Detection of a \( M, 24,000 \) Estrogen-regulated Protein in Human Breast Cancer by Monoclonal Antibodies

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ABSTRACT

Monoclonal antibodies have been prepared that can specifically detect an estrogen-regulated protein with a molecular weight of 24,000 in the MCF-7 human breast tumor cell line and in human breast tumor biopsies. These antibodies are of the immunoglobulin G1 subclass, exhibit a high affinity for the \( M, 24,000 \) protein, and recognize an antigenic site that is stable to sodium dodecyl sulfate gel electrophoresis and electrophoretic transfer techniques. These monoclonal antibodies have been used to confirm estrogen regulation of the \( M, 24,000 \) protein in MCF-7 cells and to detect \( M, 24,000 \) protein in certain human breast tumors.

INTRODUCTION

Endocrine therapy is an effective treatment for human breast cancer, providing that patients with hormone-sensitive breast tumors can be identified. Analysis of estrogen receptor and progesterone receptor status in breast tumor biopsies is widely used for this purpose (11, 22) and is capable of selecting a subset of patients (with tumors retaining both receptors), who have a response rate to endocrine therapy approaching 80% (25). The presence of a functional pathway of estrogen action is thought to account for this remarkable response rate, since progesterone receptor has been shown to be an end product of estrogen action in cultured human breast cancer cells (16). Although receptor assays are a valuable diagnostic tool, inherent properties of the receptor proteins, principally the lability of hormone ligand binding and the small amount of receptor per cell, have prompted the search for other estrogen-regulated proteins in human breast cancer.

Several laboratories have now described estrogen-regulated proteins associated with human breast tumors or tumor cell lines. In addition to progesterone receptor activity, we have measured the enzymes lactate dehydrogenase (7) and DNA polymerase (12), while Bronzert et al. (5) have studied thymidine kinase in the MCF-7 human breast tumor cell line. MCF-7 cells have also been shown to secrete proteolytic enzymes under the influence of estradiol (8, 28), an effect that appears to occur in breast tumors (26). In addition, Westley and Rochefort (30, 31) have used methionine labeling and 2-dimensional gel analysis to identify and characterize a \( M, 46,000 \) glycoprotein that is rapidly secreted by a number of breast tumor cell lines in response to estradiol. Although the exact relationship of the \( M, 46,000 \) glycoprotein and the secretory proteases is unknown, at least one facet of their regulation may be similar in that estradiol has been found recently to influence the secretory process itself in MCF-7 cells (29).

We have also used the resolving power of gel electrophoresis to search for estrogen-regulated proteins in MCF-7 cells but have chosen the double isotope gel electrophoresis method (18) to compare specific protein synthesis in antiestrogen-treated cells to that in cells stimulated by estradiol following antiestrogen pretreatment. Using this methodology, we have identified a \( M, 24,000 \) cytoplasmic protein that is specifically stimulated by biologically active estrogens in a dose-dependent manner in parallel with processing of nuclear estrogen receptor (9, 10). We estimate that the \( M, 24,000 \) protein represents 1.6% of newly synthesized protein, making it a major product of estrogen stimulation and thus a good potential marker for hormone-sensitive breast tumors.

Further study of the \( M, 24,000 \) protein as both a tumor marker and a model for hormone regulation in human breast cancer requires development of a highly sensitive assay, such as a competitive radioimmunoassay. To this end, we have used the hybridoma technique of Köhler and Milstein (19) to prepare monoclonal antibodies against the \( M, 24,000 \) protein that are suitable for radioimmunoassay. In this paper, we describe the preparation and characterization of monoclonal antibodies that react specifically with the \( M, 24,000 \) protein. Furthermore, we demonstrate that these antibodies can be used to measure estrogen stimulation of the \( M, 24,000 \) protein in MCF-7 cells by immunoprecipitation and to detect the \( M, 24,000 \) protein in human breast tumor cytosols using gel electrophoresis and immunoblotting techniques.

MATERIALS AND METHODS

Materials and General Methods. Growth of MCF-7 cells, in vitro labeling with \(^{3}H\)leucine, preparation of cytosols, and protein analysis by gel electrophoresis were performed as described previously (9, 10). Proteins were assayed by the method of Bradford (4), using bovine \( \gamma \)-globulin as standard (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, Calif.). Typing antisera for mouse IgG1, IgG2a, IgG2b, IgG3, and IgM were obtained from Litton Bionetics, Charleston, S. C.; that for mouse IgA was from Miles Laboratories, Inc., Elkhart, Ind. Polyethylene glycol 1500 was obtained from Koch-light, Research Products International, Elk Grove, Ill. Protein standards for SDS\(^4\) gel electrophoresis were those of Bio-Rad.

Preparation of \( M, 24,000 \) Immunogen. The \( M, 24,000 \) protein was partially purified from MCF-7 cytosols by 2 methods. Since our laboratory routinely prepares large-scale cytosols for the purification of estrogen receptors from MCF-7, it was possible to take a side fraction from these preparations as a source of \( M, 24,000 \) protein. In the initial method, cytosol from 80 ml of packed MCF-7 cells was prepared in 50 mm Tris (pH 7.4):1 mm disodium EDTA:1 mm diithiothreitol:5 mm sodium molyb-

\(^{4}\) The abbreviations used are: SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.
date and passed over a heparin-Sepharose column prepared by the method of Iverius (17). The flow-through fraction from this column (normally discarded in estrogen receptor purifications) was then fractionated batchwise on 100 ml of hydroxylapatite (Bio-Gel HTP, Bio-Rad), prepared as described by the manufacturer. The hydroxylapatite was washed with 4 volumes of 0.01 M phosphate buffer (0.01 M sodium phosphate, pH 6.8, containing 5 mM sodium molybdate and iodoacetamide, 50 mM/m) and then eluted with 2 volumes of 0.05 M sodium phosphate, pH 6.8. This eluate was next passed over a 50-ml column of DEAE-cellulose (Whatman DES2; Whatman, Inc., Clifton, N. J.), washed with 4 volumes of 0.05 M phosphate buffer, and eluted with 2 volumes of the same buffer containing 0.2 M NaCl. The eluate was concentrated by ultrafiltration (immexible CX10 ultrafilters; Millipore Corp., Bedford, Mass.) and stored at −20°C. Ten female BALB/c mice were immunized with this partially purified M, 24,000 protein (120 mg/mouse s.c. in complete Freund’s adjuvant) and given a 100-μg booster injection in incomplete Freund’s adjuvant approximately 1 month later.

In subsequent purifications, MCF-7 cytosols were prepared in 50 mM Tris (pH 7.4):1 mM disodium EDTA:1 mM dithiothreitol:5 mM sodium molybdate buffer, adjusted to 0.7 M KCl, and passed slowly over an estradiol affinity adsorbent (15) (about 100 ml of cytosol were run through in 18 hr at 4°C). The flow-through fraction was dialyzed against 0.01 M sodium phosphate, pH 6.8, containing phenylmethylsulfonyl fluoride, 20 μM/μl, and then fractionated batchwise on DEAE-cellulose using a 4-volume wash step followed by elution with 3 volumes of 0.10 M sodium phosphate, pH 6.8. The eluate was diluted 10-fold, rechromatographed on a 10-ml DEAE-cellulose column, and then passed over a Sephacryl S-200 gel filtration column where M, 24,000 protein appears in the void volume. Eluted material was aliquoted and stored at −20°C. The immunized mice described above were given a booster injection (50 μg i.p.) of this M, 24,000 protein preparation. One month later, 3 animals were given daily injections (25 μg i.v.) 3 days before fusion.

**Hybridoma Procedure.** Our hybridoma procedure has been described in detail previously (3). Briefly, splenocytes from 3 mice were combined and fused with NIS-1.Ag4.1 (NS-1) mouse myeloma cells (final concentration, 31.8 × 10⁶ splenocytes plus 8 × 10⁷ myeloma cells per ml) using 50% polyethylene glycol as fusing agent. Fused cells were plated in 96-well microtiter plates at a concentration of 5 × 10⁶ cells/well, Two weeks after plating and hybridoma selection on Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 20% heat-inactivated fetal bovine serum, 0.10 mM hypoxanthine, aminopterin (350 mg/ml), and 16 μM thymidine, plates were screened for anti-M, 24,000 protein activity (see below), and 5 cultures showing highest antibody production were cloned by limiting dilution using a mouse T-cell feeder layer. The fusion well with the highest initial activity gave the most positive stable clones were cloned by limiting dilution using a mouse T-cell feeder layer. The fusion well with the highest initial activity gave the most positive stable clones, 3 of which were subsequently grown and maintained in culture as the stable lines C11, G3, and H7. After 2 to 3 months in culture, these lines were recloned with the sublines designated C11.1, G3.1, and H7.1. Ascites fluid was prepared from each subline by i.p. injection of 1 ml per well for at least 4 weeks. Hybridoma cells were grown and maintained in culture as the stable lines C11.1, G3.1, and H7.1. Ascites fluid was prepared from each subline by i.p. injection of 1 ml per well for at least 4 weeks. The cell harvest was then used to aspirate the wells and to wash 3 times with PBS:azide [0.02 mM NaNO₃ (pH 7.6):0.15 mM NaCl:0.02% NaN₃]. Any free binding sites were blocked by at least a 1-hr incubation with 5% bovine serum albumin (200 μg/ml) in PBS:azide. The plates were washed and if not used immediately, were stored 4°C with PBS:azide in each well. Test antisera or hybridoma supernatants (50 μg/ml) were incubated overnight at 4°C. The plates were then washed and dried, and 50 μl of iodinated M, 24,000 protein (50,000 cpm) in 1% bovine serum albumin in PBS:azide were added and incubated at 4°C for at least 3 hr. Finally, the plates were washed, dried, and stored at −20°C. A 2-fold increase in counts above background (spent growth medium from parent myeloma cells or preimmune serum) was considered positive with values ranging as high as 12-fold.

**Isolation of M, 24,000 Monoclonal Antibodies.** Spent hybridoma culture media and mouse ascites fluid were centrifuged at 1,000 x g for 15 min, and the supernatants were stored at −20°C. Prior to salt fractionation, ascites fluid was further centrifuged at 105,000 × g for 90 min to remove contaminating debris and lipid. Antibody was partially purified by successive 34% ammonium sulfate precipitations at room temperature, and then, the globulin pellet was dissolved in PBS:azide (5 to 10 mg/ml) and stored at 4°C. To obtain samples for iodination, antibody isolated from ascites fluid was further purified by gel filtration on Sephacryl S-300 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). Fractions in the peak migrating at an apparent molecular weight of 150,000 were pooled and stored at 4°C.

**Iodination Procedure.** Both the M, 24,000 protein and M, 24,000 monoclonal antibody were iodinated by solid phase lactoperoxidase (Enzymobeads; Bio-Rad) according to the manufacturer’s protocol, except that 2 μCi of Na¹²¹I (100 μCi/ml; Amersham) were added per 50 μg of protein, and the incubation time was extended to 1 hr at room temperature. Free iodine was initially removed by gel filtration on Sephadex G-25-150 (Sigma Chemical Co., St. Louis, Mo.). However, once an antibody affinity matrix was available (1), iodinated M, 24,000 protein was purified on a 2-ml affinity column to ensure removal of both free iodine and any protein the antigenicity of which was damaged by the iodination reaction. Specific activities of 10⁶ cpm/mg were typically achieved.

**Immunoprecipitation of M, 24,000 Protein.** MCF-7 cytosols (125 μg of protein) were mixed with 50 μl of C11 hybridoma supernatant and 1% normal mouse serum in a total volume of 100 μl. The assay was incubated overnight at 4°C, followed by addition of 25 μl of goat anti-mouse IgG antiserum (University of Texas Science Park, Smithville, Texas), and incubated for an additional 4 hr at 4°C. The immunoprecipitates were then centrifuged at 1500 x g for 30 min through a 200-μl cushion of 1 M sucrose in PBS. The pellets were dissolved in SDS sample buffer and electrophoresed on 10% polyacrylamide gels using the method of Laemmli (20) as modified by Wykoff et al. (32).

**Transfer of Proteins to Diazoephthioether Paper.** Proteins separated by SDS gel electrophoresis were transferred to diazoephthioether paper (APT form; Schleicher and Schuell, Keene, N.H.) essentially as described by Alwine et al. (2) and Reiser and Wardale (27), using the Electrobolt system of E-C Apparatus Corp., St. Petersburg, Fla. Gels were transferred at 0.65 amp for 4 hr at room temperature. Unreacted diazo groups were then quenched by a 2-hr incubation in 0.1 M Tris-HCl, pH 9.0, containing 0.25% gelatin (175 bloom; Sigma). The paper was washed in binding buffer [50 mM Tris-HCl (pH 7.4):150 mM NaCl:5 mM disodium EDTA:0.25% gelatin:0.05% Nonidet P-40], sealed in a plastic boiling bag (Seal-N-Save; Sears, San Antonio, Texas) containing iodinated anti-M, 24,000 protein (500,000 cpm per 5 ml of binding buffer per gel lane), and incubated overnight at 4°C on a rocker platform. After successive washes with binding buffer, wash buffer [50 mM Tris (pH 7.4):1 mM NaCl:5 mM disodium EDTA:0.4% sodium lauryl sarcosinate], and water, the paper was dried and autoradiographed for 48 hr at room temperature using Kodak X-OMat XP-1 film (Eastman Kodak Co., Rochester, N. Y.) and Du Pont Cronex Quanta III intensifying screens (Du Pont, Wilmington, Del.). Efficiency of the electrophoretic transfer technique was monitored by silver staining (23) of gels following transfer and was routinely 90 to 100% for proteins with molecular weights of 100,000 or less. In addition, an aliquot of iodinated C11 antibody was mixed with the protein standards as an internal control for protein transfer, as a monitor of antibody integrity, and as a molecular weight and orientation marker on the autoradiogram.

**RESULTS**

**Partial Purification of the M, 24,000 Protein and Preparation of Monoclonal Antibody.** To obtain an enriched fraction of M,
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24,000 protein suitable for production of monoclonal antibodies, cytosol proteins from MCF-7 cells were separated by ion exchange and gel permeation chromatographies using SDS-polyacrylamide gel electrophoresis to monitor the purification. The M, 24,000 protein eluted from DEAE-cellulose with 0.1 M salt at pH 7.6 and appeared in the void volume on Sephacryl S-200 or S-300. These 2 properties of M, 24,000 protein were used to produce a partial purification (Chart 1, Gel-staining Profile A) that was significantly enriched in M, 24,000 protein compared to unfractionated MCF-7 cytosol (Gel-staining Profile B). This enriched M, 24,000 protein fraction was used both as immunogen in mice and as a probe in the screening assay for M, 24,000 antibodies. Fortuitously, when the partially purified M, 24,000 protein was iodinated, a single major radioactive peak was produced (Chart 1, Profile A), which represented 93% of the applied counts. Using this specific probe, 480 hybridoma culture wells were screened by the solid-phase microtiter plate assay at applied counts. Using this specific probe, 480 hybridoma culture wells were screened by the solid-phase microtiter plate assay at an input level of antigen that selects primarily high-affinity antibodies (50,000 cpm, 5 to 10 ng). Twelve % of the wells were positive in the initial screening. However, of the 4 highest positive wells that were subsequently cloned, only the highest gave stable, antibody-producing colonies. From this original fusion well, 3 clonal hybridoma lines have been derived, C11, G3, and H7. The clonality of these lines has been established by repeated cloning at limiting dilution, yielding subclones that all secrete antibody against M, 24,000 protein and sublines that have remained stable for 6 months.

Characterization of Monoclonal Antibodies. The monoclonal antibodies were characterized according to immunoglobulin subclass by binding subclass-specific rabbit anti-mouse antisera (diluted 1:10) to flexi-vinyl plates in place of the polyvalent rabbit immunoglobulin normally used in the assay. The results of this experiment indicate that C11, G3, and H7 are all of the IgG1 subclass (data not shown). This result was confirmed by Protein A-Sepharose chromatography using pH gradient elution (24). Mouse IgG1 subclass does not bind efficiently to Protein A (13).

Accordingly, when anti-M, 24,000 C11 was fractionated on Protein A-Sepharose, most of the antibody activity appeared in the flow through with only a small fraction eluting at pH 5.5, a characteristic of IgG1 (data not shown). Thus, Protein A-Sepharose chromatography, frequently the method of choice for antibody purification (14), could not be applied. Consequently, the monoclonal antibodies were partially purified by ammonium sulfate precipitation to give concentrates that, when adjusted to 1 mg/ml, each had titers of approximately 1:1000.

The affinities of the monoclonal antibodies were assayed by reacting rabbit anti-mouse IgG-coated plates with a constant amount of antibody and then incubating with increasing amounts of iodinated M, 24,000 protein, purified by antibody affinity chromatography (1). Assuming a monomer molecular weight of 24,000, Scatchard analyses of the resulting saturation curves (Chart 2) gave K_s of 3, 4, and 3 x 10^-6 for C11, G3, and H7, respectively, with correlation coefficients of ≥0.95. The 3 antibodies are therefore of the high-affinity type, consistent with our original screening strategy.

Specific Detection of M, 24,000 Protein in Breast Tumor Cells and Biopsies. With M, 24,000 monoclonal antibodies in hand, we next asked whether these antibodies could specifically detect M, 24,000 protein in complex protein mixtures. In one approach to this question, a cytosol was prepared from estrogen-stimulated MCF-7 cells pulse labeled with tritiated leucine (see below). The M, 24,000 protein was immunoprecipitated either with media from C11 hybridomas or with control medium from the parent myeloma line as described in "Materials and Methods." Immunoprecipitates were then electrophoresed, producing the gel profile shown in Chart 3. A single, major radioactive peak was observed with a molecular weight of 24,000 that was not precipitated by control medium. Using a second approach, cytosols were prepared from a series of breast tumor biopsies, and aliquots were electrophoresed on replicate SDS gels. Resolved proteins were then either stained with Coomassie blue (Fig. 1, top) or electrophoretically transferred to diazophenylthioether paper and incubated with iodinated C11 antibody. The autoradiogram (Fig. 1, bottom) shows a single M, 24,000 immunoreactive band of variable intensity in some (Lanes 2, 3, and 5 to 8) but not all (Lanes 1 and 4) breast tumor cytosols. Using this immunoblot technique, we have assayed 94 breast tumor cytosols and have detected M, 24,000 protein in 56 cases with a threshold of detection of approximately 30 ng.

**Chart 1.** Partial purification of the M, 24,000 protein. Cytosol from MCF-7 cells (SDS Gel-staining Pattern B) was fractionated on DEAE-cellulose and Sephacryl S-200. Protein eluting in the S-200 void volume (Gel-staining Pattern A) was iodinated by the lactoperoxidase technique to yield the specific M, 24,000 radioactive probe shown at the top.

**Chart 2.** Affinity of M, 24,000 monoclonal antibodies. In A, using the plate assay, a constant amount of M, 24,000 monoclonal antibodies C11 ( ), G3 ( ), or H7 ( ) was probed with increasing amounts of iodinated M, 24,000 protein, purified by antibody affinity chromatography. In B, Scatchard analysis of the resulting saturation curves gave dissociation constants of 2.7, 4.0, and 2.9 x 10^-6 M for C11, G3, and H7, respectively.
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Chart 3. Specificity of M, 24,000 monoclonal antibody C11. MCF-7 cells were treated for 6 days with 10^-6 M nafoxidine and then switched to medium containing 10^-8 M estradiol for an additional 4 days. The cells were then pulse labeled with tritiated leucine, cytosol was prepared, and an aliquot (125 μg) was incubated with medium from C11 hybridomas (---) or from the parent myeloma line (----). Immunoprecipitates obtained by the double-antibody method (see "Materials and Methods") were analyzed by SDS gel electrophoresis.

![Chart 3](image)

Fig. 1. Detection of M, 24,000 protein in human breast tumor cytosols. Proteins from 8 human breast tumor cytosols were denatured in SDS, electrophoresed (50 μg/lane), and stained with Coomassie blue (top). A replicate gel was electrophoretically transferred to diazophenylthioether paper which was then incubated with iodinated M, 24,000 antibody C11 and autoradiographed (bottom) Standards are: phosphorylase B, M, 92,500; bovine serum albumin, M, 66,200; ovalbumin, M, 45,000; carbonic anhydrase, M, 31,000; and soybean trypsin inhibitor, M, 21,500.

**Estrogen Regulation of M, 24,000 Protein in MCF-7 Cells.**

In previous work (9, 10), we used the double-isotope ratio technique of Katzenellenbogen and Gorski (18) to demonstrate estrogenic stimulation of the M, 24,000 protein in MCF-7 cells. Development of a monoclonal antibody permits direct quantitation of this effect. MCF-7 cells were treated by an estrogen rescue protocol: after 6 days of treatment with the antiestrogen nafoxidine (10^-6 M), cells were either continued on antiestrogen or switched to medium containing estradiol (10^-8 M) or ethanol vehicle alone. Cells were labeled for 4 hr with [3H]leucine, cytosols were prepared and immunoprecipitated with M, 24,000 monoclonal antibody, and the immunoprecipitates were analyzed by SDS gel electrophoresis. Quantitation of radioactivity in the M, 24,000 peak indicates that estradiol treatment produces about a 2-fold increase in M, 24,000 protein above that seen in cells continued on nafoxidine or rescued with ethanol vehicle alone (Chart 4). Cells in these 2 control groups do, however, synthesize significant amounts of M, 24,000 protein, a result consistent with our previous double-isotope ratio experiments (9, 10).

**DISCUSSION**

Recently, several laboratories have identified estrogen-regulated proteins or protein activities in human breast cancer as potential markers for hormone-responsive breast tumors (5, 7, 8, 12, 16, 30, 31). In our search for such proteins, we used the double-isotope electrophoresis technique to identify a cytoplasmic protein with a molecular weight of 24,000 synthesized in abundance by MCF-7 human breast tumor cells (10). The discovery of M, 24,000 protein was aided by the fact that the protein can be readily observed on Coomassie blue-stained SDS:polyacrylamide gels as an isolated band with a molecular weight of 24,000 (Chart 1B). By exploiting the behavior of M, 24,000 protein on gel filtration and ion exchange chromatographies, we were able to obtain a partial purification suitable for monoclonal antibody production. The power of the monoclonal antibody technique is clearly illustrated with the M, 24,000 protein, since the partially purified fraction shown in Chart 1 would probably be insufficient to produce monospecific antibodies by traditional methods.

The 3 M, 24,000 monoclonal antibodies characterized in this paper are very similar based on subclass typing, immunological titer, and affinity for M, 24,000 protein. Competitive binding experiments further suggest that C11, G3, and H7 recognize the same or highly similar antigenic sites. These antibodies may therefore be identical, considering that they were all derived from the same original polyclonal fusion well. We are currently pro-

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6 D. J. Adams, unpublished observation.
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Introducing additional monoclonal antibodies against M, 24,000 protein with different site specificities for use in a double determinant quantitative assay, the assay most appropriate for monoclonal antibodies (6).

The immunoprecipitation and immunoblotting data (Fig. 1; Chart 4) not only demonstrate the specificity of the antibodies but suggest some characteristics of the M, 24,000 protein. (a) The protein probably consists of a single monomer with a molecular weight of 24,000. The much larger apparent molecular weight exhibited on gel filtration may be due to aggregation (even in the presence of non-ionic detergents) rather than association with larger subunits. (b) The blot experiments indicate that M, 24,000 protein antigenicity is not destroyed by treatment with SDS sample buffer [0.125 M Tris-OH (pH 6.8):5% β-mercapto-ethanol:1% SDS:10% glycerol; 90° for 10 min] or by the electrophoretic and diazo-binding processes. This result led to the detection of M, 24,000 protein in human breast tumor cytosols, an observation not possible by mere inspection of gel-staining patterns.

Monoclonal antibodies against M, 24,000 protein should facilitate studies of hormone action in human breast cancer. Estrogen stimulation of M, 24,000 protein in antiestrogen-pretreated MCF-7 cells, first observed by the double-label gel technique, has been confirmed using M, 24,000 monoclonal antibodies. Furthermore, these antibodies may prove useful in the identification of hormone-responsive breast tumors. Since the evidence presented with the immunoblot technique is qualitative and subject to experimental variation, this application will require development of a quantitative immunoassay for M, 24,000 protein and analysis of a large number of tumors for which response data are known. Nevertheless, our present results suggest that the M, 24,000 protein may be a potential marker for hormone-sensitive breast cancer.

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