Novel monoclonal antibodies against human uterine progesterone receptor

Mapping of receptor immunogenic domains

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Progesterone receptor was purified in a single step from human uteri using immunoaffinity chromatography with monoclonal antibodies raised against the rabbit receptor. A total of 39 monoclonal antibodies were prepared against the human receptor and characterized. Immunoblot experiments using crude uterine cytosol or purified receptor showed that the antibodies belonged to three groups: they recognized either a single receptor species (apparent molecular mass 110 kDa), two species (110 and 79 kDa) or three species (110, 79 and 65 kDa). The species specificity of the antibodies was very variable; some recognized only the human receptor, others interacted with several mammalian receptors (rabbit, guinea pig and rat), while a single one also cross-reacted with the chick receptor. The epitopes recognized by 15 of the antibodies showing the strongest affinity for the human receptor were mapped using a method recently described [Lorenzo, Jolivet & Milgrom (1988) *Eur. J. Biochem.* **176**, 53–60] which involves immunoprecipitation of C-terminally truncated proteins obtained by transcription and translation of cloned cDNA *in vitro*. The antibodies recognized five different regions of the receptor, all localized on the N-terminal half of the protein. None of the antibodies interacted with an epitope present in the DNA-binding or steroid-binding regions of the receptor. Comparison of the pattern of receptor species recognized by the antibodies and the localization of their epitopes showed that the 79 and 65 kDa receptor species were derived from the 110 kDa form by deletion of its N-terminal part. The N-terminus of the 79 kDa species was found to lie between amino acids 121 and 208, and that of the 65 kDa species between amino acids 208 and 296.

INTRODUCTION

Initial purification of rabbit progesterone receptor allowed first the preparation of polyclonal [1] and then monoclonal [2] antibodies. These antibodies crossreacted with varying affinities with receptors from most mammalian species and especially from humans. Some have thus been used for immunocytochemical [3] and immunochemical [4] studies of human receptors in various tissues. More recently, some monoclonal antibodies have been prepared against chicken [5], and human [6–8] progesterone receptors. However, it appeared interesting to prepare a large panel of monoclonals against the human receptor in order to define and study its immunogenic domains. This analysis has been made possible by the cloning and sequencing of the human progesterone receptor gene [9] and by the description of a simple method of epitope mapping [10]. Moreover, it is known that each monoclonal antibody displays different properties in terms of affinity for the antigen when the latter is soluble, or fixed in histological preparations or on membranes for immunoblotting. The availability of a large number of monoclonals thus allows the antibody having the optimal characteristics for each specific use to be selected.

EXPERIMENTAL

Immunoaffinity purification of progesterone receptor

Fragments of human uteri were obtained from hysterectomies. They were pooled and kept in liquid N₂ until use. The tissue was homogenized with a Waring Blender (Bioblock, Illkirch, France) in 10 mM-Tris/50 mM-NaCl buffer, pH 8 (2.5 g/ml of buffer). The buffer contained the following proteolysis inhibitors: pepstatin (1 µg/ml, Sigma), phenylmethanesulphonyl fluoride (1 mM, Sigma), leupeptin (0.1 mM, Sigma), bacitracin (0.1 mg/ml, Sigma) and aprotinin (36.5 µg/ml, Biosys., Compiègne, France). Cytosol obtained by centrifugation for 90 min at 0 °C and 105 000 g was incubated for 2 h at 0 °C with 20 nM-[³H]ORG.2058 (16α-ethyl-21-hydroxy-19-nor[6,7-³H]pregn-4-ene-3,20-dione) [specific activity (2.5–4.0) × 10⁸ Ci/mol; Amersham International, Bucks., U.K.].

Purified monoclonal antibody Mo482, raised against rabbit receptor but cross-reacting with human receptor, was linked to Affigel-10 (15 mg of antibody/ml of gel) as described in the Bio-Rad (Richmond, CA, U.S.A.) instruction bulletin.

During the immunopurification procedure, uterine cytosol (500–700 ml, flow 24–30 ml/h) was successively

Abbreviation used: ORG.2058, 16α-ethyl-21-hydroxy-19-nor[6,7-³H]pregn-4-ene-3,20-dione.

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passed on three columns to decrease non-specific binding to the immunomatrix (20 ml of Protein A-Sepharose CL-4B; 30 ml of Affigel-10; 15 ml of Affigel-10-IDA10, a non-receptor-related antibody [11]). The steroid-receptor complexes were retained on Affigel-10-Mo482 immunomatrix (4 columns of 1 ml each). After extensive washing performed as previously described [12], the receptor-steroid complexes were eluted in 50 mM-ammonium carbonate buffer, pH 10.8 [12].

Assay of steroid-receptor complexes

This was performed with dextran-coated charcoal [13] or by the hydroxyapatite method [14]. The latter was only used when protein concentration was < 1 mg/ml.

Protein concentration

This was measured by the Amido Schwartz method [15].

Preparation of monoclonal antibodies

Two BALB/c mice (Pasteur Institute, Paris, France) were immunized. The first mouse received, successively, 900 pmol of receptor subcutaneously in complete Freund's adjuvant; a second subcutaneous injection of 900 pmol in incomplete Freund's adjuvant 2 weeks later; and the final boost of 600 pmol intravenously and of 600 pmol intraperitoneally after a further week. The second mouse received successively 600 pmol of receptor subcutaneously in complete Freund's adjuvant; a second subcutaneous injection of 500 pmol in incomplete Freund's adjuvant 2 weeks later; and two subcutaneous injections of 900 pmol and 200 pmol 3 and 4 months later respectively (in incomplete Freund's adjuvant). The last boost was of 800 pmol, equally divided between intraperitoneal and intravenous routes.

For both mice, the fusion was performed 3 days after the last injection. The procedures used for preparation of hybridomas, selection by e.l.i.s.a. test using partially-purified receptor, and immunoprecipitation of [³H]-progesterone-receptor-antibody complexes with anti-(mouse immunoglobulin) antisera have been described in [2].

Electrophoresis and immunoblot of receptor

Cytosol (containing 200 fmol of receptor) or purified receptor (200 fmol) were electrophoresed [16] on 9% polyacrylamide gels. The procedure used for Western blots has previously been described [12]; anti-receptor monoclonal antibodies were used at a concentration of 5 µg/ml.

Study of species-specificity of anti-receptor antibodies

Cytosol was prepared from uteri or oviducts of rabbits, rats, guinea pigs and chickens. After incubation with [³H]ORG.2058 (see above), an aliquot (500 fmol of steroid-receptor complexes) was incubated with 10 µg of anti-receptor antibodies overnight at 0 °C. A second antibody was used to precipitate the complexes as previously described [2]. Immunoprecipitation was considered as positive when at least 3-fold more counts were precipitated than with control non-receptor-related antibodies.

Density gradient ultracentrifugation

Uterine cytosol was incubated for 2 h at 0 °C with 3 nM-[³H]ORG.2058 (specific activity 46×10^3 Ci/mol). An aliquot (200 µl) was incubated for 4 h at 0 °C with

550 µg of monoclonal antibody (50 µl), then 200 µl of the latter incubation mixture was centrifuged in a SW50.1 rotor for 20 h at 105000 g and at 0 °C on a 5–20% sucrose gradient containing 0.3 M-KCl.

Immunocytochemical staining

Staining of breast cancer biopsies was performed as previously described [3].

Epitope mapping by transcription and translation of cloned cDNA *in vitro*

A fragment of 3750 nucleotides of cloned human progesterone receptor cDNA (extending from nucleotide -175 to nucleotide +2977, nucleotide +1 being the beginning of the coding sequence) was inserted into the *Eco*RI site of vector pGEM4 (Promega Biotec, Madison, WI, U.S.A.).

The DNA of the resulting vector was either used intact or after restriction with *Xba*I (site in polylinker following the insert), *Hinc*II (site at nucleotide 1367), *Mlu*I (site at nucleotide 1115), *Bal*I (site at nucleotide 887), *Apa*I (site at nucleotide 624) and *Sac*I (site at nucleotide 363). Transcription *in vitro* was performed according to the instruction bulletin of Promega Biotec. Translation of the resulting mRNA was done in a reticulocyte lysate system in presence of L-[4,5-³H]leucine (specific activity 177 Ci/mmol; Amersham International). The proteins obtained by cell-free translation were immunoprecipitated with anti-receptor monoclonal antibodies, electrophoresed and submitted to autoradiography in the presence of EN³HANCE (New England Nuclear).

RESULTS

Immunopurification of the human uterine progesterone receptor

An immunomatrix was prepared using antibody Mo482. This monoclonal antibody has been raised against the rabbit progesterone receptor but has been shown to have a high affinity for the human receptor [12] (A. Jolivet, unpublished work). Uterine cytosol incubated with the tritiated progesterone ORG.2058 was chromatographed through the column and receptor-steroid complexes were eluted at alkaline pH.

The specific activity of the starting material (steroid-receptor complexes in uterine cytosol) varied between 0.3 and 0.6 pmol of receptor/mg of protein in the various experiments. After elution the specific activity was about 1800–3000 pmol of receptor/mg of protein and the receptor yield was 20–25%. Polyacrylamide-gel electrophoresis in the presence of SDS showed two major bands of molecular mass of about 110 and 79 kDa, and a minor band at 65 kDa (Fig. 1). Immunoblotting with a polyclonal antibody prepared against highly-purified rabbit 110 kDa receptor [4] showed that all three bands reacted with the antibody and were thus probably different species of the human receptor molecule. This was later confirmed with monoclonals raised against the human receptor (see Fig. 4). The relative proportions of the bands varied in different experiments.

Preparation and characterization of monoclonal antibodies against the human uterine progesterone receptor

Purified receptor preparations were used to immunize mice. Hybridomas were prepared and screened as de-

Fig. 1. Immunoaffinity-purified human uterine progesterone receptor

Purified receptor (0.4 pmol/lane) was electrophoresed on three lanes of a 9% polyacrylamide gel [16]. Lane 1 was silver-stained. Lanes 2 and 3 were used for immunoblot with a specific anti-(rabbit progesterone receptor) polyclonal antibody prepared in goat [4] and the corresponding preimmune serum respectively.

scribed in the Experimental section. In two separate fusions, 39 monoclonal anti-(human progesterone receptor) antibodies were obtained (Table 1). Immunoprecipitation of [3 H]progesterin-receptor complexes from different species showed great variety. Many of the antibodies only recognized human receptor but not rabbit, rat, guinea pig or chicken progesterone receptors. Several others cross-reacted with receptor from the different mammals but only one recognized the receptors from all species which were tested, including chicken.

Immunoblot experiments were performed either with purified receptor or with crude uterine cytosol. All antibodies labelled the purified receptor, but some of them failed to detect the receptor in crude cytosol showing that they did not display the very high affinity necessary to interact with the rare receptor protein (Table 1).

The number of protein bands detected by the antibodies with purified receptor or crude cytosol varied between one and three (Fig. 2). Some antibodies recognized only a 110 kDa protein; others interacted in addition with a 79 kDa protein and a third group also recognized a 65 kDa form. The heterogeneity of antibodies in terms of the receptor species with which they

Fig. 2. Monoclonal antibodies' recognition by immunoblot of the 110 kDa (lane 2), the 110 and 79 kDa (lane 3), or the 110, 79 and 65 kDa (lane 4) species of human progesterone receptor

Purified progesterone receptor was submitted to immunoblot analysis (see Experimental section) with IDA 10 (non-receptor-related) (lane 1), Li689 (lane 2), Li506 (lane 3) and Li353 (lane 4) antibodies.

Fig. 3. Changes in sedimentation patterns of [3 H]progesterin-receptor complexes incubated with monoclonal antibodies recognizing various species of receptor

Human uterine cytosol was incubated with [3 H]ORG.2058 (see Experimental section). A 200 μ l aliquot was incubated for 4 h at 0 $^{\circ}$ C with 50 μ l of antibodies (11 mg of protein/ml); 200 μ l were centrifuged for 20 h at 0 $^{\circ}$ C and at 105000 g in a SW50.1 rotor on a sucrose gradient containing 0.3 M-KCl. The following antibodies were used: a, antibody IDA10 [11] (unrelated to receptor); b, antibody Li367 which only interacts with the 110 kDa species of receptor; c, antibody Li386 which interacts with all three receptor species (110, 79 and 65 kDa).

Table 1. Characteristics of monoclonal antibodies raised against the human progesterone receptor

The table shows 32 monoclonal antibodies obtained by two fusions. The number in square brackets indicates the number of receptor bands detected by immunoblot of immunopurified receptor: [1], one band (110 kDa); [2], two bands (110 and 79 kDa); [3], three bands (110, 79 and 65 kDa). ND, not done.

Antibody	Class	Species crossreaction (immunoprecipitation)*				Western blotting of:		Immuno- cytochemical studies	Epitope no. of amino acid
		Rabbit	Rat	Guinea pig	Chicken	Immuno- purified receptor	Cytosol receptor†		
Li10	IgG ₁	+	—	—	—	+ [2]	—	ND	ND
Li42	IgG ₁	+	—	—	—	+ [2]	+ / —	ND	121–208
Li45	IgG ₁	+	—	—	—	+ [2]	—	ND	ND
Li77	IgG _{2b}	—	—	—	—	ND	ND	ND	ND
Li152	IgG ₁	+	+	—	—	+ [2]	+ / —	ND	ND
Li169	IgG ₁	+	+	+	+	+ [1]	+ / —	+	1–121
Li199	IgG ₁	—	—	—	—	+ [3]	—	ND	371–455
Li216	IgG ₁	—	—	—	—	+ [3]	—	+ / —	371–455
Li226	IgG ₁	—	—	—	—	+ [2]	+ / —	ND	ND
Li317	IgG ₁	+	+	—	—	+ [2]	+ / —	+	ND
Li353	IgG ₁	—	—	—	—	+ [3]	—	ND	208–296
Li367	IgG ₁	—	—	+	—	+ [1]	+	ND	1–121
Li386	IgG ₁	+	—	—	—	+ [3]	—	+	208–296
Li417	IgG ₁	—	—	—	—	+ [2]	+	+	208–296
Li506	IgG ₁	—	—	—	—	+ [2]	+ / —	ND	121–208
Li523	IgG ₁	+	+	—	—	+ [2]	+	+	121–208
Li533	IgG ₁	+	+	—	—	+ [2]	+	+	208–296
Li689	IgG ₁	—	—	—	—	+ [1]	—	ND	1–121
Li826	IgG ₁	+	—	—	—	+ [2]	+ / —	ND	121–208
Lu17	IgG ₁	+	ND	ND	ND	+ [2]	—	ND	ND
Lu63	IgG ₁	+	ND	ND	ND	+ [1]	—	ND	ND
Lu85	IgG ₁	+	ND	ND	ND	+ [1]	—	ND	ND
Lu96	IgM	+	ND	ND	ND	+ [3]	—	ND	ND
Lu158	IgM	+	ND	ND	ND	+ [3]	—	ND	ND
Lu189	IgM	+	ND	ND	ND	+ [3]	—	ND	ND
Lu194	IgG ₁	+	ND	ND	ND	+ [1]	—	ND	ND
Lu251	IgM	+	ND	ND	ND	+ [3]	—	+ / —	ND
Lu265	IgM	+	ND	ND	ND	+ [3]	—	ND	ND
Lu282	IgG ₁	+	ND	ND	ND	+ [3]	—	+ / —	296–371
Lu290	IgG _{2a}	+	ND	ND	ND	+ [1]	—	+	ND
Lu406	IgG ₁	+	ND	ND	ND	+ [1]	—	ND	ND
Lu415	IgG _{2a}	+	ND	ND	ND	+ [1]	—	ND	ND

* See description in Experimental section.

† The intensity of the receptor bands was arbitrarily evaluated as: +, strong labelling; + / —, visible but not very strong labelling; —, no visible receptor bands. The conditions of immunoblot were as described in [12].

interacted was confirmed by displacement studies of [³H]progesterone–receptor complexes on density gradients (Fig. 3). When uterine cytosol containing steroid–receptor complexes was incubated with excess antibodies recognizing only the 110 kDa form, only a fraction of complexes was shifted to more rapidly-sedimenting species. On the contrary, when an antibody recognizing the three forms of receptor was used, nearly all of the complexes were displaced. The result with an antibody recognizing two bands (110 and 79 kDa) was intermediate (results not shown). The proportions varied from experiment to experiment since the proportion of each receptor species changed in different preparations of cytosol. It was also observed that a strong correlation existed (except in one case) between the ability to detect the receptor by immunoblot in crude cytosol and to label it *in situ* by immunocytochemistry of breast cancer biopsies.

Mapping of the epitopes recognized by antireceptor antibodies

Fourteen of the antibodies raised against the human receptor, and two antibodies raised against the rabbit receptor which cross-reacted with human, were studied using a method previously analysed in detail [10]. Briefly, this method, which allows the study of continuous (by opposition to conformational) epitopes involves insertion of the cDNA coding for the receptor in a vector containing signals necessary for acellular transcription and translation. This allows synthesis, totally *in vitro*, of radioactive receptor and study of its immunoprecipitation. The cDNA inserted in the vector is then cut at various sites with restriction enzymes. After transcription and translation *in vitro* this yields receptor truncated to various extents in its C-terminal region. The truncated receptor is immunoprecipitated until the sequence encoding the epitope recognized by the monoclonal antibody

Fig. 4. Mapping of the epitopes recognized by the monoclonal antibodies: immunoprecipitation of C-terminally truncated receptor

Receptor cDNA in vector pGEM4 was restricted with enzymes *HincII* (amino acid 455), *MluI* (amino acid 371), *BamI* (amino acid 296), *ApaI* (amino acid 208), *SmaI* (amino acid 121). The resulting cDNAs were transcribed and translated *in vitro* (see Experimental section). The proteins were immunoprecipitated with antibodies Li367 (a), Li42 (b) and Lu282 (c). The immunoprecipitated proteins were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography.

Fig. 5. Mapping epitopes recognized by monoclonal antibodies against the human progesterone receptor

Map of the epitopes recognized by the monoclonal antibodies. The legend to Fig. 4 describes the methodology used to determine this map.

has been deleted from the gene. For instance, in Fig. 4(b), antibody Li42 immunoprecipitates receptor fragments containing amino acids 1–296 and 1–208 but does not immunoprecipitate the receptor fragment containing amino acids 1–121. Its epitope must, therefore, be localized between amino acids 121 and 208. Antibodies Li367 and Lu282, shown in Figs. 4(a) and 4(c), interact with epitopes situated between amino acids 1 and 121 and 296 and 371 respectively. Some shorter, usually minor bands [except in Fig. 4(c) after digestion with *HincII*] arise mainly by premature termination of transcription or translation [10].

Fig. 5 shows the localization of the different epitopes recognized by the antibodies which were tested. All of the antibodies interacted with the N-terminal half of the molecule, in which at least five different immunogenic regions could be defined. None of the antibodies recognized the DNA- or hormone-binding regions localized in the C-terminal half.

The 79 and 65 kDa species arise by N-terminal deletions of the receptor

In Western blots some monoclonal antibodies detect three species of receptor of apparent molecular masses 110, 79 and 65 kDa (see Table 1). Some others interact only with the two largest species and a last group only with the 110 kDa protein. Comparison of this classification of monoclonal antibodies with the results of the mapping of their epitopes (Fig. 5) shows that the latter group of monoclonals corresponds to the N-terminal part of the protein (amino acids 1–121). The 79 and 65 kDa species of receptor thus lack this region. Similarly, the antibodies recognizing 110 and 79 kDa forms but not the 65 kDa form recognize epitopes localized between amino acids 121 and 208. Some such antibodies recognize the region lying between amino acids 208 and 296 but other antibodies interacting with epitopes localized in this region label the three receptor species by immunoblot. Moreover, all antibodies which

interact with epitopes localized downstream (towards the C-terminus) from amino acid 296 label the three receptor species. Thus the *N*-terminal limit of the 65 kDa species must lie between amino acids 208 and 296.

DISCUSSION

We have previously devised a method for immunoaffinity purification of rabbit progesterone receptor [12]. The receptor could be recovered in its native state by using elution with high-pH buffer, conditions in which the receptor is not inactivated. This method was later applied by Estes *et al.* [6] to the purification of human receptor from T47D cells using an antibody raised by Sullivan *et al.* [5] against chick receptor. The antibody interacted only with the 110 kDa form of receptor and thus only this form was purified.

In the present study we have used an antibody which recognizes all three species of receptor and therefore allows purification with relatively high yields of large amounts of receptor from uteri obtained from hysterectomies. Hundreds of micrograms of receptor may easily be purified from this source allowing structural studies and analysis of interactions between receptor and hormone-responsive elements of genes (A. Bailly, unpublished work).

A total of 39 monoclonal antibodies was obtained (in addition to the antibodies against the rabbit receptor which strongly cross-reacted with the human receptor). They were of a variety of subclasses and exhibited varying affinities towards the receptor. Their epitopes were mapped and found to correspond to several sites, all of which were localized in the *N*-terminal half of the receptor molecule. Prediction of antigenicity suggests the existence of such sites mainly in this region although, theoretically, some of these sites should have been present in the C-terminal half. The lack of experimental antigenicity of this part of the molecule as observed in the present study may be consistent with its extremely high conservation between species. A similar observation has been made for the rabbit progesterone receptor [10]. The monoclonal antibodies raised against the rat glucocorticoid receptor were also found to interact with the protein in a region located outside the hormone- and DNA-binding domains [18]. It cannot however be excluded that the lack of immunogenicity of these regions of receptor is due to other mechanisms, e.g. differences in catabolism in mice of the various parts of the injected protein, or a genetically restricted immunological response of this specific strain of mice. The method of epitope localization which was used in these experiments is only applicable to epitopes consisting of linear and relatively short stretches of amino acids and cannot allow the study of conformational epitopes including amino acids located at a large distance in the primary structure of the protein. Studies on the additivity of binding of antibodies (F. Lorenzo & M. T. Vu Hai, unpublished work) and construction of receptor cDNA deletion mutants (H. Loosfelt, unpublished work) confirmed the epitope localization described in this manuscript and did not give evidence of the existence of antibodies recognizing such conformational epitopes.

Three species of human progesterone receptor were detected in varying proportions by immunoblot. The

larger forms (apparent molecular masses 110 and 79 kDa) were in most cases more abundant than the 65 kDa form. The monoclonal antibodies recognized either all three forms, or only two or one of them. Comparison of these characteristics and the results of epitope mapping experiments clearly showed that the 79 and 65 kDa species are derived from the 110 kDa receptor by deletion of its *N*-terminal part. We have presented evidence that this is due to artefactual proteolysis in the case of the rabbit progesterone receptor [12,19]. In the case of the human receptor this point is still debated [6].

The cDNA for the human progesterone receptor has been cloned [9]. To analyse the function of the protein, mutagenesis experiments *in vitro* are now being undertaken. The availability of monoclonal antibodies recognizing well defined immunogenic domains will be of great value in such studies.

This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Centre National de la Recherche Scientifique (CNRS), the Association pour la Recherche sur le Cancer (ARC), the Fondation pour la Recherche Médicale Française (FRMF) and the Unité d'Enseignement et de Recherche Kremlin-Bicêtre. We are grateful to Dr. La Douche (Hôpital Kremlin-Bicêtre, France), and Dr. Papiernik (Hôpital A. Beclère, France) for their help. We thank A. Ferrard for technical assistance and N. Malpoint for typing the manuscript.

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