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IMMUNOHISTOCHEMICAL DETECTION OF HUMAN ESTROGEN RECEPTOR WITH REGION D-SPECIFIC ANTIPEPTIDE ANTIBODIES

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Summary—To study the possibility of using antipeptide antibodies for the immunohistochemical determination of human estrogen receptors (ER), three peptides corresponding to the putative major antigenic regions of the human ER (Met^{12}–Leu^{26}, or ERP1; Thr^{227}–Gln^{267}, or ERP2; Leu^{256}–Gly^{275}, or ERP3) were used to produce site-specific rabbit polyclonal antipeptide antisera. High titer antibodies were obtained against all the peptides used, as judged by time-resolved fluoroimmunoassay. The antibodies against region D (ERP3) specifically immunoprecipitated the ER proteins *in vitro*, as did the antiERP2 antibodies to a much smaller extent. With one of the region D-specific antibodies (antiERP3 Ab2) ER could also be immunohistochemically detected. When benign and malignant human breast and normal endometrial tissues were used, the immunohistochemical staining observed with these antipeptide antibodies correlated well with the staining obtained with an established method. Thus, the results reported here show that this part of region D in ER is a potential antigenic epitope for the production of site-specific antibodies against ER. Antipeptide antibodies produced against this region can be used to immunolocalize the ER in various normal and pathological human tissues.

INTRODUCTION

The estrogen receptor (ER) belongs to a superfamily of related nuclear proteins that includes the receptors for other classes of steroid hormones, thyroid hormone, vitamin D and retinoids, in addition to a number of proteins presenting sequence homology, but whose ligands are as yet unidentified ([1, 2] and refs therein). Clinically, the most important application for the assay of ER is to identify patients who may benefit from endocrine therapy. In addition, the ER status may provide prognostic information [3].

Recently, cDNAs for all known steroid hormone receptors have been isolated and sequenced ([1, 2] and refs therein). On the basis of the deduced amino acid sequences, together with various mutation analyses, the receptor proteins can be divided into six regions [4], and have been shown to contain a number of functionally independent domains [5–8]. The ligand-binding domain of ER comprises about 300 residues at the carboxy terminus of the protein. The DNA-binding domain has been shown to be located between amino acid residues 180–262 of the molecule [4, 9]. Between the ligand- and DNA-binding domains of the steroid hormone receptors, there is a short region of about 40 amino acids (region D), which has been suggested to function as a hinge facilitating the folding of the ligand-binding domain onto the DNA-binding region [6].

The availability of the amino acid sequences of the steroid receptors has provided the possibility of using synthetic peptides as immunogens for the production of receptor-specific antibodies. We report here the production and characterization of monospecific polyclonal rabbit antibodies to synthetic peptides representing different regions of the human ER. Region D-specific antipeptide antibodies were employed for the immunohistochemical localization of ER in human breast and endometrial tissues.

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EXPERIMENTAL

Chemicals and reagents

[2,4,6,7-^{3}H]estradiol (85 Ci/mmol) and 16α-iodo-[^{125}I]3,17β-estradiol (2200 Ci/mmol) were

purchased from Amersham Int. plc. (Amersham, Bucks., England) and from Du Pont NEN (Boston, MA), respectively. 17β-Estradiol was obtained from Steraloids Inc. (Wilton, NH). Glutaraldehyde was from TAAB Labs (Berks., England), carbodiimide and *N*-(gamma-maleimidobutyryloxy)-succinimide were from Boehringer (Mannheim, Germany), glycerol from BDH Chemicals (Poole, England), Norit A-charcoal (AMEND, Irvingstone, NJ) and scintillation liquid from Lumac (Landgraaf, Netherlands). Freund's complete and incomplete adjuvants were from Difco Labs (Detroit, MI). Goat antirabbit antibody was obtained from Scantibodies (San Diego, CA), europium-labeled antirabbit antibody from Wallac Oy (Turku, Finland), Biotin-labeled goat antirabbit antibody and FITC-labeled Streptavidin from Dakopatts (Copenhagen, Denmark), and DEAE-Sepharose CL 6B and Dextran T70 from Pharmacia (Uppsala, Sweden). Other reagents were either from Sigma Chemical Co. (St Louis, MO), or Merck A.G. (Darmstadt, Germany) and were of the highest purity grades available.

Synthetic peptides

The synthetic peptide corresponding to amino acids Met^{12}–Leu^{26} (ERP1) of the human ER was purchased from Cambridge Research Biochemicals (Cambridge, England). Peptides corresponding to amino acids Thr^{227}–Gln^{267} (ERP2) and Leu^{256}–Gly^{275} (ERP3) of the ER with an additional cysteine at the amino terminus of the peptides, were purchased from Boots Peptides (Nottingham, England).

Coupling of the peptides to the carrier protein

The peptides were coupled to thyroglobulin with carbodiimide (ERP1 Ab1), *N*-(gamma-maleimidobutyryloxy)-succinimide (ERP2 Ab1, ERP3 Ab1), or with glutaraldehyde (ERP1 Ab2, ERP2 Ab2, ERP3 Ab2), employing a 1000:1 molar ratio of peptide and thyroglobulin. The efficiencies of the coupling reactions were tested by adding radiolabeled peptide to the reaction mixture (10,000 cpm).

Carbodiimide. 1.5 nmol thyroglobulin, 1.5 μmol peptide and 13.5 μmol carbodiimide were dissolved in 10 mmol/l K-phosphate buffer, pH 6.0. The reaction was incubated overnight at +4°C and free peptides were removed by dialysis against 10 mmol/l K-phosphate buffer, pH 6.0. The dialyzed conjugate was used for antibody production.

Glutaraldehyde. 1.5 nmol thyroglobulin and 1.5 μmol peptide were dissolved in 600 μl 0.1 mol/l K-phosphate buffer, pH 7.0. 400 μl 0.25% glutaraldehyde in 0.1 mol/l K-phosphate buffer, pH 7.0 was added and the mixture was incubated at room temperature for 2 days. The soluble conjugate produced with the peptides ERP1 and 3 was dialyzed against 0.1 mol/l K-phosphate buffer, pH 7.0 and used for immunization. The thyroglobulin–ERP2 peptide complex was partially insoluble. The insoluble complex was centrifuged (at 4000 *g*, for 15 min) and the soluble conjugate was dialyzed as indicated above. After dialysis the insoluble and soluble conjugates were mixed for immunization.

N-(gamma-maleimidobutyryloxy)-succinimide (GMBS). 1.5 nmol thyroglobulin was dissolved in 460 μl 10 mmol/l K-phosphate buffer, pH 7.5. GMBS (1.5 μmol) dissolved in 40 μl tetrahydrofuran was mixed with the thyroglobulin and the reaction was carried out for 30 min at room temperature with mixing. The reaction mixture was applied to a PD-10 column (Pharmacia), equilibrated with 10 mmol/l K-phosphate buffer, pH 6.0. The sample was eluted from the column with the same buffer and 1 ml fractions were collected. The fraction having the highest concentration of maleimido groups (measured at 300 nm) was incubated with 1.5 μmol of peptide at room temperature for 1 h. The insoluble conjugate was centrifuged (at 4000 *g*, for 15 min) and resuspended in 0.9% NaCl for immunization.

Immunization and screening

New Zealand white rabbits were used to raise antibodies against the peptide conjugates. The antigen was subcutaneously injected to the animals at 5 separate sites in the back (200 μl each). About 400 μg of conjugate was emulsified with an equal volume of Freund's complete adjuvant for the initial immunization of each animal. Three subsequent booster injections of 400 μg of antigen in equal volume of incomplete adjuvant were administered at 2-week intervals, after which the interval between consecutive injections was 4 weeks. Blood was collected from the ear vein 10 days after each injection, and serum was separated by low speed centrifugation and stored at −20°C.

The reactivity of each antiserum towards the immunogen was tested by time-resolved fluoroimmunoassay (TR-FIA). 96-Well plates were coated with the corresponding peptide

(1–2 μg/ml, 200 μl/well) in 10 mmol/l K-phosphate buffer, pH 7.5 containing 0.1% glutaraldehyde. After 3 h incubation at room temperature, the plates were washed four times with 10 mmol/l K-phosphate buffer and saturated overnight at room temperature with 50 mmol/l Tris–0.9% NaCl–0.5% bovine serum albumin (BSA)–5% sucrose, pH 7.5, washed with 0.9% NaCl and twice more with H_2O, dried and stored at +4°C until use.

The plates were used for the screening of the antibodies against the corresponding peptides, and all the steps were done at room temperature. 200 μl of the antisera diluted in TR-FIA buffer (Wallac) were applied to the wells and incubated with shaking for 2.5 h. After washing the wells 5 times with washing solution (Wallac) 200 μl europium-labeled antirabbit antibody (diluted 1:150, Wallac) in TR-FIA buffer was added and the plates were further incubated for 2 h at room temperature, with shaking. After washing the wells 5 times with washing solution, 200 μl of enhancement solution (Wallac) was added to each well, the plates was incubated for 10 min, after which the plates were analyzed with an Arcus Fluorometer model 1230 (Wallac).

Immunoprecipitation of the estradiol-labeled ER

ER was partially purified (5- to 10-fold) from the 9000 *g* fraction of the human myometrium tissue homogenate. Immediately after surgical removal, the tissue was homogenized in 10 mmol/l K_2HPO_4, pH 7.5, 1 mmol/l EGTA, 12 mmol/l monothioglycerol, 10 mmol/l Na_2MoO_4, 10% glycerol (buffer A), and the supernatant was applied to a DEAE-Sepharose column. The ER-containing fractions were eluted with a 0–1 mol/l KCl gradient. The receptor eluted with 200 mmol/l KCl concentration. The receptor-containing fractions were pooled and dialyzed against buffer (A) and the sample was then labeled with 5 nmol/l [^{3}H]estradiol for 1 h at room temperature, and the protein-bound and free steroids were separated by suspending the sample with dextran-coated charcoal. After incubation for 10 min at +4°C, the charcoal was removed by centrifugation and the supernatant was used as the labeled ER sample.

One hundred microliters of labeled [^{3}H]estradiol–ER complex and 100 μl of different dilutions (1:20, 1:40, 1:80, 1:160, 1:320, 1:640) of the antiserum in PBS–0.1% gelatin, pH 7.4, were incubated overnight at +4°C. 50 μl of antirabbit antiserum were then added to the mixture and further incubated for 7 h at +4°C. The mixture was then centrifuged at 6000 *g*, for 30 min, the supernatants were aspirated, the pellet was suspended in 4 ml of scintillation liquid and its radioactivity was measured with a scintillation counter (Ultra Beta, LKB Wallac).

For the determination of the specificity of the immunoprecipitation of the ER, different quantities of ERP3 peptide (0–600 pmol/ml) were added to the reaction mixture together with the ERP3 antibodies (1:20 dilution) and the [^{3}H]estradiol-labeled ER. Immunoprecipitation was then carried out as described above.

The affinity of the ERP3 Ab2 antibody towards the receptor was calculated from a Scatchard type analysis. The receptor was labeled as described above with 16α-iodo-[^{125}I]3,17β-estradiol, a ligand with a high specific activity. Different dilutions of the labeled receptor (100 μl) were incubated with the antibody (100 μl, 1:40 final dilution in buffer A–0.1% BSA) overnight, at +4°C. The samples were then diluted to 3 ml with buffer A–0.1% BSA and the immunocomplexes were bound to 200 μl Protein A–Sepharose with rotation at +4°C for 4 h. After washing the matrix two times with buffer A–0.1% BSA, the matrix-bound receptor–antibody complexes were counted in a gamma-counter. The method of Scatchard [10] was used to calculate the binding data, corrected for non-specific binding.

Immunohistochemical localization of human ER

Ten breast lesion specimens were obtained from patients under surgical operation. Fifteen endometrial tissue samples of regularly cycling volunteer women operated for tubal sterilization were obtained by Strich curettage on different days of their cycle. The day of the cycle was estimated from the latest menstrual period and confirmed by the characteristic morphological features of the endometrium during the cycle [11].

Frozen 4–6 μm sections were fixed in 4% formaldehyde for 12 min and, after washing with PBS two times for 5 min each, they were further fixed with methanol (4 min, −20°C) and acetone (2 min, −20°C). The sections were washed with PBS three times, for 5 min each, and stained in a moisture chamber at room temperature as follows: non-specific binding of the antibodies was blocked by incubating the slides in 1% BSA–PBS for 30 min, the 1:100 dilution of antipeptide antiserum in 1%

BSA–PBS was added and the slides were further incubated for 2 h. After washing the slides with PBS three times, for 5 min each, a 1:50 dilution of biotin-labeled antirabbit antibody was added and the slides were incubated for 2 h, washed again with PBS three times for 5 min each, and then incubated for 1 h in a 1:50 dilution of streptavidin-conjugated FITC in 1% BSA–PBS. The slides were then washed five times with PBS, for 5 min each, mounted with 5% (w/v) *n*-propylgallate-glycerol, pH 7–8, and stored at +4°C. For the control slides, the immunoreactivity of the antiserum was blocked by preincubation of the ERP3 Ab2 antibody (dilution 1:100) with peptide (1 mg/ml) overnight, after which the antiserum was used for the immunohistochemical localization of ER as described above.

The receptors were also stained using a commercial kit supplied by Abbot Labs (North Chicago, IL) according to the manufacturer's instructions.

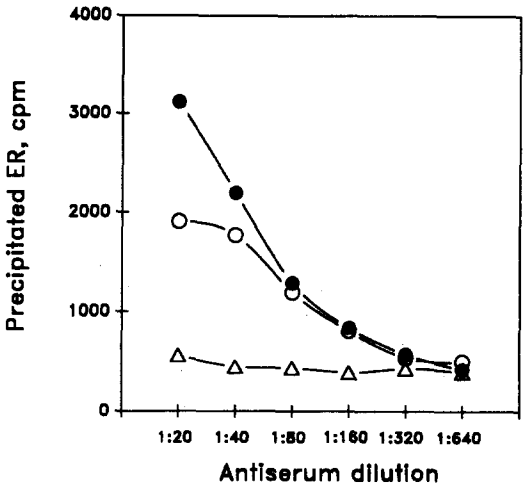

Fig. 2. Immunoprecipitation of [^{3}H]estradiol-labeled human ER. Different dilutions of antipeptide antiserum prepared against region D with GMBS (ERP3 Ab1, ○), glutaraldehyde (ERP3 Ab2, ●), or preimmune antiserum (△) were incubated with [^{3}H]estradiol-labeled human ER. The antibody–antigen complexes were then precipitated with antirabbit antibody and the radioactivity of the pellet was measured by scintillation counting.

RESULTS

High-titer polyclonal antibodies towards three peptides corresponding to sequences of the human ER (Met^{12}–Leu^{26}, ERP1; Thr^{227}–Gln^{267} ERP2; Leu^{256}–Gly^{275}, ERP3), were raised in rabbits. The antisera were screened by TR-FIA and all the antisera exhibited binding with the corresponding peptide, coated to microtiter wells, at dilutions of 1:10,000 to 1:20,000 (Fig. 1). The most antigenic peptide was ERP3, corresponding to part of region D of the ER. All the antibodies were further tested against soluble [^{3}H]estradiol-labeled ER. Using antirabbit antibody precipitation of the antipeptide antibody–ER complex, both region D-specific antisera (ERP3 Ab1 and ERP3 Ab2) recognized the native receptor at dilutions of up to 1:200 (Fig. 2). The binding of the ERP3 Ab1 and

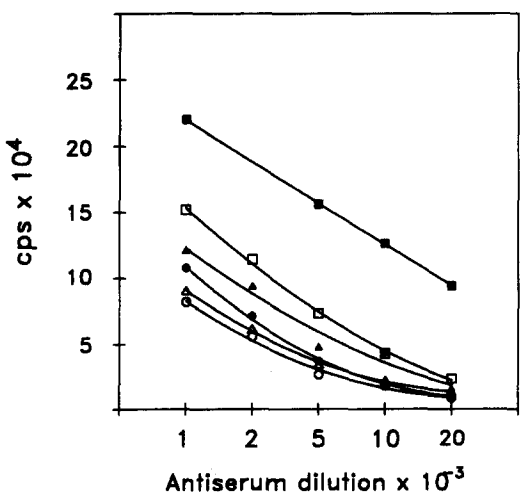

Fig. 1. TR-FIA assay of antisera prepared against synthetic peptides derived from putative immunogenic regions of the human ER. Synthetic peptides were produced and used for the production of antisera as described in the text. The reactivity of each antisera was tested against the peptide used as immunogen. (○) ERP1 Ab1, (●) ERP1 Ab2, (△) ERP2 Ab1, (▲) ERP2 Ab2, (□) ERP3 Ab1, (■) ERP3 Ab2.

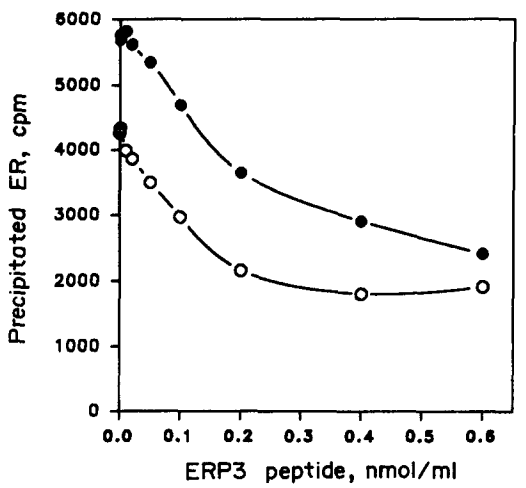

Fig. 3. Specificity of the immunoprecipitation of [^{3}H]estradiol-labeled human ER with antiERP3 antibody. The labeled ER samples were incubated with a 1:20 dilution of antiERP3 antibodies (ERP3 Ab1, ○; ERP3 Ab2, ●) and increasing concentrations (0–0.6 nmol/ml) of the ERP3 peptide. The antibody–antigen complexes were precipitated by antirabbit antibody and the radioactivity of the pellet was measured by scintillation counting.

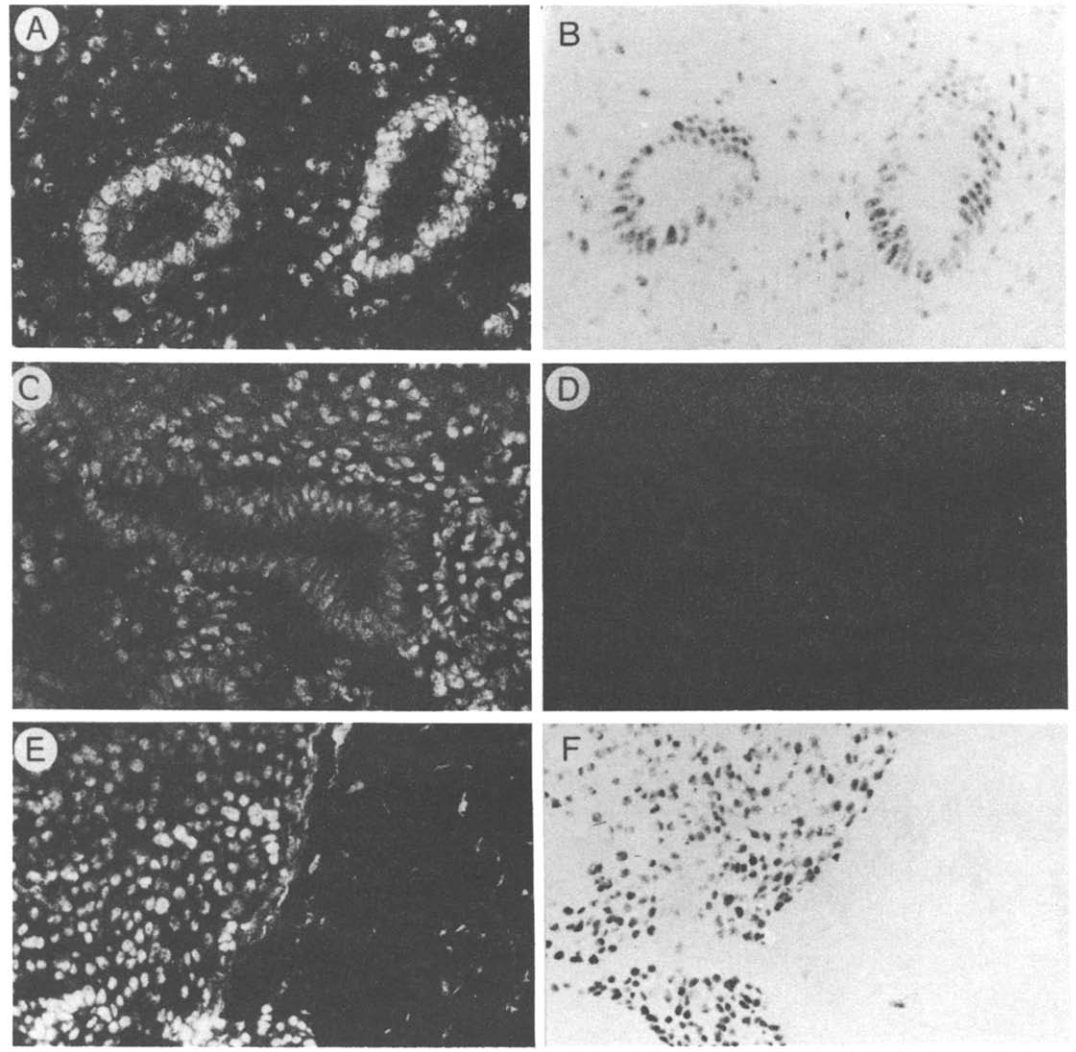

Fig. 4. Comparative staining of frozen sections (×250) showing the immunohistochemical detection of the human ER with antipeptide antibodies (ERP3 Ab2) raised against region D (A, C, D, E), and with the established method from Abbot Labs (B, F). A–D, normal human endometrium. A and B, during the early proliferative phase, there is strong nuclear staining in most of the epithelial and stromal cells. C, during the secretory phase, the staining is disappearing from the epithelial cells, while the stromal cells still stain strongly. D, control for A. The antibody was preincubated with 1 mg/ml of the corresponding peptide, which completely abolished the staining. E and F, breast carcinoma sample where ER is expressed in epithelial tumour cells.

ERP3 Ab2 antibodies did not inhibit the steroid-binding of the receptor, as measured by a Scatchard-type analysis after preincubating the receptor sample with the antipeptide antibodies. A Scatchard-type analysis was also used to calculate the affinity of the ERP3 Ab2 antibody towards ER and the apparent K_d was 1.3×10^{-10} mol/l. The ERP2 Ab2 antibody also immunoprecipitated the native receptor, but to a much lower extent (data not shown). ERP1 antibodies did not react with the [^{3}H]estradiol–ER complex in our assay conditions. Immunoprecipitation of the [^{3}H]estradiol-labeled ER with both anti-ERP3 antibodies was decreased in a dose-dependent manner by the addition of increasing concentrations of the peptide to the reaction mixture (Fig. 3).

Frozen sections of benign and malignant human breast and normal human endometrial tissues were used to evaluate the use of the antibodies for the immunohistochemical localization of ER. In these tissues, ER could be detected immunohistochemically with ERP3 Ab2 antibodies (Fig. 4). The specific staining detected with the antipeptide antibodies could be blocked with the peptide, thus showing that the corresponding epitope of the receptor protein was responsible for the staining (Fig. 4).

The staining observed in breast and endometrial samples correlated well with the results observed with the commercial ICA kit from Abbot Labs. However, in some breast samples, some additional cells were stained with the ERP3 Ab2 antibody, which were not detected with the kit from Abbot. When normal endometrium was used, the intensity of the staining observed with both methods varied with the stage of the menstrual cycle. In the specimen taken during the early proliferative phase of the menstrual cycle, there was a strong staining of the ER in both epithelial and stromal cells (Fig. 4). During the secretory phase, the staining started to disappear from epithelial cells, while it remained relatively intense in the stromal cells (Fig. 4). During the late secretory phase, the staining intensity decreased further. With both methods, the staining was exclusively detected in the cell nuclei.

DISCUSSION

Since the primary structure of human ER has been determined [12] it has been possible to produce site-specific antibodies against different regions of the receptor. On the basis of the hydropathic profile of the receptor, we chose three different peptides from different regions of the protein as possible epitopes for the production of polyclonal rabbit antiER antibodies (Met^{12}–Leu^{26}, ERP1; Thr^{227}–Gln^{267}, ERP2 and Leu^{256}–Gly^{275}, ERP3).

The results reported here show that, of the three peptides studied, the peptide correponding to region D (Leu^{256}–Gly^{275}, ERP3) was the most antigenic. The antibodies produced against this peptide also recognized the corresponding native protein, as evaluated by immunoprecipitation and immunohistochemistry. The data show that this peptide sequence is located at the surface of the protein and can be used to immunorecognize the native protein. This is further supported by the findings of a recent study, in which antipeptide antibodies were produced against region-D of the rat ER [13]. The antibodies raised against a peptide sequence corresponding to amino acids 265–280 in the human sequence, were shown to recognize the native and denaturated rat ER. In addition, the immunogenicity of the hinge region has been shown to be a common feature of different steroid receptors, since antibodies against region D of the chicken progesterone receptor [14], as well as against the human mineralocorticoid receptor [15], also recognize the corresponding receptor protein. Thus, the highly hydrophilic [12] and flexible [5] region D, which is rich in turn and coil structures, is most likely one of the strong immunological epitopes of the steroid receptor family.

The immunohistochemical staining obtained with the ERP3 Ab2 antibodies was exclusively located in the cell nuclei, which is in line with the previously reported immunohistochemical [16] and biochemical data [17]. In addition to the antibodies studied here, antipeptide antibodies to human mineralocorticoid [15] and human androgen receptor [18] have been reported to immunolocalize the corresponding native human receptor.

The staining in the human endometrium showed characteristic variations for ER during the menstrual cycle, as reported previously [19–21]. This result, together with the good correlation observed between the immunohistochemical staining with ERP3 Ab2 and the results obtained using an established method, show that the staining protein was most likely the human ER. In addition to the cells stained with the established method, in some breast samples region D-specific antipeptide antibodies also stained other cells. As no significant homology exists between ER and other steroid receptors, or known proteins, in the region used for antibody production, this could be due to the more sensitive detection system (biotin–streptavidin–FITC) employed to visualize the primary antibody, compared to the indirect peroxidase technique used in the established method. However, we can not exclude the possibility that this additional specific staining detected in some samples was due to an unknown protein having an identical immunological epitope. The results presented here demonstrate the possibility of using the peptide sequence Leu^{256}–Gly^{275} of the ER to produce antipeptide antibodies that can be employed for the immunohistochemical detection of ER in human tissues.

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