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Subunit structure of the nonactivated human estrogen receptor

(affinity labeling/chemical cross-linking/heat shock proteins/steroid receptors)

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ABSTRACT **The nonactivated estrogen receptor of human MCF-7 mammary carcinoma cells was investigated with respect to stoichiometry of protein subunits. The native receptor complex stabilized by molybdate had a molecular mass of ≈300 kDa. Chemical cross-linking with several bifunctional reagents resulted in complete stabilization of the same receptor form of ≈300 kDa and was achieved both in cell extracts and in intact cells. Incubation of the cross-linked receptor with a receptor-specific monoclonal IgG1 antibody increased the molecular mass by ≈135 kDa—i.e., no more than one immunoglobulin molecule bound to the complex. Partial and progressive cross-linking of affinity-labeled receptors revealed patterns of labeled bands upon denaturing gel electrophoresis indicative of a heteromeric structure. The completely cross-linked receptor was purified to homogeneity and analyzed for protein components. In addition to the receptor polypeptide of ≈65 kDa, we detected the heat shock proteins hsp90 and p59; the hsp90 band was roughly twice as intense as the p59 band. The heat shock protein hsp70 and the 40-kDa cyclophilin were not detected as components of the highly purified cross-linked receptor of ≈300 kDa. We suggest a heterotetrameric structure consisting of one receptor polypeptide, two hsp90 molecules, and one p59 subunit, for which the molecular mass adds up to ≈300 kDa. Thus, the nonactivated estrogen receptor has a molecular architecture homologous to those of glucocorticoid and progesterone receptors, even though phylogenetically the estrogen receptor gene forms a distinct subgroup within the gene family of nuclear hormone receptors.**

Steroid hormone receptors preexist in target cells in molecular forms able to bind the respective hormone but have not yet the potential to interact with specific DNA elements. In this "nonactivated," non-DNA-binding state the receptors are complex structures. The general consensus now is that these receptors contain a dimer of the heat shock protein hsp90 (for reviews, see refs. 1–3). However, other proteins may also be associated, most notably a 59-kDa protein (p59); this protein was first detected immunochemically in the progesterone receptor (4) and was later identified as a component of several steroid receptors, including the estrogen receptor (5–10). Furthermore, estrogen receptors were reported in association with various other proteins of a wide range of molecular masses, including a 14-kDa (11) and a 22-kDa protein (12, 13), a 40-kDa cyclophilin (12, 13), a 50-kDa (14) and a 58-kDa protein (11), and finally the heat shock protein hsp70 (13).

A similarly confusing variety of protein entities has also been described in association with the nonactivated glucocorticoid and progesterone receptors (for review, see ref. 1). However, the technique of chemically cross-linking protein subunits has contributed to sort out which polypeptides are *bona fide* receptor subunits rather than persistent contaminants of the respective purification protocol. Indeed, our data on the murine glucocorticoid receptor (7, 8) and the human progesterone receptor (9) suggest the same heterotetrameric structure for both: one receptor polypeptide in association with two molecules of hsp90 and one p59 subunit. In both instances, the molecular weights obtained for these cross-linked structures agree well with those calculated from hydrodynamic measurements of the native receptors (9, 15).

We now set out to investigate the subunit structure of the human estrogen receptor. This quest is particularly interesting because within the phylogenetic tree of nuclear receptors (16, 17), the estrogen receptor is less closely related to the glucocorticoid and progesterone receptors than these latter two are to each other. Yet the data presented here suggest that the nonactivated forms of all three receptors are quite similar in subunit composition.

MATERIALS AND METHODS

Chemicals. [2,4,6,7-^{3}H]estradiol (3.3 TBq/mmol) and [*ring*-^{3}H]tamoxifen aziridine (0.9 TBq/mmol) were purchased from Amersham. The cross-linking agents were obtained from Pierce. Laboratory chemicals were of reagent grade.

Cell Culture and Cell Extracts. MCF-7 mammary carcinoma cells (18) were maintained at 37°C in RPMI 1640 medium/10% fetal calf serum (charcoal-dextran-stripped)/ penicillin (50 units/ml)/streptomycin (50 μg/ml). For these experiments, cells were grown in medium devoid of phenol red (19). Nearly confluent cultures were harvested by incubation with 1.5 mM EDTA/medium. Cells were washed with phosphate-buffered isotonic sucrose, frozen, and stored at −80°C. Pellets were unfrozen and homogenized at 0°C in 20 mM potassium phosphate buffer, pH 7.4/1 mM EDTA/1 mM phenylmethanesulfonyl fluoride, and the high speed supernatant was obtained (20). Protein concentrations were 10 to 15 mg per ml. Incubation with 30 nM [^{3}H]estradiol or ^{3}H-labeled (*Z*)-1-{4-[2-(*N*-aziridinyl)ethoxy]phenyl}-1,2-diphenyl-1-butene (tamoxifen aziridine) was at 0°C routinely overnight, and excess hormone was removed by charcoal treatment.

Chemical Cross-Linking. We routinely used ethylene glycol-bis(succinimidyl succinate) (EGS) at 10 mM for up to 1 hr at 20°C (15). Intact cells were preincubated for 2 hr at 0°C with 30 nM [^{3}H]estradiol in medium, detached, and treated for 1 hr at 0°C with 10 mM EGS in isotonic saline. Cross-linking reactions were stopped by addition of excess lysine. Cleavage of EGS-treated receptors was by 1 M hydroxylamine at 37°C for 3–5 hr (21).

Gel Filtration and Sedimentation Analysis. Sephacryl S-300 (Pharmacia) gel filtration in 20 mM potassium phosphate buffer, pH 7.4/1 mM EDTA/10% glycerol and sedimentation in linear 10–40% glycerol gradients containing 20 mM Na_2MoO_4 and 150 mM KCl were done as before (20) with bovine catalase (11.29 S) and human hemoglobin (4.44 S) as

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Abbreviations: EGS, ethylene glycol bis(succinimidyl succinate); tamoxifen aziridine, (*Z*)-1-{4-[2-(*N*-aziridinyl)ethoxy]phenyl}-1,2-diphenyl-1-butene; hsp90, heat shock protein 90; p59, 59-kDa protein.

markers. Averages of several experiments are given ± SD, and molecular weights were calculated (22).

Receptor Incubation with Monoclonal Antibody. Cell extracts prelabeled with [^{3}H]estradiol were treated with EGS and incubated overnight at 20°C with the IgG1(κ) monoclonal antibody F3 (23) at 3 mg/ml. For immunoaffinity chromatography the F3 antibody was coupled to 1,1′-carbonyldiimidazole-activated agarose (Sigma) at ≈1 mg of antibody per ml of gel (24). Receptor samples (10–15 mg of protein per ml of immunomatrix) were incubated in 20 mM phosphate buffer, pH 7.4/1 mM EDTA/400 mM KCl/0.1% Tween 20/10% (vol/vol) glycerol for several hours in the cold. The matrix was washed extensively with buffer/1 M KCl/0.5% Triton X-100/10% glycerol. Elution was with 3.5 M sodium thiocyanate, and peak fractions were precipitated with 10% (vol/vol) trichloroacetic acid with bovine insulin at 12 μg/ml as carrier. Pellets were dissolved in 100 μl of 300 mM Tris base and reprecipitated with acetone for removal of salts.

SDS/PAGE and Immunoblotting. Cross-linked samples were analyzed in continuous 4% slab gels with incompletely cross-linked rabbit muscle phosphorylase a (subunit M_r, 97,400) as markers (25). Electroelution was as before (7). Discontinuous SDS/PAGE was in 10% gels, and proteins were detected by silver staining. Transfer of protein to Immobilon-poly(vinylidene difluoride) membranes (Millipore) was as before (9). For detection of receptor polypeptide, hsp90, p59, and hsp70 we used monoclonal antibodies F3 (23), AC88 (26), KN 382/EC1 (4, 5), and N27F3-4 (StressGen Biotechnologies, Sidney, Canada), respectively. Incubation with peroxydase-conjugated second antibodies and staining with 3,3′-diaminobenzidine (Sigma) were as described (27).

RESULTS

Molecular States of the Estrogen Receptor. Extracts of MCF-7 cells were complexed with [^{3}H]estradiol and analyzed by gel filtration. The receptor–hormone complex stabilized in the nonactivated form by molybdate has a Stokes' radius (Fig. 1*A*, closed circles) of 7.39 ± 0.16 nm (five experiments) and a sedimentation coefficient of 9.14 ± 0.1 S (four experiments); this corresponds to a molecular mass of 285 kDa. Exposure to 400 mM KCl in the absence of molybdate yielded a smaller receptor form (Fig. 1*A*, open circles) with a Stokes' radius of 4.60 ± 0.07 nm (seven experiments), which can bind to DNA. These data agree with observations by others (18, 28).

Chemical Cross-Linking in Cell Extracts and in Intact Cells. As an alternative to molybdate stabilization we used chemical cross-linking. In the experiment of Fig. 1*B* (closed circles) we treated the receptor–[^{3}H]estradiol complex with EGS and submitted the material to gel filtration under conditions that normally cause subunit dissociation. We observed a Stokes' radius of 7.38 ± 0.09 nm (four experiments). Thus, molybdate and chemical cross-linking of protein subunits stabilize the same receptor form. Similar data were also obtained with other cross-linkers [dimethyl suberimidate, dithio-bis(succinimidyl propionate), and dimethyl-3,3′-dithiobis(propionimidate)]; however, in some of these experiments we observed receptor material in various amounts eluting with the void volume of the column.

We also used cross-linking with EGS to stabilize the receptor structure in intact cells that had been preincubated with the radiolabeled ligand. After rupturing cells, we applied the extract to gel filtration in the presence of dissociating salt concentrations. Fig. 1*C* shows that the receptor was fully cross-linked. We determined in two independent experiments an average of 7.53 nm for the Stokes' radius and 10.2 S for the sedimentation coefficient. The computed molecular mass of 323 kDa agrees with data obtained for the receptor in cell extracts (see above).

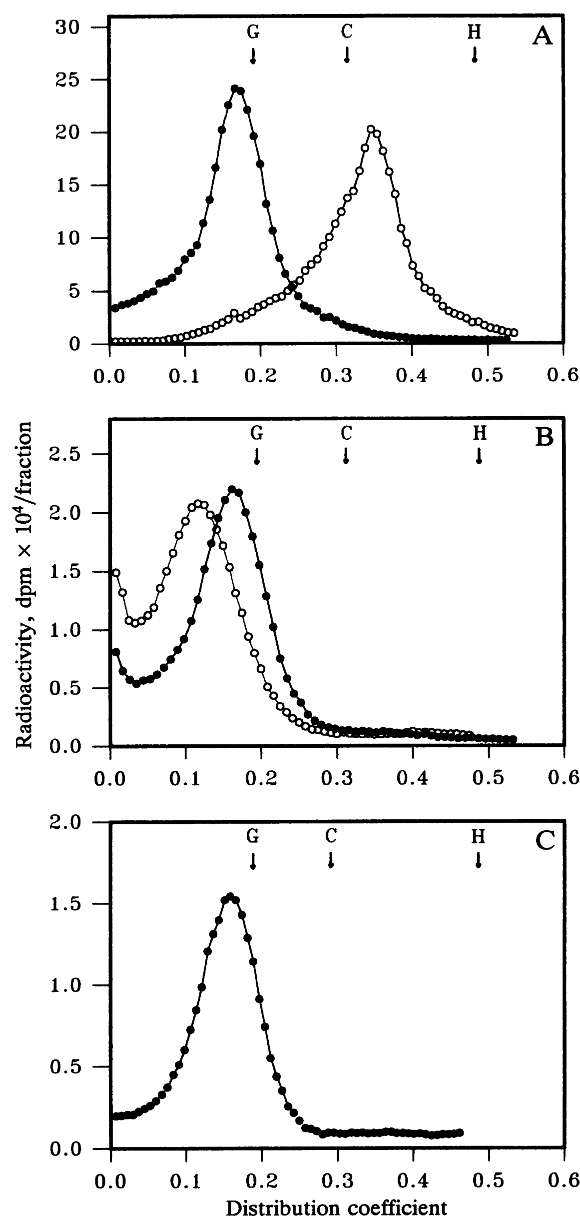

FIG. 1. Gel permeation chromatography of the estrogen receptor. Cell extracts were complexed with [^{3}H]estradiol and submitted to gel filtration on Sephacryl S-300. (*A*) Chromatography was either in the presence of 20 mM sodium molybdate (●) or 300 mM KCl (○). (*B*) The sample was cross-linked with EGS for 1 hr and either used directly (●) or treated with the antibody F3 (○) before gel chromatography in 300 mM KCl. (*C*) MCF-7 cells were preincubated with [^{3}H]estradiol in the cold and treated with EGS before preparing the cell extract which was chromatographed as in *B*. Marker proteins were *Escherichia coli* β-galactosidase [G; Stokes' radius (R_S) = 6.85 nm], bovine catalase (C; R_S = 5.23 nm), and human hemoglobin (H; R_S = 3.21 nm).

Progressive Chemical Cross-Linking. We submitted the estrogen receptor to affinity labeling with [^{3}H]tamoxifen aziridine (29) and electrophoresis in continuous SDS/polyacrylamide gels (Fig. 2*A*). A major labeled band of 67 ± 2 kDa (Table 1; 13 experiments) and a minor band of 55 kDa were obtained. The latter is probably due to partial proteolysis and varied between experiments. These findings agree with published data (29).

The affinity-labeled receptor was then submitted to progressive cross-linking. Analysis by SDS/PAGE (Fig. 2) showed increasingly complex patterns of radiolabeled material with increased time of exposure. Extensive reaction yielded the

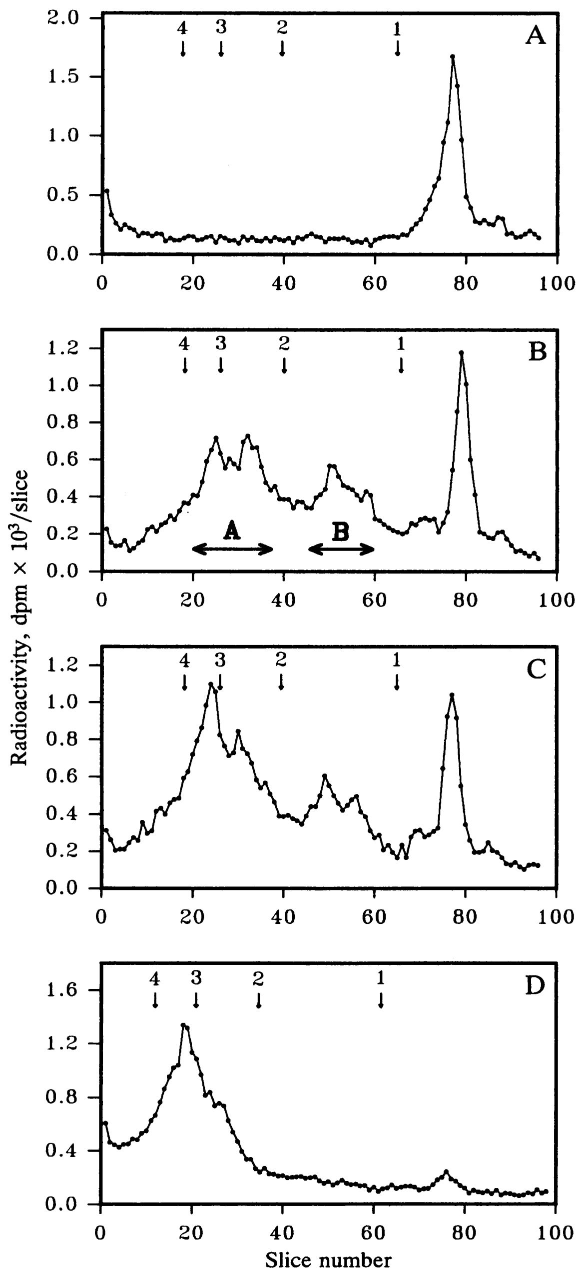

FIG. 2. SDS/PAGE of the progressively cross-linked, affinity-labeled estrogen receptor. Cell extracts were complexed with [^{3}H]tamoxifen aziridine and submitted to cross-linking with EGS for 0 min (*A*), 5 min (*B*), 15 min (*C*), or 35 min (*D*). After immunopurification, the samples were run for 2.5 hr in continuous SDS gels (1.5 mm thick) at 30 mA. Multimers of the phosphorylase a subunit are indicated by arrows 1–4. Gels were sliced, and radioactivity of the slices was counted. In *B*, the molecular mass regions 200–350 kDa (range A) and 110–180 kDa (range B) are marked.

completely cross-linked receptor of 313 ± 12 kDa (Fig. 2*D* and Table 1). We observed three major and some minor peaks between the receptor polypeptide itself (Fig. 2*A*) and the fully cross-linked structure (Fig. 2*D*). The data of several such experiments are summarized in Table 1. This table also presents two alternative subunit models for the nonactivated receptor. The molecular mass values listed cannot be used to discriminate between them due to the fact that the receptor polypeptide and the potential receptor component p59 are so similar in molecular mass. However, the main difference between these models is a qualitative one: the presence or absence of p59. The molecular mass data presented here

Table 1. Progressive cross-linking of the estrogen receptor

Labeled receptor species, kDa (*n*)	Possible subunit composition	
	Model I	Model II
67 ± 2 (13)	R	R
130 ± 6 (10)	R + p59	R + R
158 ± 6 (12)	R + hsp90	R + hsp90
226 ± 15 (3)	R + hsp90 + p59	R + hsp90 + R
253 ± 12 (12)	R + 2 hsp90	R + 2 hsp90
313 ± 12 (12)	R + 2 hsp90 + p59	R + 2 hsp90 + R

Data were obtained from experiments as in Fig. 2. Mean molecular mass and SDs are reported (number of determinations in parentheses). R refers to the affinity-labeled receptor polypeptide. A minor peak or shoulder of ≈86 kDa was seen in some experiments (compare with Fig. 2).

certainly exclude the hypothetical possibility that the fully cross-linked structure contains two molecules of the receptor polypeptide in addition to p59 and two hsp90 subunits.

Components of the Highly Purified Cross-Linked Receptor. In an attempt to distinguish between the receptor models of Table 1 we set out to identify the components of partially and completely cross-linked receptors purified by immunoaffinity chromatography with a monoclonal antibody directed against the receptor polypeptide. We conducted an experiment similar to that of Fig. 2*B* but used for preparative SDS/PAGE at least 10-fold more cell extract. The receptor material contained in the molecular mass regions indicated by ranges A and B (↔) in Fig. 2*B* was recovered, cross-links were cleaved, and the components were analyzed by immunoblotting (Fig. 3). We obtained clear signals for hsp90, the receptor polypeptide, and p59. This result unequivocally shows that p59 is a component of the cross-linked receptor and thus rules out model II (Table 1). Fig. 3, lane B, shows that p59 is also contained in the partially cross-linked material of 110–180 kDa, suggesting that the receptor peak of 130 kDa (Table 1) is composed of the receptor polypeptide plus p59. The hsp90 signal was clearly more intense in lane A than in lane B (Fig. 3). This result supports the view that the receptor peak of 158 kDa (Table 1) contains one hsp90 molecule per receptor polypeptide, whereas the peaks of 253 and 313 kDa (Table 1) contain two hsp90 molecules.

In the experiment of Fig. 4 we submitted the fully cross-linked receptor to extensive purification by immunoaffinity chromatography and subsequent SDS/PAGE. The material of ≈300 kDa was recovered and cleaved; the components were separated by gel electrophoresis and detected by silver staining (lane A) and immunoblotting (lane B). A total of three protein bands was seen—corresponding to hsp90, the receptor

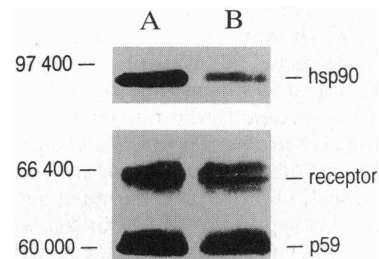

FIG. 3. Immunoblot analysis of partially cross-linked estrogen receptor. Cell extract incubated with [^{3}H]tamoxifen aziridine was cross-linked with EGS for 5 min as in Fig. 1*B* and submitted to immunopurification and continuous SDS/PAGE. Molecular mass regions 200–350 kDa (lane A) and 110–180 kDa (lane B) were excised from the gel, electroeluted, and submitted to cleavage by hydroxylamine. Both samples were analyzed by discontinuous SDS/PAGE and immunoblotting. The Immobilon membrane was cut in three parts, as indicated, and stained with the respective antibodies against hsp90, receptor polypeptide, and p59.

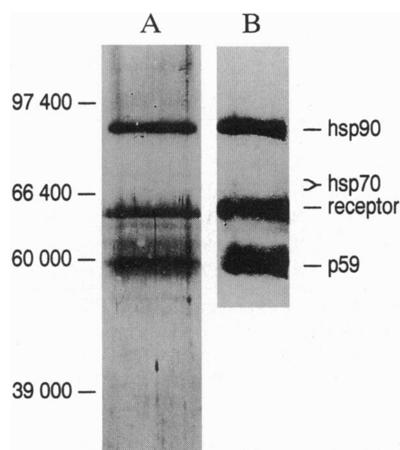

FIG. 4. Protein pattern of the fully cross-linked estrogen receptor. Cell extract incubated with [^{3}H]tamoxifen aziridine was cross-linked with EGS for 1 hr and submitted to immunopurification and continuous SDS/PAGE. The molecular mass region 220–360 kDa was excised from the gel, electroeluted, and submitted to cleavage by hydroxylamine. Analysis was in discontinuous SDS gels with rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,400), bovine liver catalase (60,000), and rabbit muscle aldolase (39,000) as molecular weight markers. Lanes: A, detection by silver staining; B, an identical sample was run on a separate gel and blotted onto Immobilon; then appropriate portions of the membrane were immunostained with the respective antibodies against hsp90, hsp70, receptor polypeptide, and p59. When a crude cell extract was used as control, the hsp70-specific antibody yielded a strong signal.

polypeptide, and p59. The molecular mass determined in these experiments was 58.3 ± 0.6 kDa (10 experiments) for p59 and 64.5 ± 0.5 kDa (10 experiments) for the receptor polypeptide. The intensity of the hsp90 band varied somewhat between experiments. Scanning of several silver-stained gels, such as that of Fig. 4, lane A, showed the intensity of the hsp90 band to be roughly twice that of the receptor and p59 polypeptides, taking into account the relative molecular masses. These data suggest a heterotetrameric structure of the nonactivated estrogen receptor, in which one receptor polypeptide is associated with two hsp90 molecules and one p59 molecule.

In experiments in which we immunopurified the molybdate-stabilized receptor we did not detect p59 by subsequent SDS/PAGE and immunoblotting, whereas hsp90 showed up (data not shown). In contrast to hsp90, this receptor component is obviously removed upon washing with 1 M salt and 0.5% Triton X-100. In some instances, such less stringently purified preparations (only immunopurification) gave immunosignals for hsp70. Also, upon protein staining an additional band of ≈40 kDa showed up (data not shown). However, neither protein is detected in extensively purified preparations previously cross-linked (compare with Fig. 4, lane A). Also the immunoblot of Fig. 4, lane B, did not show any signal with a monoclonal antibody against hsp70. We therefore conclude that both hsp70 and the 40-kDa protein are contaminants of the immunopurification protocol rather than authentic receptor components. Both proteins are removed when the fully cross-linked receptor material is further purified by SDS/PAGE.

Reaction with a Monoclonal Antibody. To obtain independent evidence for the subunit stoichiometry we incubated the cross-linked receptor with the antibody F3. This monoclonal antibody recognizes the very carboxyl-terminal portion of the receptor polypeptide (23), thus optimizing the chances that its epitope is accessible within the large receptor structure. As shown in Fig. 1*B*, the antibody shifted the receptor peak from Stokes' radius 7.38 nm (see above) to 8.43 nm (average of two independent experiments). Sedimentation analysis revealed a corresponding shift to 11.76 ± 0.15 S (three experiments). Taken together, this result corresponds to an increase in molecular mass from ≈285 to 419 kDa.

DISCUSSION

Glucocorticoid and progesterone receptors are closely related with respect to extended polypeptide sequences and subunit structures of the nonactivated forms (7, 9). Because the estrogen receptor is evolutionarily less closely related (16, 17), we became interested in finding out whether in the nonactivated state it has a similar or rather a different subunit composition, as had been proposed (30, 31). By use of two independent lines of experimentation we now provide evidence for a molecular architecture similar to those of glucocorticoid and progesterone receptors.

When we incubated the nonactivated human estrogen receptor with an IgG1 monoclonal antibody directed against the steroid-binding polypeptide, we obtained an increase in molecular mass of ≈135 kDa, as determined from hydrodynamic parameters. Because no more than one antibody molecule reacted, we conclude that only one receptor polypeptide is contained within the receptor structure. In another approach, we directly determined the subunit stoichiometry. We chemically cross-linked the nonactivated receptor and purified it to homogeneity by immunoaffinity chromatography and subsequent SDS/PAGE. After cleavage of cross-links only three polypeptide species were detected either by protein staining or immunoblotting: hsp90, the receptor polypeptide, and p59, with the hsp90 band being roughly twice as intense. We thus conclude that the human estrogen receptor is a heterotetramer composed of one receptor polypeptide in association with two hsp90 molecules and one p59 subunit. Summing up the molecular masses of these components results in 305 kDa.

We need to point out the limitations of the cross-linking technique. It completely depends on the availability of appropriate reactive groups on the individual protein subunits, and these groups need to be in steric juxtaposition. The molecular masses determined here for the molybdate-stabilized receptor (285 kDa) and for the fully cross-linked receptor by SDS/PAGE (313 kDa, compare in Table 1) are certainly in close agreement. This result argues for the proposal that all receptor components did participate in the cross-linking reaction. In addition, there is simply not enough space in terms of molecular mass to accommodate additional components of significant polypeptide size.

In less highly purified preparations that had been carried only through immunopurification we detected some additional proteins on SDS gels, most notably a band at 40 kDa. We assume that this band is the 40-kDa cyclophilin recently described in association with the bovine estrogen receptor (13). However, upon further purification, the 40-kDa protein was lost, proving that it is not a component of the cross-linked structure of ≈300 kDa. Similarly, we did not find hsp70 in association with the cross-linked and highly purified receptor. Nevertheless, hsp70 is required for the assembly of multimeric receptor structures, as shown by use of the reticulocyte lysate system for reconstitution of glucocorticoid and progesterone receptors (2, 32, 33). It is well possible that, in addition to hsp70, other proteins participate in this assembly process.

When we used molybdate for receptor stabilization rather than cross-linking, we again detected the receptor polypeptide and hsp90, but p59 was missing. The latter was obviously washed away during immunopurification, suggesting that it is less tightly integrated in the holoreceptor structure. Nevertheless, our cross-linking studies show (compare Fig. 2 and Table 1) that p59 is in close contact with the receptor polypeptide. Such a direct contact between p59 and the receptor polypeptide may contribute to the nonactivated state of the receptor (34). The p59 previously detected in human

T47D cells (9) and now in MCF-7 cells has a molecular mass very near 59 kDa, but somewhat smaller isoforms may exist in other cell types (see ref. 2). More interestingly, p59 is contained in a heat shock protein complex together with hsp90 and hsp70 (35) and is known to bind the immunosuppressive compound FK506 (see ref. 2).

The estrogen receptor has a strong tendency to dimerize (36–39) and binds as a homodimer to DNA response elements (37, 38). Our finding that the nonactivated complex contains the receptor polypeptide as a monomer, rather than a dimer, suggests that hsp90 and p59 prevent the receptor from dimerization and interacting with DNA. As soon as the receptor is released from the nonactivated complex, dimerization will occur and may not require the hormonal ligand to do so (36, 39).

The cross-linking technique used here has previously provided evidence that the heterotetrameric structure of the glucocorticoid receptor preexists in intact cells (8). We now show that the nonactivated estrogen receptor can similarly become cross-linked in intact cells. This is particularly interesting as the estrogen receptor is clearly a nuclear protein (40, 41), whether activated or not. The fact that a receptor structure of identical molecular mass is stabilized by cross-linking in whole cells and in extracts substantiates the results of detailed analyses done with receptors in cell extracts. Moreover, this observation strongly supports the view that the estrogen receptor polypeptide is associated *in vivo* with hsp90 and p59.

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