D. O., Edwards, D. P., & Horwitz, K. B. (1987) *Biochemistry* (following paper in this issue).
Welch, W. J., & Feramisco, J. R. (1984) *J. Biol. Chem. 259*, 4501–4513.
Welch, W. J., & Feramisco, J. R. (1985) *Mol. Cell. Biol. 5*, 1229–1237.
Welch, W. J., Garrels, J. I., Thomas, G. O., Lin, J. J.-C., & Feramisco, J. R. (1983) *J. Biol. Chem. 258*, 7102–7111.

Immunologic Analysis of Human Breast Cancer Progesterone Receptors. 2. Structure, Phosphorylation, and Processing†

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ABSTRACT: We have used a monoclonal antibody (MAb) directed against chick oviduct progesterone receptors (PR), that cross-reacts with human PR, to analyze PR structure and phosphorylation. This MAb, designated PR-6, interacts only with B receptors (M_r 120 000) of T47D human breast cancer cells; it has no affinity for A receptors (M_r 94 000) or for proteolytic fragments from either protein. The antibody immunoprecipitates native B receptors and was used to study the structure of native untransformed 8S and transformed 4S receptors, using sucrose density gradient analysis, photoaffinity labeling, and gel electrophoresis. On molybdate-containing low-salt gradients, PR-6 complexes with 8S B receptors, causing their shift to the bottom of the gradient while A receptors remain at 8 S. Therefore, A and B receptors form separate 8S complexes, and we conclude that A and B do not dimerize in the holoreceptor. Similar gradient studies using salt-containing, molybdate-free buffers show that there are two forms of salt-transformed 4S receptors, comprising either A proteins or B proteins, suggesting that A and B are also not linked to one another in transformed PR. The independence of A- and B-receptor complexes was confirmed by the finding that purified, transformed B receptors bind well to DNA–cellulose. Since PR-6 cross-reacts with nuclear PR, it was used to analyze nuclear PR processing—a down-regulation step associated with receptor loss as measured by hormone binding. Insoluble nuclear receptors and soluble cytosol receptors were measured by immunoblotting following treatment of T47D cells for 5 min to 48 h with either R5020 or progesterone. From 8 to 48 h after R5020 treatment, immunoassayable receptors decreased in nuclei and were not recovered in cytosols. Nuclear receptors also decreased after progesterone treatment but replenished in cytosols between 8 and 24 h after the start of treatment. Thus, processing involves a true loss of nuclear receptor protein, and not just loss of hormone binding activity, and occurs after progesterone or R5020 treatment. This loss is chronic, however, only in R5020-treated cells. Additional studies focused on the covalent modifications of receptors. We previously described shifts in apparent molecular weight of nuclear PR following R5020 treatment using in situ photoaffinity labeling. To show whether these shifts can be explained by receptor phosphorylation, untreated cells and hormone-treated cells were metabolically labeled with [^{32}P]orthophosphate, and the B receptors were isolated by immunoprecipitation with PR-6 and analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis. In both treatment states, B receptors were labeled in vivo with ^{32}P, thus demonstrating directly that human PR are phosphoproteins. Since B receptors were labeled in the absence of hormone and also after their in vivo transformation by hormone, they appear to be substrates for two phosphorylation reactions, one in the untransformed state and another after they are tightly bound to chromatin. The second phosphorylation may account for the mobility shift of the receptors on SDS gels. On the basis of these data and those in the preceding paper [Estes, P. A., Suba, E. J., Lawler-Heavner, J., Elashry-Stowers, D., Wei, L. L., Toft, D. O., Sullivan, W. P., Horwitz, K. B., & Edwards, D. P. (1987) *Biochemistry* (preceding paper in this issue)], a model of human PR structure and subcellular receptor dynamics is presented.

Two hormone binding forms have been described for the progesterone receptors (PR) of human breast cancer cells: A receptors of 94 000 daltons and B receptors which are doublets of approximately 120 000 daltons (Horwitz et al., 1985a). Their origins and relationship to one another are still unclear as is the manner in which they are organized in the 250 000–300 000-dalton native holoreceptor complex (Horwitz et al., 1985b).

Evidence is accumulating that active[1] holoreceptors contain not only a hormone binding subunit(s) but also additional proteins that do not bind steroids. At least one non-hormone binding protein (M_r 90 000) has been identified (Dougherty et al., 1984; Joab et al., 1984), and others have been implicated (Wrange et al., 1984). Moreover, the structural organization of the receptors is not immutable. Transformation of the active receptors by hormone binding and warming may involve not

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only rearrangement or loss of subunits (Nishigori & Toft, 1980; Joab et al., 1984) but also possibly covalent modification of subunits (Kurl & Jacob, 1984). Unfortunately, the study of these complex macromolecules and their hormone-induced modifications has depended almost entirely on measurement of their ligand binding properties; the need for antibodies in order to proceed with structural analysis has been essential.

The production of antibodies specific for PR has been exceptionally difficult, but polyclonal antibodies have now been described that are directed against chick oviduct (Renoir et al., 1982; Tuohimaa et al., 1984) and rabbit uterine PR (Feil, 1983; Logeat et al., 1981), and monoclonal antibodies against rabbit uterine receptors have been developed (Logeat et al., 1983). The latter reportedly cross-react with human uterine and breast cancer PR, but this has not been further characterized. The PR in human breast cancers have considerable importance, acting both as markers for estimation of hormone dependence (Horwitz et al., 1975) and for prognosis of tumor virulence (Clark et al., 1983). However, the human receptors are known to differ structurally from those of the rabbit and chick oviduct, since the human proteins are 10000–15000 daltons heavier (Horwitz et al., 1985a). Antibodies specific for human breast cancer PR are therefore critically needed for clinical as well as experimental purposes.

Recently, Sullivan et al. (1986) successfully established five hybridoma cell lines that secrete antibodies directed against PR of chick oviduct cytosols. One of these, called PR-6, is B-receptor specific, immunoprecipitates human breast cancer PR, and has been used to immunopurify B receptors and to establish four hybridoma cell lines that synthesize antibodies against the human receptors. This is described in the preceding paper (Estes et al., 1987). In this paper, we further describe properties of PR-6 and its use in studies of holoreceptor structure, nuclear B-receptor binding and processing, and B-receptor phosphorylation. These data are used to propose a model of PR structure and intracellular receptor dynamics following hormone treatment.

Materials and Methods

Reagents. Radioactive R5020 [[17α-*methyl*-^{3}H]promegestone (17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione); 87 Ci/mmol] and unlabeled R5020 were obtained from New England Nuclear Corp. (Boston, MA); Ultrapure tris(hydroxymethyl)aminomethane (Tris)–OH, ammonium sulfate, and sucrose were obtained from Schwarz/Mann, Inc. (Spring Valley, NY); disodium ethylenediaminetetraacetate (Na_2EDTA), potassium chloride, and glycerol were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ); monothioglycerol, insulin, protease inhibitors, *Staphylococcus aureus* V8 protease, trypsin, dithiothreitol, and sodium azide were obtained from Sigma Chemical Co. (St. Louis, MO). The chemicals used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were obtained from Bio-Rad Laboratories (Richmond, CA), and cell culture reagents were purchased from Grand Island Biological Co. (Grand Island, NY), except where noted. Antibodies were purchased from Cappel (Malvern, PA). Sources of other reagents are described in the text.

Cell Culture. T47D are human breast cancer cells in permanent culture that are exceptionally PR rich (Horwitz et al., 1982). Cells were plated in Falcon (Becton-Dickinson, Oxnard, CA) plastic flasks (175 cm^2) and grown in humidified 5% CO_2 and air at 37 °C. The growth medium consisted of RPMI 1640 supplemented with 0.1 mM nonessential amino acids, 2 mM L-glutamine, 5% heat-inactivated (30 min; 56 °C) fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and 6 ng/mL bovine insulin. Cells in these studies were from passages 240–300.

In Situ Photoaffinity Labeling with [^{3}H]R5020. As previously described (Horwitz & Alexander, 1983), approximately 40 × 10^6 cells were harvested with 1 mM EDTA in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution, taken to a 4 °C cold room, pelleted, and washed twice with ice-cold RPMI 1640. They were then divided into two 50-mL polyallomer tubes and incubated for 3 h at 0 °C in an ice-cold bath either with 40 nM [^{3}H]R5020 only or with a 100-fold excess of unlabeled R5020 in a total volume of 5 mL. Cells were kept in suspension with a rotary shaker set at the lowest speed. After incubation, the cells were washed with ice-cold TE buffer (10 mM Tris/1 mM EDTA, pH 7.2–7.4) containing 20 mM sodium molybdate (TEM) and resuspended in 5 mL of buffer. They were then spread over plastic film on the surface of a 300-nm UV transilluminator (Foto-UV 300, Fotodyne Inc., New Berlin, WI) and irradiated for 2 min. Coupling efficiency in situ was 8–10%. The cells were washed, then homogenized in TEM plus a cocktail of protease inhibitors (PI) designed to give a broad spectrum of specificities (TEM + PI: 1 μg/mL pepstatin A, 0.1 mM leupeptin, 100 μg/mL bacitracin, and 77 μg/mL aprotinin) in a −5 °C ice/salt water bath, and centrifuged at high speed (100000*g*; 30 min) to obtain a cytosol. Although the PI were routinely added as a precaution against receptor proteolysis, we find that proteolytic activity in T47D cells is very low, and data with and without PI are identical. For nuclear receptors, cells were incubated 10 min with 20, 40, or 80 nM R5020 (tritium labeled for photoaffinity labeling). They were then harvested, cooled to 4 °C, washed, irradiated 2 min with 300-nm UV light, rewashed, and homogenized in TEG (TE plus 10% glycerol) + PI. Nuclei were pelleted and extracted with 0.4 M KCl, and the suspension was then centrifuged at 100000*g* for 30 min to obtain the supernatant nuclear extract.

Phosphorylation. To label untransformed PR, T47D cells growing in 150 cm^2 flasks were preincubated 30 min at 37 °C with phosphate-free medium (Dulbecco's phosphate-free MEM; Gibco) and then labeled 4 h at 37 °C with 0.03 mCi/mL [^{32}P]orthophosphate (H_3PO_4, ICN Radiochemicals). To label nuclear PR, T47D cells growing in T-150 flasks were preincubated for 30 min at 37 °C with phosphate-free medium. They were then treated for 10 min at 37 °C with 80 nM unlabeled R5020 to transform receptors to the tight chromatin binding state, before the medium was charged with [^{32}P]-orthophosphate. After being labeled, cells were harvested; cytosols or nuclear extracts were prepared in the presence of protease inhibitors and processed for immunopurification as described below.

Immunoprecipitation. ^{32}P-Labeled cytosols or nuclear extracts (1 mL) were mixed with PR-6 (20 μg/mL final concentration) and incubated at 4 °C overnight. In a separate reaction, a "precoated" protein A–Sepharose slurry was prepared by incubating a well-washed protein A–Sepharose/TEDG [TE containing 1 mM dithiothreitol (DTT) and 10%

[1] "Inactive" receptors cannot bind hormone in contrast to "active" receptors which are potentially able to bind hormone. If they are "unoccupied" by hormone, or occupied by hormone treatment in the cold, they are "untransformed" and are characterized by low affinity for nuclei, chromatin, or DNA. Such receptors are easily "soluble" in isotonic buffers and are recovered in "cytosols" when cells are broken. In contrast, "transformed" hormone–receptor complexes have high affinity for nuclei in intact cells, as a result of hormone treatment plus warming. They are "nuclear" and "insoluble" in isotonic buffers when cells are broken.

glycerol] slurry (1/1) for 1 h at 4 °C with rabbit anti-mouse IgG (0.3 mg/mL final concentration) using end-over-end rotation. After three more washes in TEDG, the slurry was diluted in TEDG (1/2) and stored overnight at 4 °C. The next morning, 1 mL of the PR-6–receptor complex was mixed with the pellet from 1 mL of the anti-mouse IgG/protein A–Sepharose slurry and incubated 4 h at 4 °C with end-over-end rotation. The resin was then pelleted (5 min, 1000*g*), the supernatant was removed, and the pellet was washed extensively with TEDG + M (3 times), TEDG + M + 0.3 M KCl (3 times), TEDG + M + 0.2% NP-40 (3 times), and TEDG + M (once). After the last wash, the pellet was mixed with 50 μL of TEDG + M, then with 100 μL of SDS/DTT/BPB sample buffer (10% SDS, 0.1 M dithiothreitol, and 0.2% bromphenol blue in 50% glycerol), boiled 5 min, and centrifuged (3000*g*, 1 min), and the supernatants were subjected to SDS–PAGE immediately or stored frozen for later use.

Proteolysis. Proteases at 10× concentration prepared in 10 mM Tris, 1 mM EDTA, 0.1% SDS, and 10% glycerol were added to photoaffinity-labeled cytosol to yield final concentrations of 0.5–8.0 μg/mL (*S. aureus* V8) or 100–300 μg/mL (trypsin) and incubated at room temperature for 30 min. Cytosols were then processed for electrophoresis.

Gel Electrophoresis. Cytosol (150 μL), nuclear extracts (150 μL), or gradient fractions (200–250 μL) were mixed with 40 μL of SDS/DTT/BPB sample buffer, boiled for 2 min, and then cooled. Immune-purified receptors were prepared as described above; 100–150 μL was analyzed. Electrophoresis was performed on discontinuous 1-mm polyacrylamide slab gels (Model 220, Bio-Rad Laboratories). Running gels consisted either of linear 7.5–19% acrylamide gradients or of 7.5% gels as described by Laemmli (1971) and 0.08–0.2% *N,N'*-methylenebis(acrylamide) (0.3% for 7.5% gels); stacking gels contained 3% acrylamide and 0.08% *N,N'*-methylenebis-(acrylamide). Samples were run for 4 h at 40 mA/gel or until the dye front reached the end of the gel. ^{14}C-Labeled molecular weight standards purchased from Bethesda Research Laboratory (Gaithersburg, MD) were the following: myosin, 200 000; phosphorylase B, 97 400; bovine serum albumin (BSA), 68 000; ovalbumin, 43 000; α-chymotrypsinogen, 25 700; β-lactoglobulin, 18 400; cytochrome *c*, 12 300. These were run in a parallel lane. Gels were then used for fluorography, electroelution, or immunoblotting. For fluorography, the gels were fixed in 30% methanol/10% acetic acid. They were then soaked in En^{3}Hance^R (New England Nuclear) for 1 h and water for 1 h, dried under vacuum, and used to expose Kodak X-Omat XAR-5 film (Eastman Kodak, Rochester, NY), which was held in a metal film cassette at −70 °C for 10–30 days.

Electroelution. Electrophoretic gels containing photoaffinity-labeled receptors were stained with Coomassie Brilliant Blue, and the position of molecular weight standards was used to excise A or B receptors in 2–3-mm-wide slices. Accuracy of excision was verified by fluorography of the remaining gel. Four gel slices were homogenized in 500–1500 μL of buffer (25 mM Tris/192 mM glycine, pH 8.3), and the proteins were eluted for 8 h using an electroelution apparatus (ISCO, Model 1750) set at 3 W. Running buffer consisted of homogenization buffer containing 0.1% SDS. Elution (determined by solubilization and scintillation counting of sample slices) was 75% efficient, and A and B proteins, concentrated to 225 μL, were rerun on 7.5–19% gels and used for fluorography or for immunoblotting. B proteins were pure as judged by silver staining (Oakley et al., 1980); A proteins were occasionally contaminated by the M_r 90 000 heat shock protein as judged by immunoblotting with Ac88 antibody (Riehl et al., 1985). This protein runs just ahead of the M_r 94 000 A protein.

Immunoblotting. Unstained electrophoretic gels were incubated 30 min at room temperature in transfer buffer (20 mM sodium phosphate, 20% methanol, and 0.05% SDS, pH 6.5) and then transferred to nitrocellulose filters (S+S, 0.45 μm; Research Products International, Inc.) using a Transphor apparatus (Hoeffer Scientific, San Francisco, CA) operating at 0.4 A for 5 h (Towbin et al., 1979). Prestained molecular weight markers (Bethesda Research Laboratories) were co-transferred to the filters. After transfer, nonspecific sites on the nitrocellulose were blocked by incubating for 1–3 h at room temperature in wash buffer [10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.4, 1 mM EDTA, 30 mM NaCl, 0.5% Triton-X-100, and 0.25% gelatin] containing 3% bovine serum albumin (BSA). The filter was then incubated at 4 °C overnight with 10 μg/mL PR-6 in dilution buffer (wash buffer containing 1% BSA) and then washed 3 times for 10 min with wash buffer using a rocker platform at room temperature. The filter was then incubated at room temperature for 3 h with horseradish peroxidase conjugated goat anti-mouse IgG (heavy and light chain specific, Cappel) at a 1/1000 dilution. Unbound antibody was removed by one wash with wash buffer, followed by three washes in substrate buffer (50 mM Hepes/150 mM NaCl, pH 7.4), and immunoreactive bands were visualized by incubation with substrate buffer containing 0.5 mg/mL 4-chloro-1-naphthol/15% methanol and 0.025% H_2O_2. After the desired band intensity was achieved, the filter was washed with distilled water and photographed. If the receptors were covalently labeled with either phosphate or tritium, the filter was then air-dried and used for ^{32}P autoradiography, or for fluorography of tritium after being sprayed with En^{3}Hance.

Sucrose Density Gradient Centrifugation. A concentrated cytosol containing untransformed (8S) receptors was prepared from cells that had been photoaffinity labeled in situ at 0 °C with 80 nM [^{3}H]R5020 and then homogenized in TEM + PI. To obtain transformed (4S) receptors, cytosol was prepared from untreated cells using TE + PI. The cytosol was then incubated with 20 nM [^{3}H]R5020 for 4 h at 0 °C, photoaffinity labeled with UV, and brought to 0.4 M NaCl in order to transform receptors. To stabilize hormone–receptor complexes, free hormone was not removed. For each cytosol, a 100-μL aliquot was used to measure PR by charcoal assay, and also for protein measurements. The cytosols were then incubated with PR-6 (20 μg/mL) or a nonspecific monoclonal antibody (MOPC-21; Sigma) for 3.5 h at 4 °C, followed by incubation with a 5-fold excess of a second antibody (rabbit anti-mouse IgG, Cappel) for an additional 3 h at 4 °C to increase the mass of the complex. ^{14}C-Labeled BSA was added to each sample as an internal standard. Untransformed receptors were layered on 4 mL of 5–20% sucrose gradients containing 20 mM molybdate and PI, and transformed receptors were layered on 4 mL of 10–30% gradients containing 0.4 M NaCl and PI. Gradients were formed on a 250-μL cushion of glycerol to trap receptors sedimented to the bottom of the tube. Tubes were centrifuged 16.3 h at 297000*g* in an SW60 rotor (Beckman) and then fractionated into 200-μL aliquots of which 25 μL was counted to determine the radioactive profile, and the remainder was frozen. Fractions of interest were thawed, boiled in SDS sample buffer, subjected to electrophoresis, and transferred to nitrocellulose, and the filter was used first for blotting with PR-6 and then for fluorography. The fluorograms of photoaffinity-labeled A and B receptors are shown; immunoblotting data were identical for the B receptors and are not shown but confirm that pho-

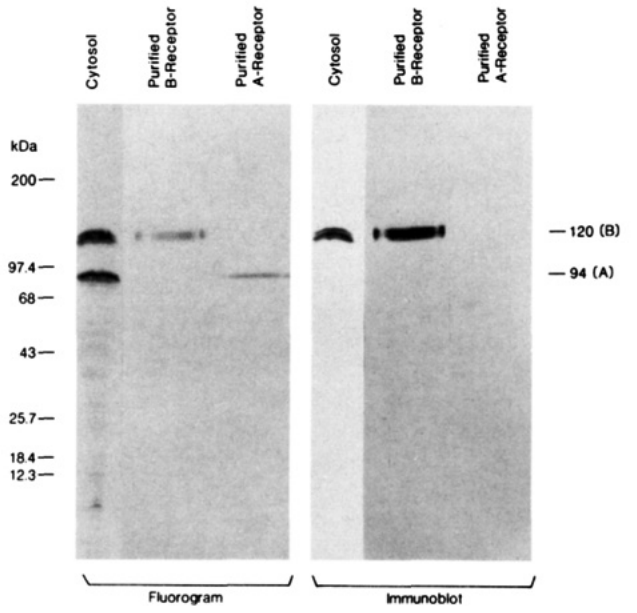

FIGURE 1: Specificity of antibody PR-6 for human progesterone receptors. Intact T47D cells were incubated with 40 nM [^{3}H]R5020 for 3 h at 0 °C, treated with UV light for 2 min to in situ photoaffinity label the receptors, and then homogenized, and a cytosol was prepared. The labeled cytosol was mixed with SDS sample buffer and boiled, an aliquot was stored frozen, and the remainder was subjected to SDS–PAGE. Proteins A and B were cut from gels, electroeluted, and, together with the stored cytosol sample, again subjected to electrophoresis. Proteins in this gel were transferred to nitrocellulose; the sheet was first probed with antibody PR-6 (immunoblot), then sprayed with Enhance, and subjected to fluorography.

toaffinity labeling data parallel protein data.

RESULTS

We have previously shown (Horwitz et al., 1985a) that human progesterone receptors have two hormone binding proteins—A receptors with a mass of 94 000 daltons and B receptors that usually appear as doublets of 116 000–120 000 daltons. To study the structure and biological activity of these proteins further, we have used an antibody called PR-6 that was produced against PR of chick oviducts (Sullivan et al., 1986). It cross-reacts with human receptor B but not A proteins as shown in Figure 1. In this study, B and A proteins from T47D cells were cross-linked to [^{3}H]R5020 by 300-nm ultraviolet light, separated by SDS–PAGE, and then purified from gels by electroelution. The purified, covalently labeled receptors were reapplied to separate lanes of an acrylamide gel and after electrophoresis were blotted to a nitrocellulose filter. The filter was first probed with antibody PR-6 to measure the immunoreactive proteins, and then the same filter was used to obtain a fluorogram of the radioactive hormone-labeled proteins. Nonpurified covalently labeled cytosol proteins were included as controls. As the fluorogram shows, B and A receptors were the major photolabeled proteins in cytosol and were separated in the purified fractions. The immunoblot shows that the antibody cross-reacts with both bands of the B-receptor doublet but has no apparent affinity for A receptors. Furthermore, it appears that the presence of A does not interfere with binding of PR-6 to B, so that the antibody can be used to study B-receptor structure in crude cell extracts by gel electrophoresis and immunoblotting.

In an attempt to map its binding site, we tested the ability of PR-6 to cross-react with proteolytic receptor fragments but found, as Figure 2 shows, that PR-6 interacts only with undegraded B receptors. In this experiment, photoaffinity-labeled cytosol PR were partially digested with *S. aureus* V8 protease or with trypsin and then subjected to electrophoresis. intact receptors and proteolytic fragments were then blotted to nitrocellulose, and the filter was first probed with PR-6 and then analyzed by fluorography. The fluorogram shows the presence of intact tritium-labeled B and A receptors in the absence of enzymes and a ladder of decreasing molecular weight hormone

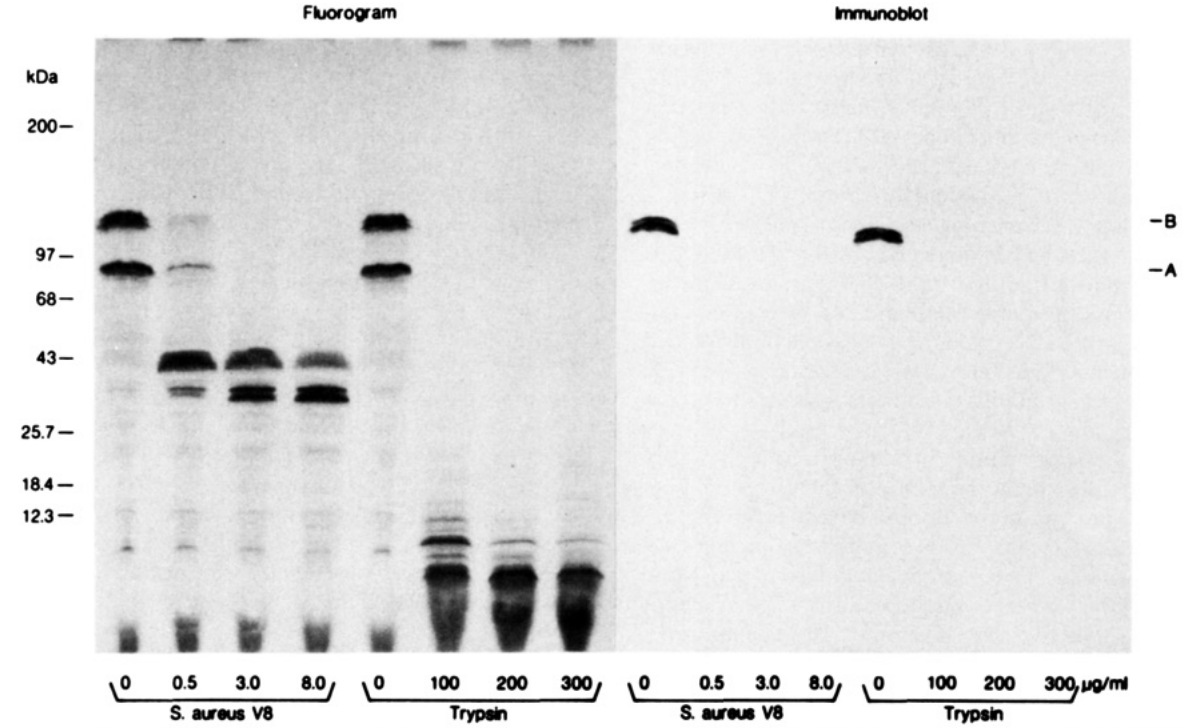

FIGURE 2: Analysis of PR-6 binding to proteolytic fragments of progesterone receptors. Intact T47D cells were incubated with 40 nM [^{3}H]R5020 for 3 h at 0 °C, the receptors were photoaffinity labeled in situ, the cells were then homogenized, and a cytosol was prepared. Aliquots of the labeled cytosol were incubated with increasing concentrations of *S. aureus* V8 or trypsin for 30 min at room temperature. After denaturation in SDS sample buffer, cytosols were subjected to SDS–PAGE on gradient gels, the proteins were blotted to nitrocellulose, and the sheet was first probed with PR-6 (immunoblot), then sprayed with Enhance, and subjected to fluorography.

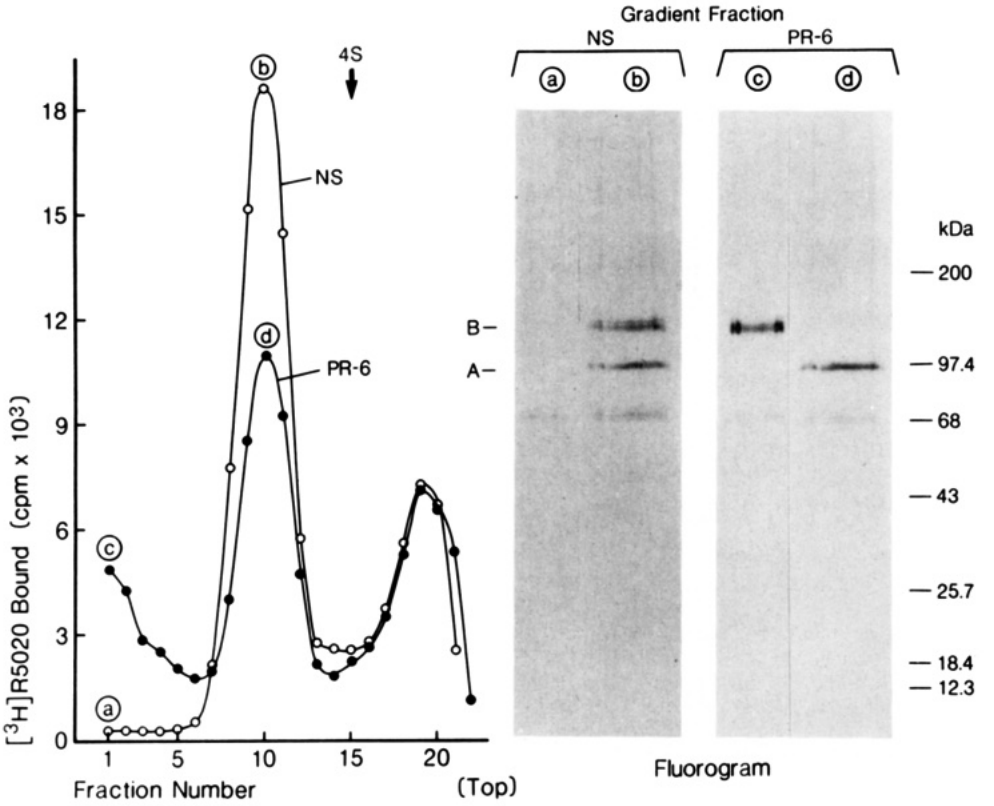

FIGURE 3: Separation of untransformed 8S B receptors from 8S A receptors by PR-6 in the presence of molybdate. T47D cells were incubated at 0 °C with 80 nM [^{3}H]R5020 and photoaffinity labeled in situ, and a labeled cytosol was prepared in buffer containing molybdate. Aliquots (200 μL) of the cytosol were incubated with antibody PR-6 or a nonspecific control antibody (NS), both sets were then incubated with a second anti-mouse IgG, and the mixtures were layered on 5–20% molybdate-containing sucose gradients and centrifuged. Gradient fractions (200 μL) were collected; 25 μL was counted to obtain the radioactive profiles (left panel), and 175 μL from the fractions of interest (fractions labeled a–d) was subjected to electrophoresis on SDS–PAGE and transferred to nitrocellulose, and the filter was used for fluorography (right panel). 3.28 mg of protein/200 μL and 711 166 specific cpm/200 μL were loaded on the gradient. ^{14}C-labeled BSA was included as a 4S sedimentation marker on gradients, and its position is marked by the arrow. ^{14}C-Labeled molecular weight markers were run in a parallel electrophoretic lane, and their position is shown on the right of the fluorogram.

binding receptor fragments following digestion with increasing enzyme concentrations. Though a set of photoaffinity-labeled fragments was generated by the two enzymes (left panel, Figure 2), only the intact B receptors were detected by immunoblotting (right panel, Figure 2), suggesting either that the antibody recognizes an epitope on B that is destroyed by both of these enzymes or that the antibody cross-reacts only with the protein in its native conformation. The latter is possible since the immunoblotting conditions would be expected to partially renature the receptors (Bowen et al., 1980; Oblas et al., 1983), and we find that PR-6 does not immunoprecipitate B receptors that have been unfolded by heat denaturation (not shown). Since PR-6 recognizes only undegraded PR, all subsequent experiments contained a cocktail of protease inhibitors in buffers to guard against proteolysis (see Materials and Methods).

Because it is B specific and binds to native PR, antibody PR-6 can be used to study the nature of the association between A and B proteins in the untransformed 8S state and in the transformed 4S state. This is of interest since there are conflicting models for the molecular interaction of the A and B proteins. One holds that A and B dimerize (Schrader et al., 1981), and the other that A and B exist as separate 8S molecules (Dougherty & Toft, 1982; Renoir & Mester, 1984). We reasoned that if the first model is correct, addition of PR-6 to receptors stabilized in the 8S state would shift both A and B proteins to the bottom of sucrose density gradients, but that A would not be shifted if the two proteins form independent 8S holoreceptor complexes. Figure 3 shows a study in which PR, covalently labeled with [^{3}H]R5020 by UV irradiation, were incubated with PR-6 or a control antibody and then sedimented on sucrose gradients in the continuous presence of molybdate and protease inhibitors to maintain intact and native PR conformation. Aliquots (25 μL) of every gradient fraction were counted to obtain the [^{3}H]R5020 binding profile (Figure 3, left), and an additional 175-μL aliquot of the bottom fractions (a, c) and peak 8S fractions (b, d) were analyzed by gel electrophoresis and fluorography (Figure 3, right). The control antibody had no effect on 8S sedimentation of PR, and electrophoretic analysis showed that all B and A receptors remained in the 8S peak. In contrast, after addition of PR-6, half of the 8S radioactivity was shifted to heavier sedimenting forms. Electrophoretic analysis of the shifted receptors at the bottom of the gradient (fraction c), as well as the residual receptors in the 8S peak (fraction d), showed only B present in the antibody-shifted fraction while A, separated from B, remained at 8S. We conclude that A and B do not dimerize but that each exists as a separate holoreceptor which is 8S because of self-association, or association with non-hormone binding proteins. Two non-hormone binding proteins of 90 and 76 kDa copurify with untransformed human B receptors (Estes et al., 1987).

PR-6 can also separate B receptors from A receptors that have been transformed to the 4S species by treatment with salt. Approximately half of the radioactivity seen in the 4S peak in the presence of control antibody was shifted to heavier aggregates upon addition of PR-6 and a secondary antibody (Figure 4, left). The control 4S peak contained both hormone binding proteins, but only A remained at 4 S after PR-6 addition while the B receptors were shifted to heavier sedi-

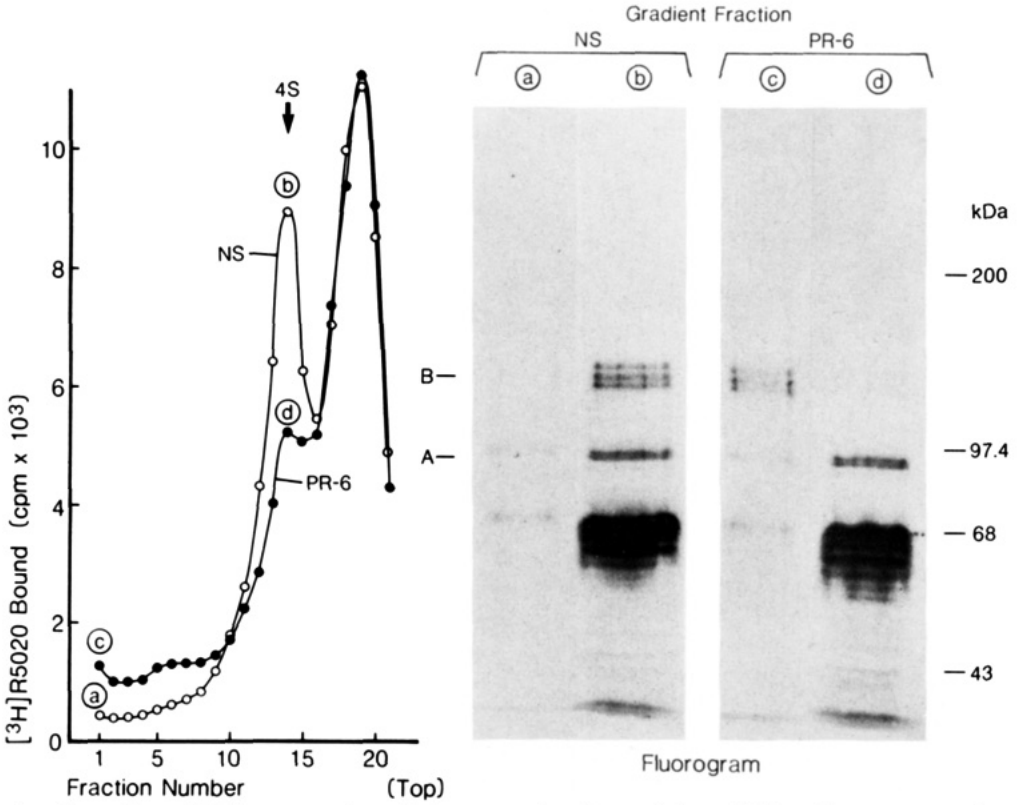

FIGURE 4: Separation of transformed 4S B receptors from 4S A receptors in salt-containing molybdate-free sucrose gradients. T47D cytosol prepared in molybdate-free buffer was incubated with 20 nM [^{3}H]R5020 at 0 °C, photoaffinity labeled in vitro, and then brought to 0.4 M NaCl to transform receptors. Aliquots (250 μL) of labeled cytosols were incubated with antibody PR-6 or a nonspecific antibody (NS), then incubated with a second antibody, layered on 0.4 M NaCl containing sucrose gradients, and fractionated as described in Figure 3. 5.18 mg of protein/250 μL and 576 948 specific cpm/250 μL were loaded on the gradient. Aliquots (250 μL) of gradient fractions were counted to obtain the sedimentation profile (left panel), and fractions of interest (fractions a–d) were subjected to SDS–PAGE, electroblotting, and fluorography (right panel) as described in Figure 3. The heavily labeled proteins below the 68-kDa marker represent 4S sedimenting, nonspecific R5020 binding fetal calf serum proteins previously described as a "50-kDa" binder. The proteins are unrelated to PR (Horwitz & Alexander, 1983).

menting fractions by the antibody (Figure 4, right). A small amount of both A and B was nonspecifically precipitated to the bottom of the gradient by the control MOPC antibody. It appears then that antibody PR-6 cross-reacts both with the native 8S form as well as with the transformed 4S form of B receptors and that, like the case for 8 S, there are two types of 4S species containing either A protein or B protein but not both. The 76-kDa non-hormone binding protein copurifies with transformed 4S B receptors [see Estes et al. (1987)].

Since A and B are not linked in transformed PR, the two-stage nuclear binding model, in which B is seen as a chromatin-specifying protein that guides A to appropriate DNA binding sites (Schrader et al., 1981), is unlikely to be correct. The alternative model is that A and B proteins form independent functional receptor complexes and that like other steroid receptors each binds to DNA. While there appears to be consensus that A receptors bind DNA, for B receptors this point has been unsettled (Schrader et al., 1981; Gronemeyer et al., 1985). We have tested the ability of immunopurified transformed human B receptors to bind DNA–cellulose and find that they do so efficiently (Estes et al., 1987).

We have previously shown that human PR in T47D cells acquire tight chromatin binding capacity within 5 min of R5020 treatment and that longer nuclear occupancy is characterized by an apparent increase in molecular weight of the receptors, suggesting that a covalent modification had occurred. This step precedes nuclear receptor down-regulation (Horwitz et al., 1985a). Since demonstration of these receptor modifications required photoaffinity labeling with [^{3}H]R5020 and electrophoresis under denaturing conditions, we have not been able to study these reactions using the natural ligand progesterone, whose binding to PR cannot be subjected to these manipulations. This is now possible by use of PR-6 and immunoblot assays, since the antibody cross-reacts with the various forms of nuclear receptors. As shown in Figure 5, PR-6 binds not only to soluble cytosol receptors but also to receptors transformed by hormone treatment and extracted from nuclei. After homogenization of untreated T47D cells, B receptors are found mainly in cytosols. This is reversed by 5 min of R5020 treatment when most B receptors are bound tightly to chromatin and are not solubilized during homogenization but must be extracted with salt-containing buffers.

We therefore used PR-6 in progesterone- and R5020-treated cells to study the covalent receptor modification and nuclear "processing"—a down-regulation step that leads to loss of nuclear receptors as determined by ligand binding assays. It has been argued that processing represents loss of hormone binding capacity or an inability to exchange unlabeled hormone for label and that it is not a real loss of protein mass. The study in Figure 6 shows that this is not the case. Is shows further that the kinetics of B-receptor replenishment differ markedly in R5020- and progesterone-treated cells. In figure 6, we have measured B receptors either in salt-extracted nuclear fractions or in cytosols, following treatment of T47D cells with unlabeled R5020 or progesterone, for times ranging from 5 min to 48 h. Proteins from the two cellular compartments were then separated by SDS–PAGE and blotted to nitrocellulose, and the B receptors were identified with PR-6.

In cells treated with R5020, the immunoblots parallel the earlier photolabeling data (Horwitz et al., 1985a): After 5 min of hormone treatment, B receptors are entirely nuclear, and the immunoreactive B protein is a doublet (Figure 6, top

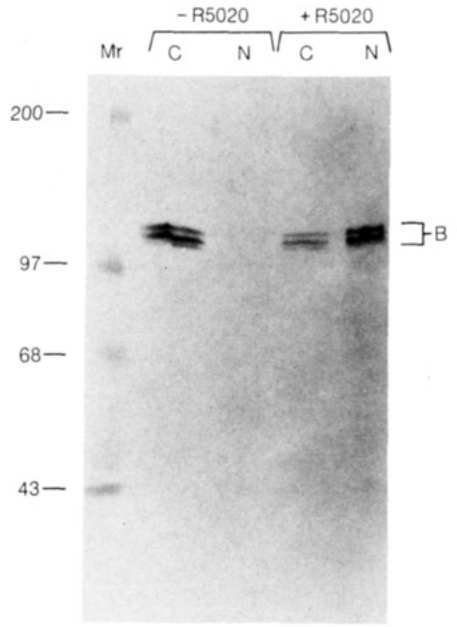

FIGURE 5: Cross-reactivity of PR-6 with nuclear B receptors. Two replicate-plated 100 cm^2 petri dishes of T47D cells were incubated 5 min with 80 nM unlabeled R5020 (+R5020); two plates were left untreated (-R5020). Cells were harvested and homogenized, nuclei were pelleted, cytosol was prepared from the supernatant, and proteins were extracted from the nuclear pellet with 0.4 M KCl. The proteins in the cytosols or nuclear extracts were concentrated by ammonium sulfate precipitation, redissolved in SDS sample buffer, and subjected to SDS–PAGE. Proteins were then electroblotted onto nitrocellulose, and the sheet was probed with antibody PR-6.

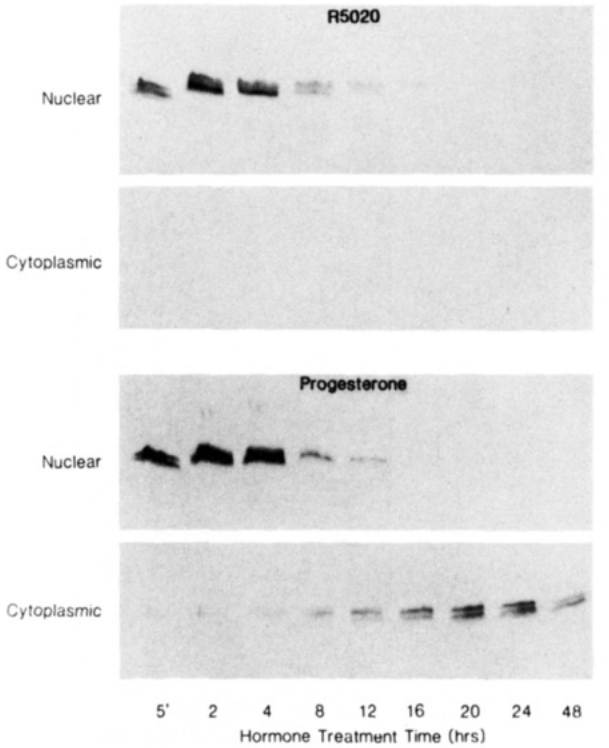

FIGURE 6: Nuclear processing and cytoplasmic replenishment of progesterone B receptors demonstrated by immunoblotting. T47D cells were replicate plated in 100 cm^2 petri dishes (two dishes per treatment set). Confluent cells were treated for 5 min to 48 h with 80 nM unlabeled R5020 (top two panels) or 80 nM unlabeled progesterone (bottom two panels). At the end of treatment, the cells were harvested and homogenized, and a cytosol and nuclear salt extract was obtained from each treatment set. Total cytosol or nuclear proteins were concentrated by ammonium sulfate precipitation, solubilized in SDS sample buffer, and subjected to electrophoresis in four separate gels. The proteins were blotted onto nitrocellulose sheets and probed with antibody PR-6. For illustrative purposes, only the region around the 120-kDa B protein is shown—the remaining parts of each lane were totally devoid of signal and were cropped.

panel). At 2 h, the B doublet changes to a heavier singlet, and then processing leads to loss of detectable protein from salt-extractable compartments. No receptors were shifted to salt-resistant compartments, as judged by extraction into SDS and immunoblotting (not shown). Thus, nuclear processing after R5020 treatment appears to represent a true decrease of receptor protein and not simply loss of hormone binding or exchange capacity. Although we have shown previously by photoaffinity labeling (Horwitz et al., 1985a), and here by immunoblotting, that nuclear PR seem to be completely gone between 8 and 24 h of R5020 treatment, we know from conventional ligand binding assays that low levels of nuclear receptors do persist during this time (Mockus et al., 1983). The discrepancy lies in the sensitivity of the assays. The low levels of persistent receptors measured by conventional ligand binding are below the levels of detection by immunoblot and affinity labeling. They can be detected following immunoconcentration (not shown). There is no apparent cytoplasmic replenishment of B receptors for at least 2 days after R5020 treatment since no immunoreactive protein was found in cytosols (Figure 6, second panel) or in microsomal pellets (not shown).

Nuclear B receptors bound to progesterone are also doublets initially and then convert to a singlet of increased mass before processing occurs (Figure 6, third panel). Thus, the covalent modification responsible for the upshift is a general effect of progestin action and is not unique to R5020. Unlike the data with R5020, detectable amounts (10–20% of control) of receptors remain in the cytosol after progesterone treatment. Also, in contrast to R5020, B receptors replenish in the soluble cytosol between 8 and 24 h after the start of progesterone treatment. We observe that it is the heavier singlet band that replenishes at early time points and that the lower molecular weight band re-forms later. Therefore, after both R5020 and progesterone treatment, tight chromatin-bound B receptors become processed in nuclei as a result of protein loss; they are quickly restored to the soluble compartment in the untransformed state after progesterone treatment, but not after R5020 treatment.

We next addressed the nature of the mobility shifts seen in nuclei in response to hormone addition. Mobility shifts on SDS–PAGE can occur when the phosphorylation state of proteins is altered (Wegener & Jones, 1984), since conformational changes due to phosphate are preserved in SDS. Moreover, such shifts have been observed associated with phosphorylation of vitamin D_3 receptors (Pike & Sleator, 1985) and rabbit uterine PR (Logeat et al., 1985). We therefore analyzed immunoprecipitated B receptors from control and hormone-treated cells that had been metabolically labeled with [^{32}P]orthophosphate to see whether phosphorylation could account for the B doublets and/or the molecular weight upshift. In general, untreated or hormone-pretreated T47D cells were incubated with [^{32}P]orthophosphate for 4 h. The labeled receptors were then immune precipitated using PR-6 or a nonspecific control antibody followed by protein A–Sepharose bound with secondary anti-mouse IgG as the absorbent. The precipitated proteins were solubilized from the resin, separated on SDS–PAGE, transferred to nitrocellulose, and then analyzed by immunoblotting and autoradiography of the same filter.

The left-hand panel of Figure 7 is a study using hormone-untreated cells. By immunoblotting, we show that a triplet protein is precipitated and detected by PR-6 which is not precipitated by the control antibody. These are the B

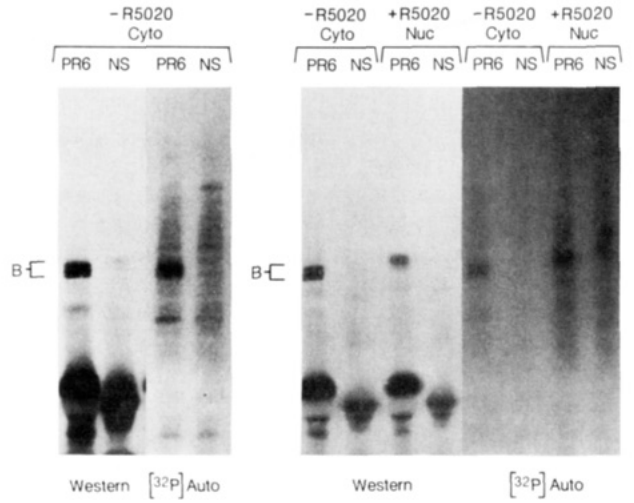

FIGURE 7: Phosphorylation of human B receptors in the untransformed and the nuclear-bound state. (Left panel) Untreated T47D cells (−R5020) were preincubated with phosphate-free medium and then incubated 4 h at 37 °C with 0.03 mCi/mL [^{32}P]orthophosphate. Cells were harvested, and a cytosol was prepared. (Right panel) Replicate-plated T47D cells were preincubated with phosphate-free medium and then incubated 10 min at 37 °C with (+R5020) or without (−R5020) 80 nM unlabeled R5020, before all the flasks were incubated 4 h at 37 °C with 0.03 mCi/mL [^{32}P]orthophosphate. Cells were then harvested and homogenized, nuclei were pelleted, the supernatant was used to obtain cytosol, and the nuclear pellet was mixed with 0.4 M KCl to obtain a nuclear extract. (Both panels) ^{32}P-Labeled cytosols or nuclear extracts were immunoprecipitated by using PR-6 or a nonspecific control antibody (NS) plus an anti-mouse IgG bound to protein A–Sepharose as described under Materials and Methods. The resin was washed extensively; the remaining proteins were then eluted with SDS sample buffer and subjected to SDS–PAGE. Proteins in the gels were blotted to nitrocellulose, and the sheets were first probed with antibody PR-6 (Western) and then used to expose an X-ray film to obtain the ^{32}P-labeled autoradiogram.

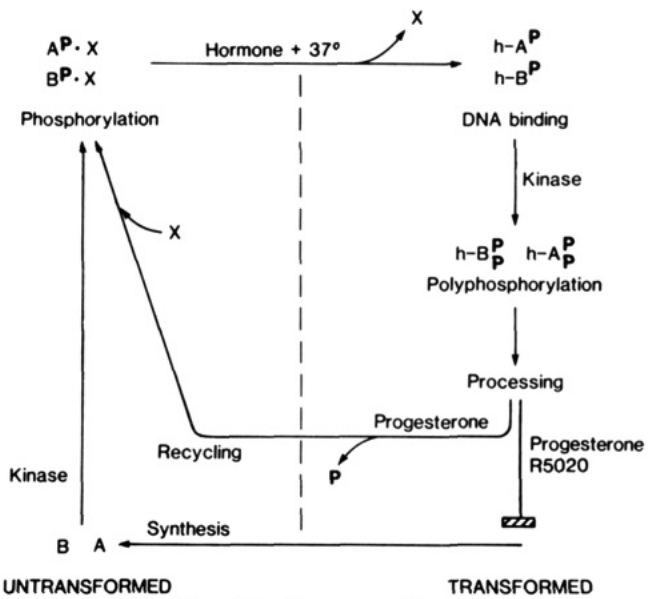

FIGURE 8: Model describing the structural organization of human PR and the place of phosphorylation and nuclear processing. Details are described under Discussion. Briefly, hormone binding receptor proteins are synthesized as the B receptor (120 kDa) or the A receptor (94 kDa). They undergo phosphorylation co- or posttranslationally and complex with one or more non-hormone binding proteins (X) to form 8S untransformed receptors (upper left). A and B proteins are contained in separate 8S species. Upon hormone treatment and warming, the receptors transform to the 4S, tight chromatin/DNA binding state (upper right), losing one or more non-hormone binding proteins in the process (−X). Once in the nucleus, both A and B receptors are subject to another round of phosphorylation to become "polyphosphorylated". This step may precede receptor processing—the down-regulation of nuclear receptors. To reset the cellular receptor machinery, untransformed receptors are restored either by resynthesis or by recycling. The latter requires dephosphorylation (−P) plus the reacquisition of non-hormone binding proteins (+X).

receptors—characteristically heterogeneous and often appearing as triplets after purification. The parallel autoradiogram shows that B receptors are clearly phosphorylated in the native state and despite the absence of hormone. Since all the bands are labeled with ^{32}P, their etiology remains a mystery. They could be due to unequal phosphorylation of a single B protein, or they could represent primary amino acid sequence heterogeneity of two or more closely related proteins that are uniformly phosphorylated. We cannot distinguish between these. The heterogeneous structure of B receptors is not unique to T47D cells. It is also seen in MCF-7 human breast cancer cells and normal rabbit uterine PR (our unpublished observations).

The right-hand panel of Figure 7 contrasts receptors from control and hormone-treated T47D. Cells from replicate platings were either untreated or treated 10 min with 80 nM unlabeled R5020. Both sets were then incubated with [^{32}P]orthophosphate for 4 h. After sets were harvested and homogenized, receptors were immunoprecipitated either from cytosols of the untreated cells (there are no detectable receptors in nuclei) or from nuclear extracts of the hormone-treated cells (there are few receptors in cytosols). In each case, proteins precipitated with PR-6 were compared to a nonspecific antibody. Eluates from the resins were analyzed by SDS–PAGE, immunoblotting, and autoradiography. The Western blot shows the specific precipitation of B receptors from control cytosols and the precipitation of a similar but heavier protein from nuclear extracts of hormone-treated cells. The autoradiogram shows again that untransformed receptors are phosphoproteins. It shows further that when these phosphorylated proteins are transformed and acquire tight nuclear binding capacity as a result of hormone treatment, at least one additional phosphorylation site becomes available for labeling with [^{32}P]orthophosphate. The mobility change could be explained by this phosphorylation. Thus, the B proteins of PR are the subject of two separate phosphorylation reactions: once while they are in the native, untransformed state; then again in nuclei after they have been transformed and are tightly bound to chromatin. More recent studies (P. L. Sheridan and K. B. Horwitz, unpublished results) show that A receptors are also phosphorylated in nuclei after transformation.

Discussion

The data in this and in the preceding paper (Estes et al., 1987), and those of other investigators, support the model of receptor structure and function shown in Figure 8. The features of the model include the following points:

(1) In the hormone-free cell, PR are in the 8S oligomeric state and are soluble in cytosols following cell lysis. Under these conditions, unoccupied receptors are phosphorylated (Figure 7, left panel). This suggests that during or after B-protein synthesis, covalent modification occurs by the addition of one or more phosphate groups. The function of this phosphorylation step is unknown, though for glucocorticoid and estrogen receptors (Nielsen et al., 1977; Migliaccio et al., 1982) phosphorylation at a similar point is thought to convert inactive receptors (unable to bind hormone) to the activated state (ready to bind hormone). If this reaction occurs sometime after translation rather than cotranslationally, it would account for the "lag" in appearance of hormone binding PR at steady state and during replenishment seen with dense amino acid labeling (Mullick & Katzenellenbogen, 1986).

(2) There is suggestive evidence from in vitro data that 8S untransformed receptors are composed of hormone and non-hormone binding proteins. The stoichiometry of these components has not been determined. We propose that 8S receptors are oligomers of one of the hormone binding proteins and additional non-hormone binding proteins. A possible structure for this complex may include dimers of the 90-kDa heat shock protein which is known to copurify with 8S untransformed PR (Dougherty et al., 1984; Joab et al., 1984; Catelli et al., 1985; Shuh et al., 1985; Estes et al., 1987) and one 76-kDa subunit which also copurifies with B receptors (Wrange et al., 1983; Puri & Toft, 1984; Estes et al., 1987). The combined molecular mass of such a tetrameric complex (≈375 000 daltons) falls into the range proposed for the mass of untransformed 8S steriod receptors (Sherman & Stevens, 1984) but is somewhat heavier than the mass of chick oviduct PR calculated by gel filtration (Puri & Toft, 1984). Hormone binding and non-hormone binding proteins alike appear to be phosphorylated (Puri & Toft, 1984).

(3) Further, it is clear from Figure 3 that A and B receptors are not associated with one another but form separate 8S complexes. This can also be predicted from the molecular weight estimates and from the number of non-hormone binding subunits, which, if arranged as described above, would preclude involvement of more than one hormone binding subunit in the 8S complex. The reason for the structural heterogeneity, unique to PR among the steroid receptors, is not known. Loosfelt et al. (1984) argue that the A protein is a proteolytic fragment formed by cell-free incubation conditions and that this breakdown can be blocked by protease inhibitors. Our data do not support this theory (Horwitz et al., 1985a,b). First, in our studies, a cocktail of protease inhibitors is routinely added to all buffers used for homogenization and for subsequent steps. Nevertheless, A proteins in unaltered concentrations are present in all PR preparations. Second, we have previously shown that A receptors are equimolar to B, are present in detergent-solubilized cells, cannot be generated during in vitro incubations, are translocated and tightly bound to nuclear chromatin after hormone treatment, and are covalently modified in nuclei in a pattern dissimilar to B (Horwitz et al., 1985b). Gronemeyer et al. (1985) suggest that underrepresentation of A in purified preparations stems from their instability compared to B on conventional chromatographic columns. On the basis of these observations, we hypothesize that A are integral intracellular proteins. How A and B are formed is unknown, however. Possible mechanisms include gene duplication, alternate processing of a single precursor for mRNA, or alternate translation start sites.

(4) Transformation of receptors to the tight chromatin binding state occurs in vivo after hormone treatment and warming or, in vitro, by a variety of treatments, as, for example, with molybdate-free buffers of high ionic strength. These receptors sediment at 4 S and may no longer be associated with the 90-kDa proteins (Estes et al., 1987) though this is not firmly established (Puri & Toft, 1984); the 76-kDa protein copurifies with transformed B receptors and may remain complexed to them (Estes et al., 1987). It is not known, however, whether this protein is found in nuclei. As shown by the study in Figure 4, there appear to be at least two classes of transformed PR—those that contain A and those that contain B.

(5) Following transformation by hormone treatment, B receptors as well as A receptors acquire tight nuclear binding capacity from which they can be recovered in solution only with buffers of high ionic strength (Horwitz et al., 1985a,b). However, the site in nuclei to which B and A bind remains unclear. The original proposal, that B is a chromatin binding protein without DNA binding affinity (Schrader et al., 1981), is no longer tenable. Binding of purified chick oviduct B receptors to DNA has now been reported (von der Ahe, 1986; Renoir et al., 1985), and our studies show that human B receptors also bind DNA well [see Estes et al. (1987)]. Further, since DNA–cellulose has been used to purify B and A receptors (Gronemeyer et al., 1985), it appears that both proteins must be classed as DNA binding proteins. Whether both receptors also bind chromatin proteins requires additional study (Spelsberg et al., 1983).

(6) We propose that after receptors acquire tight nuclear binding capacity, they undergo another round of phosphorylation (Figure 7, right panel). That PR are phosphorylated in two stages is suggested by the ability of the phosphorylated untransformed receptors to become transformed and then to be labeled again by [^{32}P]orthophosphate in nuclei (Figure 7, right panel). We hypothesize that the molecular weight upshift that occurs for both A and B receptors following 30–60 min of nuclear occupancy (Horwitz et al., 1985a) is due to this phosphorylation. Could this covalent modification after receptors are chromatin bound be the signal that initiates receptor processing? Do these receptors have lower affinity for DNA? Two phosphorylations, once to activate receptors to the hormone binding state and once on chromatin, may involve the actions of two kinases, one of which could be the receptor itself.

(7) Nuclear receptor processing, analogous to receptor down-regulation seen with cell surface receptors (Wileman et al., 1985), appears to be due to a real loss of protein (Figure 6). We postulate that this terminates the nuclear action of the receptors. The loss is equivalent in rate and extent for receptors occupied by progesterone or R5020 and resembles the loss we previously described for estrogen-occupied receptors (Horwitz & McGuire, 1980).

(8) Receptor processing and restoration of unoccupied receptors to pretreatment levels not only must be closely associated events but also must somehow be related to the treatment regimen. A hormone pulse characterized by brief hormone treatment would be expected to be followed by restoration of unoccupied receptor levels to steady-state pretreatment values. Progesterone treatment in cell culture is a prototype for this. The hormone is so rapidly metabolized (Horwitz et al., 1986) that its practical effectiveness is short-lived whether or not it is actually removed from cells.

In contrast, we postulate that prolonged hormone treatment, associated with chronic biologic stimulation, is characterized by a continuous turnover of receptors—as soon as new receptors are synthesized, they are occupied by hormone, transformed, and processed—with the overall effect that a new steady-state of receptor levels is established that is lower than pretreatment control levels and confined to the tightly bound chromatin compartment. During chronic treatment, no unoccupied (cytoplasmic) receptors exist in the cells, and only functional DNA binding forms (preprocessed receptors) are found in nuclei. We have observed this low steady-state nuclear level for estrogen (Horwitz & McGuire, 1980) and progesterone receptors (Mockus & Horwitz, 1983) using conventional ligand binding assays. These nuclear levels are below the limits of sensitivity for photoaffinity labeling or immunoblotting with PR-6 which accounts for the inability to detect nuclear PR in the 8–48-h time points shown in Figure 7 following R5020 treatment. They can be detected following immunoconcentration. R5020, unlike progesterone, is not

metabolized by T47D cells (Horwitz et al., 1986), hence its chronic stimulatory effects. We would predict that presistent progesterone treatment (requiring addition of fresh hormone to cells every 4 h) would result in an apparent lack of replenishment like that seen after R5020 treatment. This is only an "apparent lack", since replenishment is probably a continuous process, but the receptors are immediately captured if hormone is present and transformed to the chromatin-bound state.

(9) As to the mechanisms for replenishing unoccupied receptors: clearly recycling and/or resynthesis must be involved, and there is evidence for both in other steroid receptor systems (Horwitz & McGuire, 1980; Raaka & Samuels, 1983). Our data do not address this question directly. Replenishment following progesterone withdrawal (Figure 6, lower panel) is characterized by the appearance in cytoplasm first of the heavier band of the B doublet followed by formation of the lighter band. Is this due to dephosphorylation of the heavy nuclear protein accompanying recycling? On the other hand, the absolutely low levels of receptors obtained during R5020 treatment (Figure 6, top two panels) can probably only be restored by synthesis of new molecules. It would therefore not be surprising if both mechanisms—recycling and resynthesis—were involved in replenishment, depending on factors such as hormone type and treatment time. Our model predicts that recycling, if it occurs, must be more than simple reshuffling of receptor proteins from one cellular compartment to another. If all the steps that lead from the untransformed, unoccupied, but activated receptor state to the processed nuclear receptor state are to be reversed, then nuclear receptors must be dephosphorylated, must lose the hormone, and must reassociate with one or more non-hormone binding protein molecules, clearly a complex process. The availability of antibodies reactive with both A and B receptors (Estes et al., 1987) as well as antibodies to the associated non-hormone binding proteins (Riehl et al., 1985; Welch et al., 1983) should permit us to test various aspects of this model further.

Registry No. Progesterone, 57-83-0.

REFERENCES

Auricchio, F., Migliaccio, A., & Castoria, G. (1981) *Biochem. J. 198*, 699–702.

Bowen, B., Steinberg, J., Laemmli, U. K., & Weintraub, H. (1980) *Nucleic Acids Res. 8*, 1–20.

Catelli, M. G., Binart, N., Testas, I. J., Renoir, J. M., Baulieu, E.-E., Feramisco, J. R., & Welch, W. J. (1985) *EMBO J. 4*, 3131–3135.

Clark, G. M., McGuire, W. L., Hubay, C. A., Pearson, O. H., & Marshall, J. R. (1983) *N. Engl. J. Med. 309*, 1343–1347.

Dougherty, J. J., & Toft, D. O. (1982) *J. Biol. Chem. 257*, 3113–3119.

Dougherty, J. J., Puri, R. K., & Toft, D. O. (1984) *J. Biol. Chem. 259*, 8004–8009.

Estes, P. A., Suba, E. J., Lawler-Heavner, J., Elashry-Stowers, D., Wei, L. L., Toft, D. O., Sullivan, W. P., Horwitz, K. B., & Edwards, D. P. (1987) *Biochemistry* (preceding paper in this issue).

Feil, P. D. (1983) *Endocrinology* (*Baltimore*) *112*, 396–398.

Gronemeyer, H., Govindan, M. V., & Chambon, P. (1985) *J. Biol. Chem. 260*, 6916–6925.

Horwitz, K. B., & McGuire, W. L. (1980) *J. Biol. Chem. 255*, 9699–9705.

Horwitz, K. B., & Alexander, P. S. (1983) *Endocrinology* (*Baltimore*) *113*, 2195–2201.

Horwitz, K. B., McGuire, W. L., Pearson, O. H., & Segaloff, A. (1975) *Science* (*Washington, D.C.*) *189*, 726–727.

Horwitz, K. B., Mockus, M. B., & Lessey, B. A. (1982) *Cell* (*Cambridge, Mass.*) *28*, 633–642.

Horwitz, K. B., Francis, M. D., & Wei, L. L. (1985a) *DNA 4*, 451–460.

Horwitz, K. B., Wei, L. L., Sedlacek, S. M., & d'Arville, C. N. (1985b) *Recent Prog. Horm. Res. 41*, 249–316.

Horwitz, K. B., Pike, A. W., Gonzalez-Aller, C., & Fennessey, P. V. (1986) *J. Steroid Biochem. 25*, 911–916.

Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M. G., Binart, M., Mester, J., & Baulieu, E.-E. (1984) *Nature* (*London*) *308*, 850–853.

Kurl, N. R., & Jacob, S. T. (1984) *Biochem. Biophys. Res. Commun. 119*, 700–705.

Laemmli, U. K. (1971) *Nature* (London) *227*, 680–685.

Logeat, F., Hai, M. T. V., & Milgrom, E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1426–1430.

Logeat, F., Hai, M. T. V., Fournier, A., Legrain, P., Buttin, P., & Milgrom, E. (1983) *Proc. Natl. Sci. U.S.A. 80*, 6456–6459.

Logeat, F., LeCunff, M., Pamphile, R., & Milgrom, E. (1985) *Biochem. Biophys. Res. Commun. 131*, 421–427.

Loosfelt, H., Logeat, F., Hai, M. T. V., & Milgrom, E. (1984) *J. Biol. Chem. 259*, 14196–14202.

Migliaccio, A., Lastoria, S., Moncharmont, B., Rotondi, A., & Auricchio, F. (1982) *Biochem. Biophys. Res. Commun. 109*, 1002–1010.

Mockus, M. B., & Horwitz, K. B. (1983) *J. Biol. Chem. 258*, 4778–4783.

Mullick, A., & Katzenellenbogen, B. S. (1986) *J. Biol. Chem. 261*, 13236–13243.

Nielsen, C. J., Sando, J. J., & Pratt, W. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1398–1402.

Nishigori, H., & Toft, D. O. (1980) *Biochemistry 19*, 72–83.

Oakley, B. R., Kirsch, D. R., & Morris, N. R. (1980) *Anal. Biochem. 105*, 361–364.

Oblas, B., Boyd, N. D., & Singer, R. H. (1983) *Anal. Biochem. 130*, 1–8.

Pike, J. W., & Sleator, N. M. (1985) *Biochem. Biophys. Res. Commun. 131*, 378–385.

Puri, R. K., & Toft, D. O. (1984) *Endocrinology* (*Baltimore*) *115*, 2453–2463.

Raaka, B. M., & Samuels, H. H. (1983) *J. Biol. Chem. 258*, 417–425.

Renoir, J. M., & Mester, J. (1984) *Mol. Cell. Endocrinol. 37*, 1–13.

Renoir, J. M., & Baulieu, E.-E. (1985) *C. R. Acad. Sci., Ser. 3 301*, 859–864.

Renoir, J.-M., Radanyi, C., Yang, C.-R., & Baulieu, E.-E. (1982) *Eur. J. Biochem. 127*, 81–86.

Riehl, R. M., Sullivan, W. P., Vroman, B. T., Bauer, V. J., Pearson, G. R., & Toft, D. O. (1985) *Biochemistry 24*, 6586–6591.

Schrader, W. T., Birnbaumer, M. E., Hughes, M. R., Weigel, N. L., Grody, W. W., & O'Malley, B. W. (1981) *Recent Prog. Horm. Res. 37*, 583–633.

Sherman, M., & Stevens, J. (1984) *Annu. Rev. Physiol. 46*, 83–105.

Shuh, S., Yonemoto, W., Brugge, J., Bauer, V. J., Riehl, R. M., Sullivan, W. P., & Toft, D. O. (1985) *J. Biol. Chem. 260*, 14292–14296.

Spelsberg, T. C., Littlefield, B. A., Seeke, R., Dani, E. M., Toyoda, H., Boyd-Leinen, P., Thrall, C., & Kon, O. L. (1983) *Recent Prog. Horm. Res. 39*, 463–517.

Sullivan, W. P., Beito, T. J., Proper, J., Krco, C. J., & Toft,

D. O. (1986) *Endocrinology (Baltimore) 119*, 1549–1557.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Tuohimaa, P., Renoir, J.-M., Radanyi, C., Mester, J., Joab, I., Buchou, T., & Baulieu, E.-E. (1984) *Biochem. Biophys. Res. Commun. 119*, 433–439.
von der Ahe, D., Janick, S., Scheidereit, C., Renkawitz, R., Schütz, G., & Beato, M. (1985) *Nature (London) 313*, 706–709.
von der Ahe, D., Renoir, J. M., Buchou, T., Baulieu, E.-E., & Beato, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2817–2821.
Wegener, A. D., & Jones, L. R. (1984) *J. Biol. Chem. 259*, 1834–1841.
Welch, W. J., Garrels, J., Thomas, G. O., Lin, J. J.-C., & Feramisco, J. R. (1983) *J. Biol. Chem. 258*, 7102–7111.
Wileman, T., Harding, C., & Stahl, P. (1985) *Biochem. J. 232*, 1–14.
Wrange, O., Okret, S., Radojcic, M., Carlstedt-Duke, J., & Gustafsson, J.-A. (1984) *J. Biol. Chem. 259*, 4534–4541.

Distributions of Fluorescence Decay Times for Parinaric Acids in Phospholipid Membranes†

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ABSTRACT: Analysis of fluorescence decay data for probes incorporated into model or biological membranes invariably requires fitting to more than one decay time even though the same probe exhibits nearly single-exponential decay in solution. The parinaric acids (cis and trans) are examples of this. Data are presented for both parinaric acid isomers in dimyristoylphosphatidylcholine membranes collected to higher precision than normally encountered, and the fluorescence decays are shown to be best described by a smooth *distribution* of decay times rather than by a few discrete lifetimes. The temperature dependence of the fluorescence decay reveals a clear shift in the distribution to longer lifetimes associated with the membrane phase transition at 23.5 °C. The physical significance is that fluorescence lifetime measurements appear to reflect a physical process with a distribution of lifetimes rather than several distinct physical processes.

Fluorescence spectroscopy provides a convenient, sensitive, and selective tool for studying dynamic processes in biological membranes (Yguerabide & Foster, 1981; Devaux & Seigneuret, 1985). Fluorescence lifetime measurements are of intrinsic interest but are also essential to adequately interpret temperature- and concentration-dependent quantum yield changes and form the bases for correct analysis of both steady-state and time-resolved fluorescence anisotropy data. As the application of fluorescence techniques to membrane research has developed, the complexity of the data analysis and interpretation in terms of physical processes has steadily increased. The development of diphenylhexatriene as a probe of membrane disorder is a prime example (Chen et al., 1977; Kawato et al., 1977; Lakowicz et al., 1985).

Fluorescence decay data obtained from probes in membranes invariably must be analyzed by fits to two or more exponential decays, frequently with decay times differing by less than a factor of 2 or 3 (Blatt & Sawyer, 1985; Devaux & Seigneuret, 1985; Petersen et al., 1987; Vincent & Gallay, 1984; Wolber & Hudson, 1981). This is a perplexing and poorly understood feature of these systems, particularly since most of the probes exhibit nearly single-exponential decays in homogeneous solution (Wolber & Hudson, 1981; Vincent & Gallay, 1984; Parasassi et al., 1984). In many cases the set of lifetimes (two or three) has been attributed to compartmentalization of the probes in different membrane environments, and the proportion of each component has been related to the probe concentration in each environment (Devaux & Seigneuret, 1985; Klausner et al., 1980; Karnovsky et al., 1982). In a few cases it has been suggested that the set of lifetimes may arise from a distribution of lifetimes, but no evidence was presented (Petersen et al., 1987; Wolber & Hudson, 1981).

Recently James and Ware (1985, 1986) have demonstrated through extensive computer simulations that with fluorescence decay data at normal levels of precision (20 000–50 000 counts in the peak channel) it is impossible to distinguish between two or three discrete lifetimes and a continuous distribution of lifetimes by any criteria of goodness of fits. However, as the precision of the data increases (to 200 000 counts in the peak channel or more), it becomes possible to recover the true distribution of lifetimes, whether it is unimodal or bimodal or contains discrete lifetimes (James & Ware, 1986).

In this paper we apply the methods of James and Ware (1986) to the analysis of fluorescence decay curves collected to high precision for both *cis*- and *trans*-parinaric acid in dimyristoylphosphatidylcholine (DMPC)[1] membranes and show that in both cases smooth distributions of decay times are recovered. Analysis of measurements performed as a function of increasing temperature through the main phase

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[1] Abbreviations: t-PnA, *trans*-parinaric acid; c-PnA, *cis*-parinaric acid; DMPC, dimyristoylphosphatidylcholine; ESM, exponential series method; n-AS, *n*-(9-anthroyloxy)stearic acid; POPC, palmitoyloleoylphosphatidylcholine; CPC, counts in peak channel.

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