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EPITOPE MAPPING OF THE ANTI-HUMAN PROGESTERONE RECEPTOR MONOCLONAL ANTIBODY, AB-52

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Summary—The monoclonal antibody AB-52 has high affinity and specificity for the two natural human progesterone receptor forms, receptor A (hPR_A) and receptor B (hPR_B), but it does not bind the PR of chick, mice, rats or rabbits. We have used a novel method to map its epitope. Based on a series of site-directed mutants of hPR_A, together with immunoblotting, DNA gel mobility shift and antibody super shift assays, we have mapped the epitope of AB-52 to a 17 amino acid sequence lying between Val^{221} and Leu^{237} of the 933 amino acid hPR_B protein. This N-terminal sequence is common to both hPR_B and hPR_A but is missing in chick PR and differs extensively from mouse PR. No anti-rabbit PR antibodies map to the homologous rabbit PR sequence which differs from hPR by four amino acids, suggesting that one or more of these four amino acids form a critical subset of residues in hPR that define the human specificity of AB-52. Knowledge of the AB-52 epitope is useful in structural analyses of PR, and in competition studies. Additionally, this 17 amino acid peptide whose antigenicity would not be predicted from computer analysis, should be useful for generating additional hPR specific antibodies.

INTRODUCTION

AB-52 is a monoclonal antibody (MAb) having an immunoglobulin G, isotype, that cross reacts with both natural forms of human progesterone receptors (hPR), hPR_B of 120 kDa and hPR_A of 94 kDa. It was generated by injection of mice with partially purified, intact, hPR_B [1]. The antibody has proven to be extremely useful in studies of hPR quantitation, distribution, structure, DNA binding and transcriptional regulation, since it binds with high affinity and specificity to both forms of the human receptors [2–7]. Its specificity suggests that the epitope of AB-52 lies in hPR_A at a site common to hPR_B (see Fig. 1). However, AB-52 does not bind to PR of chick, mice, rats or rabbits [8], so that its epitope must either be missing or altered in the PR of these species. Precise epitope mapping is important when antibodies are used to study the functional domains of proteins, for competition studies, and for the generation of additional antibodies. However epitope mapping by conventional microsequencing of proteolytic peptide fragments is laborious. Recently, Lorenzo *et al.* [9] described a rapid epitope mapping method for a protein whose cDNA has been cloned, by using a series of cDNA deletions and *in vitro* translations to yield a set of nested polypeptides. Immunoprecipitation then allows determination of the shortest protein recognized by the MAb, thereby defining its binding site. In a modification of this mutagenesis approach, we have identified the epitope of MAb AB-52 using transfected and expressed, site-directed mutant recombinants of hPR_A, together with immunoblotting and gel mobility shift assays. Substitution of four serine residues in one hPR_A mutant totally eliminated AB-52 binding, thus mapping the epitope to a 17 amino acid region lying between Val^{221} and Leu^{237} of the receptors. We discuss the species specificity and antigenicity of this site.

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METHODS

Site-directed mutagenesis

The cDNA hPR2 cloned into the eukaryotic expression vector pSG5 encodes hPR_A and was a gift from P. Chambon [7]. An oligonucleotide-directed site-specific mutagenesis procedure similar to that described by Inouye and Inouye [10] was employed to construct a panel

of 12 serine or cysteine substitution mutants (hPR2–M1-9; MH, M-DBD) in the N-terminus, DNA binding domain and hinge region of hPR_A. Ten mutants contained 1–4 serine to alanine substitutions in hPR_A based on the sequence of Kastner *et al.* [11]. Two M-DBD mutants were cysteine or DNA binding specificity substitution mutants. Specifically, hPR2–M2 was constructed from a 50 bp oligonucleotide:

```
221                           (Ser) (Ser)       (Ser)             (Ser)
Val  Glu  Glu  Glu  Asp  Ala   Ala  Glu   Ala  Glu  Glu   Ala  Ala  Gly  Pro  Leu  Leu
GTT  GAG  GAG  GAG  GAT  GCT   GCA  GAG   GCA  GAG  GAG   GCT  GCG  GGT  CCG  CTT  CT
                         ==    = =        =                =
1404                     AG    T T        T                T
                               PstI
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containing 4 Ser to Ala substitutions (Ser 226, 227, 229 and 232) and a new *Pst*I site. Briefly, the mutagenesis procedure included annealing of mutant oligonucleotides (35–51 mers), containing appropriate codon nucleotide changes and a new restriction enzyme site, to single stranded circular wild-type hPR2 subcloned into pSG5 [12]. DNA polymerase I and T4 ligase were used to extend the oligonucleotide primer and synthesize double-stranded circular hPR2 containing one wild-type and one mutant strand. This DNA was then used to transform DH5 competent bacteria. Mutant colonies were screened by colony hybridization and restriction digest. Double-stranded hPR2 with mutations incorporated into both strands was obtained by retransforming DH5 with DNA isolated from positive colonies. Dideoxy sequencing was used for final confirmation of mutants.

Transient transfections

Transfections into Cos-1 cells were performed by calcium phosphate precipitation [13, 14] using 5 μg of the hPR2 or hPR_A–M2 expression plasmids, 3 μg of the β-galactosidase expression plasmid for monitoring transfection efficiency, and 12 μg of Bluescribe carrier plasmid. Cells were grown on 100 mm culture dishes and harvested 48 h following addition of precipitated plasmid DNA. Some dishes were treated with the progestin R5020 or the anti-progestin RU486 for 2 h prior to harvest. Cells from duplicate dishes were pooled for preparation of 0.6 M KCl nuclear extracts which were used for immunoblot or gel-mobility shift analysis. T47D human breast cancer cell cytosols were the source of wild-type hPR [2].

Immunoblotting

Immunoblots were performed as described previously [2]. Briefly, cytosols prepared from T47D breast cancer cells, or hPR_A or hPR_A–M2 receptor proteins in whole cell extracts isolated from transfected Cos-1 cells, were electrophoresed on a 7.5% polyacrylamide gel, blotted to nitrocellulose, blocked with 3% bovine serum albumin (BSA), probed with AB-52 monoclonal antibody, and visualized by using a peroxidase-labelled goat anti-mouse secondary antibody.

Gel-mobility shift assay

Gel-mobility shift assays [15] were performed as described previously using diluted nuclear salt extracts prepared from transfected Cos-1 cells treated with R5020 for 3 h prior to harvest [2, 5]. The oligonucleotide probe contained the progesterone response element (PRE) from the tyrosine amino transferase (TAT) gene promoter (5′-AAAGTCTGTACAGGATGTTCT-GATCAA-3′: 3′-TTTCAGACATGTCCTACA-AGACTAGTT-5′). Approx. 0.06 pmol hPR_A or hPR_A–M2 were incubated with 20–30,000 cpm/0.3 ng of [^{32}P]end-labelled, double-stranded oligonucleotide probe, 2 μg poly dI:poly dC, 10% glycerol, 1 mM DTT, 0.1 M KCl, 2 mM $MgCl_2$, 0.5 μg gelatin and 10 mM tris buffer, pH 7.5, in a total volume of 20 μl. Incubations were performed for 15 min at 20°C, after which either 1 μl of AB-52 (1 μg/μl) or an irrelevant IgG was added and the incubation continued for an additional 20 min. Samples were then electrophoresed on a 5% non-denaturing polyacrylamide gel. Gels were dried and autoradiographed by exposure to X-ray film at −70°C.

RESULTS AND DISCUSSION

Figure 1 shows the functional domains of hPR and the translation initiator methionines (amino acids 1 and 165) marking the synthesis start-sites of the hPR_B and hPR_A proteins [11, 14, 16]. The expression vector hPR2, which encodes wild-type hPR_A [7, 12] was mutated at twelve different sites by oligonucleotide

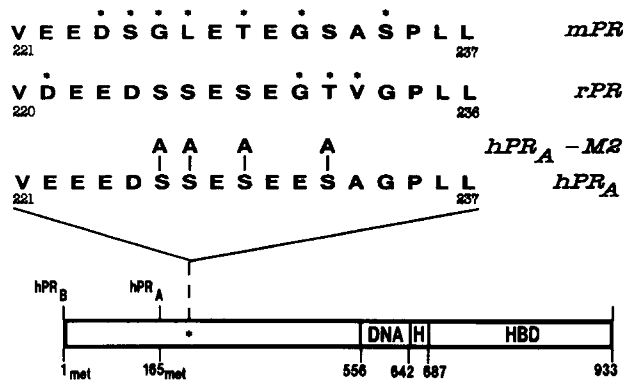

Fig. 1. hPR sequence and functional domains according to Kastner *et al.* [11] showing the location and structure of the peptide Val^{221} to Leu^{237} containing the AB-52 epitope in the wild-type receptor hPR_A, and the 4 Ser to Ala mutations which destroy the epitope in hPR_A–M2. The homologous rabbit (rPR; 23) and mouse (mPR; G. Shyamala, personal communication) sequences are also shown with the amino acids that differ from hPR marked by (*). Note that S^{226} according to Kastner *et al.* [11] is G^{226} according to Misrahi *et al.* [24]. Either amino acid at this position in wild-type hPR permits AB-52 binding.

directed methods. As shown in Fig. 1, one mutant was generated by using a 50 bp oligonucleotide containing four Ser to Ala codon substitutions to yield the expression vector hPR2–M2 encoding an hPR_A with mutant amino acids at positions Ser^{226}, Ser^{227}, Ser^{229} and Ser^{233} (hPR_A–M2) according to the hPR sequence of Kastner *et al.* [11]. Both wild-type and mutant expression vectors were transiently transfected into Cos cells, treated with or without the progestin R5020 or the anti-progestin RU486, and the expressed wild-type and mutant hPR_A were extracted and analyzed by immunoblotting with AB-52. Figure 2, lane 1 shows, as a control, the immunoblot obtained using AB-52 and endogenous hPR isolated from T47D breast cancer cells. The antibody labels both hPR_A, and the multiple phosphorylated isotypes of hPR_B [4]. The antibody also binds well to recombinant wild-type hPR_A expressed in Cos cells from hPR2, regardless of the hormone occupancy or phosphorylation state of the receptors (Fig. 2, lanes 2–4). However no protein was recognized by AB-52 when Cos cells

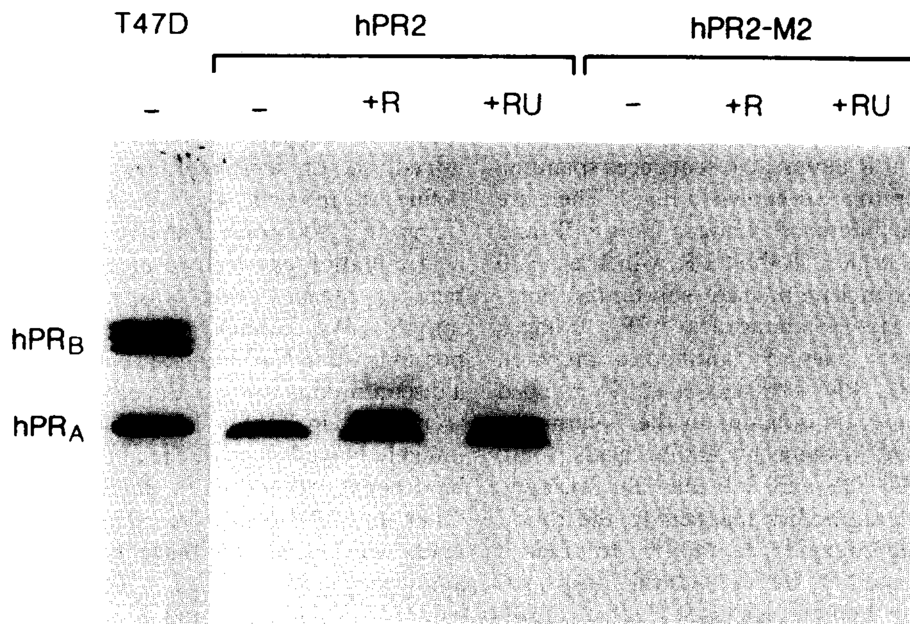

Fig. 2. AB-52 immunoblot of endogenous hPR_B and hPR_A isolated from T47D breast cancer cells, and of wild-type or mutant hPR_A expressed in Cos cells transfected with hPR2 or hPR2–M2. Cos cells were transfected with the expression vectors shown and left untreated (−) or were treated with R5020 (+R) or RU486 (+RU) for 16 h, before total cellular receptors were extracted with 0.6 M KCl. T47D cells were untreated and a cytosol was prepared. Cell extracts were subjected to SDS–PAGE, transferred to nitrocellulose, and immunoblotted with AB-52 as described in Methods.

were transfected with hPR2–M2, raising the possibility that the epitope of the antibody had been destroyed in the mutant receptor. In contrast, eleven other receptors with Ser to Ala or cysteine substitution mutations in various regions of the N-terminus, DNA binding domain, or hinge region, were efficiently immunoblotted by AB-52 (not shown), suggesting that only a very specific mutation can destroy the epitope.

To confirm that a receptor was indeed being synthesized by hPR2–M2, we used the DNA binding property of the receptors in a gel mobility shift assay to monitor the presence of the protein. Both recombinant wild-type hPR_A expressed from hPR2, and the four amino acid substitution mutant expressed from hPR2–M2, were able to bind to the progesterone response element of the TAT promoter (Fig. 3). However, only the mobility of the wild-type receptor was further retarded by AB-52, while that of the mutant receptor was not. This confirms that hPR2–M2 directs synthesis of a mutant hPR protein that retains DNA binding activity, but is invisible to the antibody AB-52. We conclude that the epitope for AB-52 involves at least one of the four mutated serine residues in hPR2–M2 and spans, at most, Val^{221} to Leu^{237}.

AB-52 is a human and primate (R. Brenner, personal communication) restricted anti-PR antibody that fails to cross react with PR from chick, rat, mouse or rabbit [8]. A computer search of the chicken PR peptide detected no homologies to the 17 amino acid sequence that generates the epitope for AB-52. Rat PR cDNA sequences have not been published. The rabbit, mouse and hPR amino acid sequences spanning the AB-52 epitope are shown in Fig. 1. There are considerable differences between mouse PR and hPR in this region. Rabbit PR, which are 930 amino acids in length, have substantial homology with the 933 amino acid hPR_B. Using a selected panel from 59 antibodies prepared against rabbit PR, Lorenzo *et al.* [9] mapped four immunogenic domains in the N-terminus of the rabbit protein to amino acids 1–60, 101–110, 295–325 and 370–396. Surprisingly, none of the antibodies mapped to the rabbit sequence homologous to Val^{221} to Leu^{237}, suggesting that it has structural properties unique to the human receptors. Four of the 17 hPR amino acids are mismatches in rabbit PR including a continuous 3 amino acid set involving human Ser^{233}. These three, Glu^{232}, Ser^{233} and Ala^{234}, may form a critical subset of residues in hPR that define the structure of the AB-52 epitope. While this analysis does not predict the full extent of the human epitope, in proteins in which the antigenic structure has been determined, six or seven continuous residues can define an antigenic site [17]. At the other extreme, in cytochrome *P*450IIC, a single amino acid mutation can lead to acquisition of MAb binding capacity that was previously missing [18]. We cannot entirely rule out the possibility that Val^{221} to Leu^{237} forms one part of a discontinuous epitope, the other part of which is missing in rabbit PR. This possibility is unlikely however, given the ability of AB-52 to bind denatured hPR [19]. Binding sites of two other anti-hPR antibodies that do not cross-react with rabbit PR have been mapped to amino acids 208–296 [20], which of course includes Val^{221} to Leu^{237}, and differs from the four rabbit epitopes listed above.

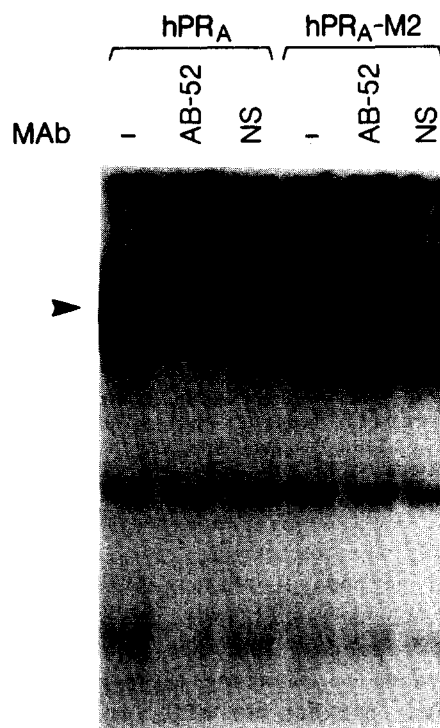

Fig. 3. Binding of hPR_A and hPR_A–M2 to the tyrosine amino transferase PRE in the presence or absence of AB-52. Cos-1 cells were transfected with hPR2 or hPR2–M2, treated with R5020, and nuclear extracts were incubated with the [^{32}P]end-labelled PRE containing oligonucleotide in the presence or absence of AB-52, or an irrelevant non-specific (NS) IgG. Protein–DNA complexes were electrophoresed on non-denaturing gels, dried, and autoradiographed. The arrowhead marks the position of hPR_A bound to DNA.

Figure 4 shows a calculation of the antigenicity index for the 17 amino acid hPR peptide based on secondary structure predictions. While the peptide is likely to be hydrophilic, flexible

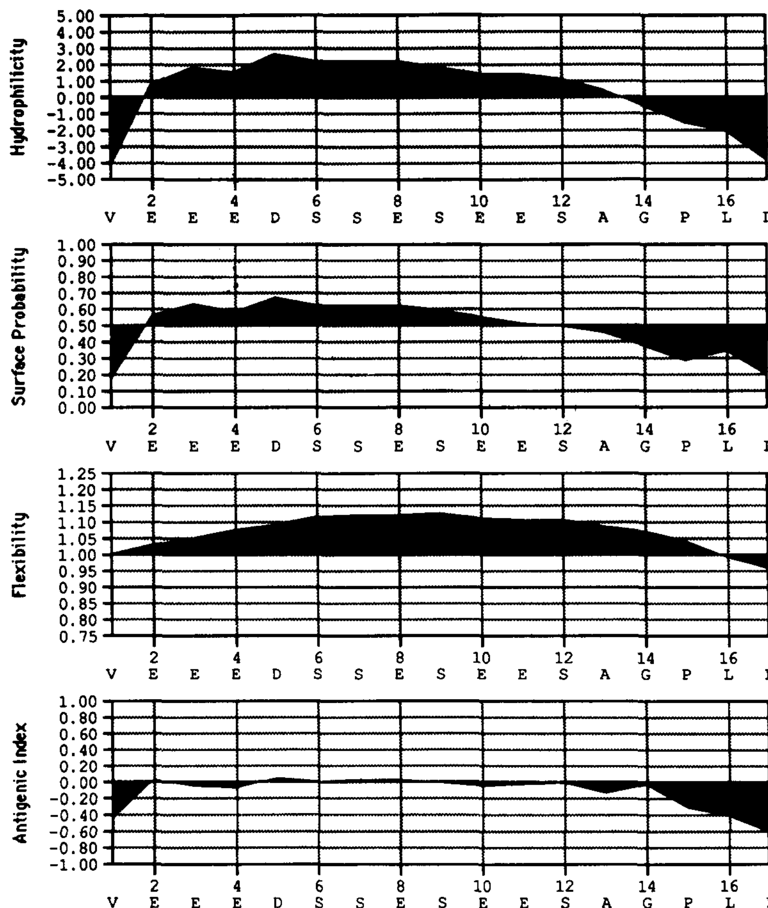

Fig. 4. Secondary structure predictions of the Val^{221} to Leu^{237} peptide and the antigenicity index based on Chou–Fasman/Robson–Garnier algorithms. The single letter amino acid code for the peptide is shown below each chart.

and on the surface of the protein (Fig. 4), it lacks amphiphilic helix or sheet behaviour (not shown). Hence the computer profile, a composite of Chou–Fasman [21] and Robson–Garnier [22] algorithms, is neutral with respect to predicted antigenicity. Theoretical introduction of covalent modifications, such as addition of phosphates to the serine residues [2, 4] did not alter the predicted antigenicity (not shown). Neither did substitution of Gly for Ser at position 226 (see note in legend to Fig. 1). Therefore, based strictly on computer generated data, this peptide would be an unlikely choice for antibody production by methods that start with a synthetic peptide. However, at best, each method has about 60% probability of being correct and the consensus of two, possibly wrong predictions, may also be incorrect. We suggest that Val^{221} to Leu^{237} could be an excellent peptide for generation of additional human specific anti-PR antibodies. Precise knowledge of its epitope will be useful to investigators who are using AB-52 in structural and functional analyses of hPR. It is also useful for control and specificity studies, since the antibody can now be blocked with the peptide rather than requiring purified receptor preparations.

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