Evidence for the Existence of a Third Progesterone Receptor Protein in Human Breast Cancer Cell Line T47D

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Abstract

We have used a new monoclonal antibody, designated C-262, directed against the last 14 amino acids of the carboxy-terminus of human progesterone receptors (N. L. Weigel et al., Mol. Endocrinol., 6: 1585-1597, 1992) to analyze progesterone receptor structure. This new antibody recognizes the previously described B-receptors (Mr 120,000) and the naturally occurring N-terminal truncated A-receptor (Mr 94,000). In addition to B- and A-receptors, C-262 detects a third progestin-binding protein with a molecular weight of approximately 60,000 in the progestin-responsive human breast cancer cell line, T47D. The 60,000 dalton protein is predominantly found in the cytosolic fraction of untreated T47D cells and binds tightly to the nucleus following progesterone or R5020 treatment of T47D cells. These dynamics are similar to the previously described progesterone receptor isoforms. The 60,000 dalton protein binds the synthetic progestin, [3H]R5020, which competes with cold R5020 as determined with the technique of in situ photoaffinity labeling. Prolonged incubation of nuclear extracts at elevated temperatures does not result in accumulation of the 60,000 dalton protein, yet the level of photoaffinity-labeled B- and A-receptors declines. These data support our hypothesis that the 60,000 dalton protein is not a degradation product of the two larger progesterone receptor isoforms but is a distinct progestin-binding protein. This is further supported by our previous study identifying at least two progesterone receptor mRNAs that do not code B- or A-receptors. These two transcripts are not unique to T47D cells and are also present in human breast cancer cells, MCF-7, and normal human endometrium. Taken together, these data provide evidence for the existence of a third progesterone receptor isozyme in progesterin-responsive tissues.

Introduction

ERs and PRs are recognized as important prognostic markers as well as valuable tools for predicting positive response to hormonal therapy in the treatment of hormone-dependent breast cancer (1). The measurement of ER is of such importance that rigorous investigation has focused on the identification, isolation, and characterization of various ER isoforms. Variant estrogen receptor messages and proteins have been identified by several research groups from both human breast cancer tumors and established breast cancer cell lines (2-9). Some of these isoforms may in part explain why approximately 50% of ER-positive tumors fail to respond to antiestrogen therapy. One variant ER isoform is present not only in select breast cancer tumors but also in normal human endometrial samples (6).

Progesterone receptor induction is a well-known estrogen response. Because of this, the measurement of PR has become as important as a prognostic factor as ER in predicting the response to endocrine therapy. Several lines of evidence also support an important role of progesterone in mammary carcinogenesis and the growth of established tumors, although its specific function remains to be elucidated (for review, see Ref. 10). Based on results correlating the response to antiestrogen treatment and estrogen receptor status, it is speculated that PR-rich tumors are especially sensitive to progestin therapy.

Progesterone receptors, unlike estrogen receptor, consist of at least two progestin-binding proteins. The smaller human PR protein, the A-receptor (Mr ~94,000), is a naturally occurring, truncated form of the larger B-receptor (Mr 116,000-120,000) (11). Both proteins can bind progestins and DNA. Recently, it has been demonstrated that these two proteins may have distinct biological properties, depending on the gene expressed and the choice of ligand (12). In certain circumstances, the activity of A-receptors suppresses the biological response to B-receptors (12-14). We report here evidence for the existence of a third PR isoform that may inhibit the activity of both A- and B-receptors in the presence of progesterone.

T47D is an established human breast cancer cell line rich in PR that has been used extensively to study normal PR structure and receptor dynamics (15). Using these cells as a source, we initially described the complex PR message profile. To date, we have demonstrated 9 distinct PR message transcripts (16). Two of these transcripts could not code the previously described A- and B-proteins but do code a smaller amino-terminally truncated receptor isoform. Using a new anti-PR antibody, C-262, directed against the last 14 amino acids of human PR, we demonstrate the presence of a third progestin-binding protein (Mr ~60,000) (17). This protein is present in T47D cells but not in PR-negative cells.

Materials and Methods

Cell Culture. T47D cells were cultured as described (15) and maintained on 5% fetal calf serum in minimum essential medium. The PR-negative MDA-231 and HeLa cells as well as the estrogen-responsive, PR-positive human breast cancer cell line, MCF-7, were all maintained in the above-described medium. Cell pellets were homogenized in TEDG buffer in the presence of a cocktail of protease inhibitors (18). Cytosols and nuclear extracts were prepared as previously described (18).

Western Blot Analysis. Cell extracts were electrophoresed on 7.5% polyacrylamide-sodium dodecyl sulfate gels, and separated proteins were transferred to nitrocellulose and probed with the C-terminal anti-PR antibody, C-262 (gift from Dr. D. P. Edwards), according to the method of Weigel et al. (17). Blots were probed with a rabbit anti-mouse immunoglobulin G linked to a peroxidase conjugate, and immunoreactive bands were visualized using 4-chloro-1-naphthol and 0.025% H2O2 (18).

In Situ Photoaffinity Labeling with [3H]R5020. Details of the technique are described by Horwitz and Alexander (19). Briefly, cells were incubated 20 min with 100 nM labeled R5020. They were then harvested, cooled, washed, and irradiated 2 min with 300 nm of UV light; rinsed with buffer; and homogenized in TEDG buffer/molybdate in the presence of protease inhibitors. Nuclei were pelleted, extracted with 0.6 M NaCl, and centrifuged at 100,000 × g for 30 min to obtain the supernatant nuclear extract. Extracts were then concentrated by ammonium sulfate precipitation, and pellets were resuspended and subjected to gel electrophoresis. Gels were stained with Coomassie Blue and destained to visualize lanes and protein bands. Subsequently, lanes were frozen and then sliced. Each gel slice was incubated in 0.5 ml of water and 0.5

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The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; TEDG buffer, 10 mM Tris (pH 7.4)-1 mM EDTA-1 mM dithiothreitol-10% glycerol; cDNA, complementary DNA; NLS, nuclear localization signal; DD, dimerization domain.

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ml of Solvable (New England Nuclear) and baked at 50°C for 3 h. The gel slices were neutralized with 100 µl of 0.1 M glacial acetic acid, and 10 ml of scintillation cocktail were added. Samples were then counted. For degradation studies, molybdate and protease inhibitors were excluded from the buffer. Extracts were incubated at 20°C for 10 to 40 min and then prepared for gel electrophoresis.

Northern Analysis. RNA was isolated, and Northern blots were performed according to the method of Wei et al. using a nick-translated partial human PR cDNA provided by Dr. B. W. O’Malley (20).

Results and Discussion

Western blots of cell extracts from T47D cells probed with the new monoclonal antibody, C-262, specific to the extreme 14 amino acids of PR proteins, detected B- and A-receptors and three major additional lower molecular weight proteins. The 60,000 dalton protein was present only in cytosols from T47D cells and not from the two PR-negative cell lines, MDA-231 and HeLa (Fig. 1A). The lower molecular proteins are nonspecific, since they are detectable in all three cell lines tested, detected only by direct blot, and not immunoprecipitated in solution, suggesting that they are not receptor related (17).

Hormone treatment for 20 min with either the natural ligand, progesterone, or the synthetic progestin, R5020, results in tight nuclear binding of B-, A-receptors and the 60,000 dalton protein (Fig. 1B). Nuclear extracts from MDA-231 and HeLa cells did not contain the 60,000 dalton protein. Note that this 60,000 dalton protein would not be detected by N-terminal anti-PR antibodies, such as AB-52, B-30, or PR-6.

To determine if the 60,000 dalton protein binds progestins, we used the technique of photoaffinity labeling. T47D cells were incubated in the presence of either [3H]R5020 alone or 100-fold excess cold hormone. Three peaks of [3H]R5020 labeling were detected (Fig. 2). The first two peaks (left to right) represent B-receptors and A-receptors. A third peak is detectable at a molecular weight of approximately 60,000, which we have named the C-receptor. In seven experiments, C-receptors represent ~50% of either A- or B-receptors and ~25% of the total PR content as determined by [3H]R5020 in situ photoaffinity labeling. All three peaks are eliminated in the presence of excess R5020 (Fig. 2), progesterone, or the antiprogestin RU486 (data not shown). To confirm that the 60,000 dalton protein is not a proteolytic degradation product of A- or B-receptors, receptors were covalently labeled with radioactive ligand and purposely allowed to degrade for 0 to 40 min at 20°C (Fig. 3). With time, both B- and A-receptor levels diminish but without an accumulation of the 60,000 dalton protein. This suggests that the 60,000 dalton protein is not a degradation product of the two larger PR isoforms but a distinct progestin-binding protein.

We then asked the question whether this third PR isoform is unique to T47D cells. Based on previous RNA-mapping studies of human PR transcripts, we characterized two unique messages (11.4III and IV), which do not contain the translation start sites for either B- or A-receptors but could code a third progesterone receptor protein with a molecular weight of approximately 60,000. We postulate that the 60,000 dalton protein is synthesized from these two unique messages (11.4III and IV) utilizing a downstream in-frame methionine translation start site. To assess if other cells may potentially synthesize the 60,000 dalton protein, mRNA was isolated from the human breast cancer cell line, MCF-7, and human endometrial specimens (Fig. 4A). All three of the samples contained the 11.4III and IV transcripts. Multiple human endometrial specimens from three different patients
gave the same results. The presence of bands 11.4III and IV suggests that the 60,000 dalton protein or putative C-receptor may exist in both normal and abnormal progestin target cells. Future studies are aimed at demonstrating the translation of these two transcripts in other progestin target cells. Whether the expression and regulation of this third progestin-binding protein are identical in all tissues remains to be determined.

From our mapping data, we postulate that the C-receptor would have only the second zinc finger of the DNA-binding domain (exon 3), would contain the NLS and DD sequences, and bind progestins with high affinity (see Fig. 4B). We estimate the molecular weight to be approximately 60,000. The protein would be detectable using a C-terminal antibody. The 60,000 dalton protein recognized by C-262 fits several of these criteria: (a) it is present in the cytosolic extracts of untreated cells and in the nuclear compartment of hormone-treated cells and, thus, has similar characteristics to those of the previously identified A- and B-proteins; (b) the \textit{in situ} photoaffinity data suggest that the 60,000 dalton protein binds $[^3H]\text{R}5020$ which is displaceable by progestins and the antiprogestin RU486; (c) it is of the approximate size of a protein initiating translation at MET 595 and is not found in PR-negative cells; (d) this protein is detectable with an anti-PR antibody specific to the last 14 amino acids of PR and not recognized by antibodies to PR directed at amino-terminal epitopes; and (e) the existence of another PR protein form is supported by the discovery of several variant estrogen receptors present in breast tumor tissue as well as established mammary carcinoma cell lines (2-9). One such variant ER is also found in both select breast cancer specimens and in normal human endometrial samples (6). The discovery of a progestosterone receptor isoform which does not bind DNA, but dimerizes with the two other PR forms, could antagonize the biological activity of B- and A-receptors. This potentially could have important implications in the design of endocrine therapies against breast cancer, especially progestin/antiprogestin treatment. Future studies are aimed at further characterization of C-receptors and their possible role in progestin-mediated effects on breast cancer.

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