuclear origin, should be operationally defined as cytosoluble.

Chemical crosslinking has allowed stabilization of oligomeric estrogen-receptor complexes in intact MCF-7 cells (Figs 1–5), leading to detection of a 220 kDa species and a minor 100 kDa form (Figs 3–5), and the analysis of components in immunoprecipitates has revealed the presence of estrogen receptors (Figs 5 and 6) and a second protein with an estimated molecular mass of 50 kDa (Fig. 6). As our measurements

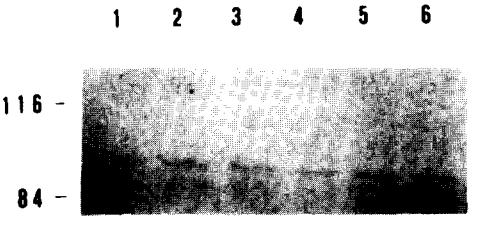

Fig. 7. Detection of hsp90 in immunoprecipitates of cytosolic extracts from MCF-7 cells. MCF-7 cells were incubated for 2 h at 2°C with 5 nM nonradioactive 17β-estradiol and were (lanes 4–6) or were not (lanes 1–3) subjected to crosslinking with DSP. After crosslinking, cells were processed to prepare cytosolic extracts, which were subjected to immunoprecipitation using either normal rat serum (lanes 1 and 4), H222 (lanes 2 and 5) or D75 (lanes 3 and 6) anti-estrogen receptor antibodies, as described in the legend to Fig. 3. The material extracted from immunoprecipitates was then fractionated by SDS–PAGE with a 6% separating gel and immunoblotting was performed using the AC88 anti-hsp90 antibody. Molecular mass markers were as described under Experimental.

indicated that the stoichiometry of estrogen receptor and p50 in 220 and 100 kDa oligomers appeared to be the same, it seems likely that larger forms may be derived by association of smaller ones. Indeed, with the lack of information regarding the methionine content and the turnover rate of p50, the data we have obtained can provide only a preliminary indication on the stoichiometry of components in oligomers, whose estimated molecular masses are consistent with a 1:1 ratio between estrogen receptor and p50.

The nature of p50 and its possible functional role are presently speculative. This protein is not a proteolytic product of estrogen receptor [56, 57], as no specific estrogen binding sedimented in the 2.5–4 S region of sucrose gradients after estrogen-receptor complexes were shifted by monoclonal anti-estrogen receptor antibodies (Fig. 2). Furthermore, no 50 kDa component was specifically detected in our immunoblotting experiments using H222 (Fig. 5), or D547 (not shown), anti-estrogen receptor antibodies, which recognize epitopes present in receptor fragments [44, 56]. The data we obtained, therefore, would be in line with the conclusion that non-steroid binding proteins with molecular masses of 45–60 kDa can associate with estrogen-receptor complexes [22, 24, 58–60]. In agreement with Tai *et al.* [22], we could not detect 70 kDa proteins in estrogen-receptor complex oligomers and this finding is in line with the contention that hsp70 is not associated with receptors [18, 20, 21].

The absence of hsp90 in the oligomers we have analyzed, instead, should be reconciled with the fact that hsp90 has been found associated with estrogen receptors under cell-free conditions [5, 61, 62].

One explanation might be that the oligomeric estrogen receptors we have analyzed represent a specific subset of cellular forms which do not contain hsp90. This possibility should be considered in the light of the fact that, as we have already pointed out, the experimental procedure employed in this study allowed the analysis of a portion of total estrogen receptors. If this interpretation is correct, our data would indicate that multiple supramolecular arrangements of estrogen receptors exist in intact MCF-7 cells.

We cannot exclude, however, the possibility that nuclear estrogen receptors [53–55] in intact cells might not be associated with hsp90, which is believed to be a cytoplasmic component [63]. Thus, most estrogen receptors could interact with hsp90 after cell rupture and nuclear leakage, as suggested by others [64, 65]. If estrogen receptors are stabilized by chemical crosslinking when they are inside nuclei and before cell rupture, as has been done in the present study, any rearrangement of components in the oligomers could be prevented and receptors might not be available to binding of hsp90 once they are released into the cytosol upon homogenization. In line with this contention, recent data show that progesterone receptor is not associated with hsp90 in nuclei of intact cells [66]. Those considerations, instead, might not hold true in the case of the glucocorticoid receptor pool present in the cytoplasm of target cells [67], where it could be associated with hsp90 *in vivo* [12, 15, 19, 20]. Thus, the high degree of structural homology among members of the steroid and thyroid hormone receptor superfamily [2] might not have been maintained in the case of receptor interaction with hsp90, as is indicated by the findings that neither thyroid hormone [68] nor retinoic acid [69] receptors associate with hsp90.

The lack of detection of hsp90 in oligomeric estrogen receptors is in line with the proposal by Gorski's group that the interaction between these two proteins might not reflect the normal state of receptor in intact nuclei, which would involve other nuclear components [65].

A model of the structure and topological arrangement of oligomeric estrogen receptors can then be proposed on the basis of the data we have obtained with the four monoclonal anti-estrogen receptor antibodies. The epitopes recognized by these antibodies have been mapped to defined regions of estrogen receptor proteins [44, 52] and are located in the steroid binding domain (H222), near the DNA binding domain (H226), close to the carboxy-terminus (D75) and between the steroid binding domain and the DNA binding domain (D547). The H antibodies were able to bind the three forms of estrogen-receptor complexes we have detected, whereas the epitopes for D antibodies were not accessible in 220 kDa receptor complexes and, in the case of D75, were partly accessible in 100 kDa forms (Fig. 3). Furthermore, the stoichiometry of p50 and estrogen receptor appeared to be the same in both 220 and 100 kDa oligomers. The simplest interpretation of these data, therefore, is that the estrogen-receptor complex interacts with one molecule of p50, giving rise to a 100 kDa heterodimer and the association of two dimers results in the formation of 220 kDa heterotetramers. In line with this interpretation, the hypothetical supramolecular arrangement of receptor complex oligomers (Fig. 8) could involve the association of two molecules of estrogen receptors, through their steroid binding domain, and one molecule of p50 interacting with each receptor monomer in a region including the DNA binding domain and the adjacent D domain.

Our model of oligomeric estrogen-receptor complex is similar to that proposed by Giambiagi and Pasqualini [70] but we have represented receptor dimers interacting by the steroid binding domains, as the epitopes for the H226 monoclonal were perfectly accessible in the oligomers obtained from crosslinked cells. The two models, however, differ with regard to the non-steroid binding component of heterotetramers, which in that study was assumed to be hsp90 [70]. These discrepancies most likely reflect the different experimental conditions of the two studies, as in that investigation oligomers were not subjected to crosslinking.

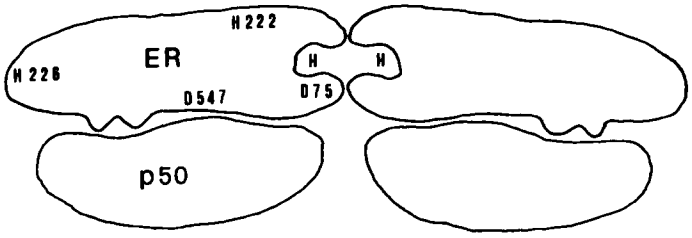

Fig. 8. Hypothetical model of the supramolecular arrangement of components in cytosoluble estrogen-receptor complex oligomers. The location of epitopes recognized by anti-estrogen receptor antibodies on estrogen receptor protein (ER) are indicated. H, estrogen; p50, 50 kDa non-steroid binding protein.

A notable aspect of this model is that it matches a number of established characteristics of estrogen receptor and is coherent with several aspects of the molecular mechanism of functioning of this *trans*-acting transcription factor. On a structural ground, it has been shown that estrogen receptors form homodimers [71–73] through the interaction of a portion of receptor proteins colocalizing with their steroid binding domain [74, 75] and this type of arrangement corresponds to the core unit of our model. In terms of estrogen receptor functioning, in turn, the emerging picture involves the nuclear location of hormone-free receptors in association with proteins [65, 76], giving rise to structures which are incapable of modulating transcription [65, 73]. In the presence of hormone, a temperature-dependent step [65, 77, 78] would induce a change in the structures of estrogen-receptor complexes [76, 79, 80], possibly involving the removal of non-steroid binding proteins covering the DNA binding region of estrogen receptors [73], accompanied by conversion of receptor complexes to transcriptionally competent forms, interacting with estrogen responsive elements as homodimers [71–73]. The mechanistic basis for this productive interaction with the genome would also involve the interaction of receptor homodimers with other proteins in the highly ordered environment of the intact nucleus [65]. Interestingly, a nuclear protein of about 45 kDa has been shown to promote receptor binding to an estrogen responsive element [60], and it is tempting to speculate that it might be one of the nuclear components in the model proposed by Gorski's group [65].

By crosslinking of intact cells incubated with hormone at 2°C, we have probably stabilized one of the structures assumed by estrogen receptors in the course of their functioning *in vivo* and the model we have proposed is coherent with most features of the currently accepted view of their molecular mechanism of action. One structural aspect which might not be directly accounted for by our model, however, is the second weak constitutive dimerization domain contained in the DNA binding region of estrogen receptor [71, 81]. Indeed, this feature could be included in a more complex model involving a direct interaction between two receptor molecules through the steroid binding domain and the second zinc finger. This arrangement would imply that the two molecules of p50 are located on opposite sides of the homodimer, covering the epitopes for the D547 anti-estrogen receptor antibody and preventing the interaction of estrogen receptors with DNA. In any case, both models would be coherent with the view that the temperature-dependent transformation of estrogen-receptor complexes should involve a topological rearrangement of subunits in oligomers, accompanied by the attainment of high ordered structures competent to modulate gene transcription in target cells. The DNA-binding form of forceps-shaped homodimers, in fact, would result from dissociation or rearrangement of p50 and, in the case of the model shown in Fig. 8, after a rotation of the amino-terminal half of receptor proteins, bringing in contact the second zinc finger of each monomer.

More information is certainly required for a better evaluation of these structures. Our working hypothesis is that p50 might have a role in the control of estrogen receptor interaction with the genome.

Acknowledgements—We thank Dr Geoffrey L. Greene, and Dr David O. Toft for kindly providing the monoclonal antibodies used in this investigation. This work was supported by a grant from the Italian Ministero della Università e della Ricerca Scientifica e Tecnologica (Quota 60%).

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