14C-Labeled Proteins as Markers for Gradient Analysis of Steroid-Hormone Receptors

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Two useful [14C]marker proteins—[14C]human serum albumin (4.6S) and [14C]glucose oxidase (7.9S)—can be simply prepared. Both may be used as molecular-mass standards in polyacrylamide gel electrophoresis and sucrose density-gradient centrifugation. The utility of these markers for estrogen receptor studies was investigated under a variety of conditions, to ensure that they do not interfere with current assay procedures. Their use as internal markers allows more samples to be analyzed per rotor, a significant factor because each centrifugation run requires 16 h and two bucket spaces for each sample assayed; improves accuracy and overall quality control by eliminating any problems resulting from variations among individual gradients; and facilitates evaluation of changes in gradient profiles, which may provide clinically and biochemically relevant information concerning the microheterogeneity of estrogen receptors.

Additional Keyphrases: ultracentrifugation in density gradient • breast-cancer detection • estrogen receptors • albumin • glucose oxidase • internal standards

The steroid-receptor content of mammary tumor cytosols is an indicator of the endocrine responsiveness of breast cancers (1-3). Patients with estrogen-receptor-negative tumors rarely respond to hormonal manipulations, whereas 50 to 60% of patients with measurable amounts of estrogen receptor are responsive to endocrine therapies of the additive or ablative types. Clearly, the estrogen-receptor assay, when used in combination with established clinical guidelines, provides a better means of determining the therapeutic approach for the patient with breast cancer.

Several procedures have been developed for determining the presence of cytosolic estrogen receptors (e.g., multipoint titration with dextran-coated charcoal (4-6), the hydroxylapatite procedure (7-11), the protamine sulfate precipitation method (12, 13), the DEAE filter method (14), and sucrose density-gradient techniques (1, 15-17)). Of these procedures the titration assay with use of dextran-coated charcoal and the sucrose density-gradient technique are the most commonly used clinically. Many laboratories have shown that these assays are quantitatively equivalent; however, the density-gradient centrifugation technique has the unique advantage that estrogen receptor can be separated into its various molecular forms (1, 15, 18, 19). Sucrose density-gradient analysis has revealed that the cytosolic estrogen receptors exist in at least two distinct molecular forms: one sediments in the 3-5S region, the other of higher molecular mass, sediments in the 7-9S region. Recent studies indicate that there may be microheterogeneity—i.e., multiple forms of estrogen receptors within each molecular-mass class (20). Furthermore, it has been suggested that the distribution of these molecular species is related to the responsiveness of breast-cancer patients given hormonal therapy (18, 20).

Usually the S-value of estrogen receptors is estimated by comparison with marker proteins for which the molecular mass and sedimentation coefficient are known and which are separated on gradients run in parallel with the sample. Sedimentation coefficients determined under these conditions may be inaccurate, owing to variations in sucrose density gradients between different laboratories or even within a given laboratory. Because forces exceeding 300 000 × g for 16 h must be used to separate various species, the need for a separate gradient for standards substantially limits the number of samples that can be analyzed during any one centrifugation. In addition, recent clinical data suggest that it is the relative amount of each receptor form that may have prognostic value (18, 20). Therefore unequivocal identification of these steroid-binding proteins, based on their sedimentation coefficients, appears essential.

To improve clinical steroid-receptor assays in which [3H]estradiol-17β is used, we have prepared [14C]human albumin monomer (HSA, 4.6S) and [14C]glucose oxidase (GlOx, 8S), which can be added in trace amounts to each tumor cytosol.2 Such standards allow for the absolute determination in each gradient of any estrogen-receptor species that may be present. We describe here the preparation of such [14C]marker proteins and their utility as internal standards. Additionally, these internal [14C]marker proteins do not interfere with estrogen-receptor assays done by sucrose density-gradient procedures.

Materials and Methods

Apparatus

Breast-tumor cytosols were prepared by use of a Type 40 fixed-angle rotor in an LS-65 preparative ultracentrifuge (both from Beckman Instruments, Inc., Palo Alto, CA 94304) and polycarbonate centrifuge tubes. For sucrose density-gradient centrifugation we used a SW60 Ti rotor (Beckman) with 4.5-mL cellulose nitrate tubes. Gradients were fractionated by means of a mechanical fractionation/recovery system (Beckman). The radioactivity of each fraction was determined with an LS-9000/liquid scintillation counter equipped with programs for [3H] and [14C]. Details of these methods are contained in earlier reports (e.g., 20).

All chromatographic procedures were performed on columns obtained from Pharmacia, Piscataway, NJ 08854. Various column fractions were concentrated in an ultrafiltration system (Amicon Corp., Lexington, MA 02173).

Reagents

Unless otherwise noted, all chemicals were reagent grade or better and obtained from either Fisher Scientific, Cincinnati, OH 45242, or Sigma Chemical Co., St. Louis, MO 63178.

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2 Nonstandard abbreviations used: HSA, human serum albumin; GlOx, glucose oxidase; and DES, diethylstilbestrol.
Radioisotopes [2,3,6,7-3H]estradiol-17β (105 kCi/mol), [1-14C]iodoacetamide (15.8 Ci/mol), [14C]formaldehyde (42 Ci/mol), and Omnifluor scintillant were obtained from New England Nuclear Corp., Boston, MA 02118. Affi-Gel Blue (Cibacon Blue F3GA–agarose) was obtained from Bio-Rad Laboratories, Richmond, CA 94804. Purified glucose oxidase (Grade III) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250. Outdated human plasma was donated by the American Red Cross, Louisville, KY 40202.

Procedures

Albumin purification. Commercial preparations of HSA are contaminated to various degrees with α-globulins and albumin polymers. Therefore, we purified HSA from outdated plasma by salt fractionation, affinity chromatography, and ion-exchange chromatography, as follows. Initially, a crude albumin preparation was obtained by ammonium sulfate fractionation of outdated human plasma. The protein that precipitated at between 50 and 75% saturation of ammonium sulfate at pH 4.7 was collected by centrifugation. The precipitate was dissolved and dialyzed exhaustively against glass-distilled water at 4 °C. The dialysate was adjusted to 0.1 mol/L sodium phosphate, pH 7.4, in preparation for affinity chromatography.

A sample of the crude HSA (~500 mg) was applied to an Affi-Gel Blue affinity-chromatography column (2 x 15 cm) that had been previously equilibrated with 0.1 mol/L phosphate buffer, pH 7.4. The unbound serum glycoprotein contaminants were eluted with the same buffer. The bound HSA was eluted from the affinity column with the phosphate buffer containing NaCl, 2 mol/L. The fractions containing the HSA were pooled, then concentrated, with a subsequent buffer change, by ultrafiltration with a PM-10 membrane.

We enriched sulfhydryl-containing HSA monomer and further purified the affinity-purified albumin preparation by ion-exchange chromatography on DEAE-Sephadex A-50, using a sodium phosphate buffer system at pH 7 according to the method of Janatova et al. (21). The free sulfhydryl content of the purified HSA was determined (21) by use of the 5,5'-dithiobis-2-nitrobenzoic acid reaction. This highly purified HSA was concentrated and the buffer system changed to 0.13 mol of KCl and 0.1 mol of K2HPO4 per liter (pH 7.7) by ultrafiltration.

[14C]Albumin preparation. To carboxyamidomethylation the lone free sulfhydryl of HSA (Cys-34), we mixed the purified HSA (250 mg/20.5 mL) with [14C]iodoacetamide (250 μCi, 15.8 Ci/mol, in 2.5 mL of ethanol), adjusted the final volume with the above pH 7.7 buffer system to 25 mL (HSA concentration 10 g/L), and allowed the reaction to proceed for 4 h at 23 °C. Excess [14C]iodoacetamide was removed and the reaction was terminated by ultrafiltration. The [14C]albumin preparation was exhaustively rinsed by ultrafiltration until the ultrafiltrate was negligibly radioactive. We prepared the [14C]HSA sample for gel filtration chromatography at acid pH by changing the buffer system from K2HPO4, pH 7.7, to 0.25 mol/L ammonium formate, pH 3.

The [14C]HSA was applied to a 2.5 x 87 cm G-150 column that had been previously equilibrated with the ammonium formate buffer, and it was eluted at a flow rate of 24 mL/h. The [14C]HSA monomer fraction was first neutralized and the medium then changed by ultrafiltration to 50 mmol/L NaCl at a final albumin concentration of 16 g/L. This [14C]albumin monomer had a specific activity of 4.3 x 10^9 dpm/mg.

[14C]Glucose oxidase preparation. Purified GIOX (Grade III) was labeled with [14C]formaldehyde by using a modification of the method of Winkelhake (22). Briefly, GIOX, 11 g/L in 10.0 mL of 0.2 mol/L sodium borate buffer, pH 9, was incubated at 0 °C for 5 min with 250 μCi of [14C]formaldehyde under nitrogen. The Schiff base was reduced with a 2.1-fold molar excess of sodium borohydride for 5 min at 0 °C under nitrogen. The reaction was terminated by adding Tris buffer (1 mol/L, pH 6) to give a final concentration of 0.3 mol/L, and the [14C]methylated GIOX was dialyzed against 50 mmol/L Tris buffer, pH 8, at 4 °C. The chemically modified GIOX was concentrated by ultrafiltration and analyzed by sucrose density-gradient ultracentrifugation, as described for the estrogen-receptor assays. At least four minor contaminants were found in the [14C]GIOX preparation, indicating that further purification was necessary. The [14C]methylated GIOX sample was subject to G-150 gel filtration chromatography with ammonium formate pH 3 buffer. The symmetrical peak corresponding to [14C]glucose oxidase was collected and concentrated, and for the buffer was substituted, by ultrafiltration, 50 mmol/L NaCl.

Analysis of [14C]labeled proteins. Both the [14C]HSA and [14C]GIOX preparations were stored frozen at -20 °C in 50 mmol/L NaCl. Human serum albumin concentrations were determined by measuring the absorbance at 280 nm, based on the value 0.53 (23) for a 1 g/L solution at 280 nm. Glucose oxidase concentrations were determined according to the method of Waddell (24). The purity of the labeled protein preparations was assessed by polyacrylamide gel electrophoresis and sucrose density-gradient ultracentrifugation. Electrophoresis on polyacrylamide gel was performed in 7.5% gels under both denaturing and non-denaturing conditions according to the methods of Weber and Osborn (25) and Davis (26). The samples in a 300 g/L sucrose solution were layered under the running buffer and directly onto the top of the 5 x 55 mm separation gel in a constant volume (25 μL), which contained 25 to 125 μg of protein.

Estrogen receptor assay. Tissue sample preparation and separation of estrogen receptors, by sucrose density-gradient centrifugation, was according to Wittliff et al. (18). Briefly, cytosols of human breast carcinomas were prepared by homogenization in Tria • HCl buffer (10 mmol/L, pH 7.3) containing, per liter, 1.5 mmol of EDTA, 100 mL of glycerol, and 10 mmol of monothioglycerol, followed by centrifugation in a Beckman Type 40 rotor for 30 min at 105 000 X g. Breast-tumor cytosols were incubated with 5 mmol of [3H]estradiol-17β in the absence (total binding) and presence (non-specific binding) of a 200-fold excess of DES for 5 h at 4 °C (charging). Some samples were subjected to an activation step, which involved an additional 30-min incubation at 28 °C. Before centrifugation, we removed unbound steroid with dextran-coated charcoal. Cytosols, marker proteins, or mixtures of the two were layered onto linear sucrose gradients (100 - 350 g/kg) and centrifuged for 16.5 h at 4 °C in a Beckman SW60 Ti rotor at 60 000 rpm (360 000 x g). Gradients were fractionated into five-drop (~100 μL) fractions, to which 300 μL of distilled water and 4 mL of scintillant was added, and the radioactivity of each fraction was determined by liquid scintillation counting.

Results and Discussion

Figure 1 shows the sedimentation profiles of [14C]HSA (4.6S), [14C]GIOX (7.9S), and a mixture of the two. All samples were layered onto linear sucrose gradients (100 - 350 g/kg) and centrifuged at 4 °C under conditions identical to those described for the estrogen-receptor assay (18). Both 14C-labeled protein preparations sedimented as single peaks, as shown in Panels A (HSA) and B (GIOX). Figure 1C shows that a mixture of these labeled proteins can be readily resolved into two separate peaks having sedimentation properties identical to those of marker proteins separated individually. In general, these data indicate that both protein preparations exhibit two properties that are essential for internal protein standards in gradient centrifugation: homogeneity, as evidenced by a
symmetrical peak, and no interaction by either protein with the sedimentation properties of the other. In addition, the purity of the labeled marker proteins was assessed by polyacrylamide gel electrophoresis; both protein preparations exhibited single bands under denaturing and non-denaturing conditions (data not shown).

Sedimentation coefficients of the various estrogen receptor species currently are determined by the method of Martin and Ames (27), with use of unlabeled protein standards of known S values, which are run on parallel gradients. As noted above, estimating sedimentation coefficients in this way has two major disadvantages: (a) S values may be inaccurate, owing to variations between individual gradients, and (b) the procedure is inefficient because it severely limits sample analysis by requiring separate gradients for standards. To improve the interpretation of steroid receptor assays, we have developed a modified procedure utilizing [14C]HSA and [14C]GOx as internal protein standards. The two techniques were compared in several experiments, with typical results presented in Figure 2. The unlabeled proteins chosen were ribonuclease (1.3S), hemoglobin (4.2S), and catalase (11.3S). Various combinations of labeled and unlabeled proteins were separated on different gradients. Each gradient was fractionated and the positions of the unlabeled protein markers were determined spectrophotometrically by measuring the absorbance at 280 nm. Aliquots of each fraction were also counted to determine the positions of the 14C-labeled proteins.

The sedimentation coefficients of both the labeled and unlabeled marker proteins were plotted as a function of gradient position (Figure 2A). Although the data indicate an apparent linear relationship for S values in the region of 1 to 11, a thorough analysis of labeled and unlabeled marker proteins has demonstrated a nonlinearity in the high-molecular-mass region (11S). To resolve this apparent discrepancy, we ran labeled and unlabeled proteins individually and as mixtures under high- and low-salt conditions. One of the unlabeled proteins, catalase (11.3S), failed to sediment as expected when compared with the four other proteins included in this study (Figure 2B). In addition, catalase cannot be recommended for routine use as a marker protein because, in the presence of ribonuclease and hemoglobin, it tends to form aggregates of high molecular mass. Figure 2B shows the data obtained for a mixture containing both unlabeled and labeled protein standards separated on a single gradient. The results suggest that under routine assay conditions (low ionic strength), a linear relationship between S value and gradient position exists for those marker proteins, labeled or unlabeled, having sedimentation coefficients between 1 and 8. Comparable results were obtained when an identical mixture of marker proteins were separated at high ionic strength (0.15 mol/L KCl, data not shown). These results verify the utility of the internal marker procedure. In fact, use of 14C-labeled internal proteins standards will not only increase the efficiency of the estrogen receptor assay by increasing the number of samples per centrifugation but will also increase the accuracy of the sedimentation coefficients determined, because the technique compensates for variations between individual gradients.

Because the radiolabeled protein standards were prepared for use as internal markers in the estrogen-receptor assays,

**Fig. 1. Separation of 14C-labeled marker proteins by sucrose gradient centrifugation**

Aliquots of [14C]HSA (10 µg), [14C]GOx (20 µg), or both, corresponding to 3500 cpm were layered in a sample volume of 200 µL onto 100-350 g/10 ml linear sucrose gradients and centrifuged (360 000 × g, 16 h, 4 °C) in a Beckman SW 60 Ti rotor. Gradients were fractionated and analyzed as described in Materials and Methods.

**Fig. 2. Separation of marker proteins by sucrose gradient centrifugation**

Relationship between sedimentation coefficients and gradient positions for 14C-labeled and unlabeled marker proteins. (A) Unlabeled ribonuclease (RNase, 1.3S), hemoglobin (Hb, 4.2S) and catalase (Cat, 11.3S) were layered separately onto linear sucrose gradients and centrifuged as described in Fig. 1. (B) A mixture containing [14C]HSA (4.8S) and [14C]GOx (7.8S) and unlabeled marker proteins (RNase, Hb, and Cat) was layered onto a single linear sucrose gradient and centrifuged as described in Materials and Methods. The open circle (O) in panel B represents catalase that had to be centrifuged on a separate gradient for reasons described in the text.
it was necessary to determine whether the presence of either protein affected other aspects of the assay. Therefore, we assessed the influence of the individual markers on total and nonspecific estrogen binding in tumor cytosols. [14C]HSA and [14C]GIOx were examined at three different concentrations: 0.5-, 1.0-, and 1.5-fold the amount routinely used in a single gradient. Typical data are depicted in Figures 3 and 4. Figure 3 shows the sedimentation profiles of estrogen receptors in the presence of 10, 20, and 30 μg of 14C-labeled GIOx. Clearly, there was no difference in the nonspecific binding of the sample.

Our use of albumin as an internal standard presented an interesting problem because HSA binds steroids, in particular estradiol, with low affinity and high capacity (23). In fact, HSA represents about 25% of the total cytosol protein (Hess.

Fig. 3. Influence of [14C]GIOx on distribution of nonspecific binding in breast cancer cytosol
Cytosols from human breast carcinomas were incubated with 5 nmol/L [3H]-estradiol-17β in the presence of a 200-fold excess of diethylstilbestrol for 5 h at 4°C. After removal of unbound steroid by dextran-coated charcoal, the steroid-receptor complexes were mixed with 10 μg (A), 20 μg (B), and 30 μg (C) of [14C]GIOx and layered onto 100-350 g/kg linear sucrose gradients, centrifuged, and analyzed as described in Fig. 1. [3H]estradiol, (Φ); [14C]GIOx, (Ο)

Fig. 4. Influence of [14C]HSA on the distribution of estradiol binding in breast cancer cytosol
Cytosols from human breast carcinomas were incubated with 5 nmol/L [3H]-estradiol-17β in the presence (A and C) and presence (B and E) of a 200-fold excess of diethylstilbestrol for 5 h at 4°C. After removal of unbound steroid by dextran-coated charcoal, the steroid-receptor complexes were mixed with 5 μg (A and B) and 15 μg (D and E) of [14C]HSA, centrifuged, and analyzed as described in Materials and Methods. Specific binding (C and F) is calculated by subtracting nonspecific binding from total binding. [3H]estradiol, (Φ); [14C]HSA, (Ο)

Fig. 5. Estimation of sedimentation properties of estrogen receptors by using marker proteins on sucrose gradients
Cytosols from human breast carcinomas were prepared as described in Fig. 3, except that two samples (C and D) were activated by an additional 30-min incubation at 28°C before unbound steroid was removed with dextran-coated charcoal. Aliquots of 14C-labeled marker proteins were layered in a constant sample volume of 200 μL onto 100-350 g/kg linear sucrose gradients, centrifuged, and analyzed as described in Materials and Methods. [3H]estradiol, (Φ); 14C-labeled marker proteins, (Ο)
Feldhoff, and Wittliff, unpublished results), and endogenous albumin may be responsible for some of the nonspecific estrogen binding in the 4–5S region of the assay (e.g., 15). However, when [14C]HSA was added to the same reference powder used in Figure 3 at an amount 0.5- or 1.5-fold the amount routinely used in the receptor assay (10 pg), no difference in total or nonspecific estrogen binding was observed (Figure 4). The amount of [14C]albumin added to each sample represented less than 5% of the endogenous amount usually present in tumor cytosols. Clearly, the results of these experiments (Figures 3 and 4) show that neither [14C]marker protein affects these aspects of estrogen-receptor assays. For economy, marker proteins were added at the time of loading onto each gradient; however, the 14C-labeled proteins can also be added before the dextran-coated charcoal step (data not shown).

Currently, 14C-labeled marker proteins are added routinely to preparations of estrogen receptors that are to be analyzed by sucrose density-gradient centrifugation. A typical example is depicted in Figure 5. As shown in panel A, after charging with [2,3,6,7,14H]estradiol-17β (5 h at 4°C), the two major forms (4S and 8S) of estrogen receptors in this human tumor cytosol were readily resolved by sucrose density-gradient centrifugation. However, as shown in panel C, when an identical sample was charged as above and subsequently activated (30 min at 28°C), there was a dramatic change in the relative amounts of 4S and 8S species. Clearly, the use of the marker proteins shows that the shift is real and is not an experimental artifact, because the internal marker proteins sedimented normally. Perhaps of even more importance to current research efforts (e.g., 18, 20) is the unequivocal demonstration of the heterogeneity present in the 4S region of panel C, the apparent shoulder region of the 4S peak was observed with a slower migration and resolved from the 14C internal marker in a manner significantly different from that observed in panel A. The profiles in Panels B and D indicate the amounts of nonspecific binding under the different experimental conditions and demonstrate the usefulness of the 14C-labeled internal markers for routine quality control.

We recommend that both 14C-labeled marker proteins are added routinely to preparations of estrogen receptors in clinical material when sucrose gradient centrifugation is used. Addition of these labeled-marker proteins in studies of species distribution of steroid receptor, such as in the evaluation of activation or DNA-binding phenomena, appears useful.

Recently 14C-labeled proteins of defined molecular mass have become available commercially (New England Nuclear Corp., Boston, MA 02118). However, some care must be exercised in the choice of these 14C proteins, because several are supplied in buffered solution containing sodium dodecyl sulfate or β-mercaptoethanol, or both, which may alter the sedimentation and binding properties of estrogen receptors.

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