Fusion Proteins Containing Androgen Receptor Sequences and Their Use in the Production of Poly- and Monoclonal Anti-Androgen Receptor Antibodies

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ABSTRACT. Complementary DNA segments that encode different domains of human and rat androgen receptors were fused to the Escherichia coli trpE gene using pATH expression vectors. Fusion proteins expressed by the bacteria were used to immunize rats and rabbits to obtain polyclonal antibodies to androgen receptors. Spleen cells of immunized rats were fused with myeloma cells to obtain stable hybridomas that produced monoclonal antibodies. Gradient centrifugation and immuno-preciptitation assays indicated that the antibodies interacted with androgen receptors specifically.

Monospecific antibodies have been important in cloning cDNAs for various steroid receptors (1) and in the cytochemical localization of these steroid receptors (2,3). Most of these antibodies were raised by using receptors purified from target organs as antigens. Since the purification of ARs has been very difficult, this approach has not been feasible for raising AR antibodies. In 1985, Liao and Witte found high titer autoantibodies to androgen receptors (ARs) in the serum samples of some prostate cancer patients (4). Recently, Young et al. (5) transformed lymphocytes from blood of prostate cancer patients by Epstein-Barr virus and cloned anti-AR antibody producing cells. Transformed B-cells, however, were unstable and lost antibody producing capacity.

Since we have constructed cDNAs that can encode full-length rat and human ARs (6,7), we have employed molecular fusion techniques to obtain large quantities of specific AR peptides and used them as antigens in raising AR specific poly- and monoclonal antibodies.

MATERIALS AND METHODS

Materials. [35]DMNT (87 Ci/mmol), [6,7-3H]dexamethasone (49 Ci/mmol) and 17β-[6,7-3H]estradiol (53 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [3H]ORG 5028 (52 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Affinity-purified goat anti-rat and anti-rabbit immunoglobulins were the product of Cappel Laboratories (Malvern, PA). Agarose linked anti-rat IgG antibodies were purchased from HyClone Laboratories (Logan, UT). pATH expression vectors were kindly provided by Dr. Alexander A. Tzagoloff. Radioactive steroid receptor complexes were prepared by the methods described previously (4) using cytosol fractions of rat ventral prostate for AR, human breast tumor MCF-7 cells for ER, rat liver for GR, and human breast tumor T47D cells for PR respectively.

Construction and production of the AR fusion proteins. We selected the E. coli trpE gene using the expression vectors pATH (8) for this purpose because the trpE-fusion proteins were insoluble and could be easily isolated. Three fusion proteins (A, B, and C) were made for this purpose (Fig. 1). For production of fusion protein-A the 2.6 kb cDNA Cla I-Nde I fragment of human AR (6) was digested with Sac I to obtain a 726 bp fragment. This fragment was ligated to a Sac I-digested pATH 10 vector (chimeric vector A). For the production of fusion protein-B, the 835 bp DNA fragment obtained from the Sau 3A-digested 2.83 kb cDNA insert of rat AR (7) was ligated to BamHI-digested pATH 1 vector (chimeric vector B). For the production of the fusion protein-C, the 435 bp fragment isolated from Eco RI- and Pst I-digested 2.83 kb rat AR (7) was ligated to Eco RI- and Pst I-digest. -ATH 11 vector (chimeric vector C). The vectors were used to transform E. coli RR 1 and the transformed clones selected by ampicillin resistance. The clones containing chimeric pATH were grown in M9 medium supplemented with 0.5% acid hydrolyzed casein and induced by 38-indoleacrylic acid (8,9). Cells containing fusion proteins were lysed with lysozyme and sonicated. The soluble fusion protein was isolated by centrifugation, solubilized by heating in SDS and separated from other proteins by SDS-PAGE (6-9).

Production of AR antibodies. For the primary immunization, fusion proteins were separated on SDS-PAGE (Fig. 2). The protein bands that contained fusion proteins or the electroeluted proteins (100 µg) were emulsified with Freund's complete adjuvant and injected intradermally to a 2-month-old male Lewis rat. This was followed by biweekly intradermal boosts using the incomplete adjuvant mixture. For the last boost, the fusion protein (100 µg) was electroeluted from the gel and injected intravenously in saline. The rat serum was diluted (1:1600) and tested by peroxidase-linked immunosay using the fusion protein as the antigen. Positive serum was then assayed by a double antibody precipitation method using [3H]DMNT-receptor complexes of rat ventral prostate (4). Polyclonal antibodies to ARs were also raised in New Zealand white female rabbits by the same procedure. For cloning monoclonal antibody producing hybridomas, spleen cells from immunized rats were fused with SP2/0 rat myeloma cells in the presence of 50% polyethylene glycol 1500 in DMEM. Cells

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were incubated in DMEM containing 20% fetal calf serum and then selected in growth medium containing hypoxanthine, aminopterin, and thymidine (3). Cells from positive wells were cloned by limiting dilution and the media taken from the cell culture wells were assayed for anti-AR antibodies. The monoclonal antibodies in the hybridoma culture media were purified by affinity isolation with agarose-linked second antibodies to rat IgG. On SDS-PAGE, purified IgG fractions showed only two distinct bands representing heavy and light chains of IgG. For purification of polyclonal antibodies anti-trPE antibodies could be removed from serum by co-precipitation with purified trPE protein (insoluble) as described by Hoffman et al. (9).

Other methods. In vitro transcription and translation of cloned DNAs were carried out as described before (6, 7). Gradient centrifugation of radioactive steroid-receptor complexes was performed by centrifugation at 257,000 g for 16 h at 4°C on 3.8 ml linear 5%-20% (w/v) sucrose gradients containing 1.5 mM EDTA, 2 mM dithiothreitol, 0.4 M KCl, and 20 mM Tris-HCl buffer, pH 7.5. Gradients were fractionated from the bottom and 0.2 ml/fraction collected. The protein markers for sedimentation coefficients were the same as described previously (10,11).

RESULTS AND DISCUSSION

The structural constructs of the three fusion proteins are shown in Fig. 1. Based on the procedure used for the construction of vectors, each of the three fusion proteins contained 323 amino acids of the amino terminal portion of trPE protein (8,9). This sequence was followed by a linker oligopeptide which preceded the AR segment. Fusion protein-A or -B had another linker oligopeptide at the C-terminal. As expected, SDS-PAGE analysis of fusion proteins produced by E. coli and isolated as insoluble products (Fig. 2) showed that fusion protein-A (M, about 62 k), protein-B (M, about 70 k) and protein-C (M, about 47 k) were present as major proteins. As shown in Fig. 1, fusion protein-A should have a segment that represents 242 amino acid sequence of human AR starting from the human AR amino acid No. 331 (7). The equivalent region in rat AR had 37 amino acid changes and lacked an 18 polyglycine segment. The homology between human AR and rat AR at this region was about 77% (7). Fusion protein-B should have a segment that represents 279 amino acid sequences of rat AR, starting from the amino acid No. 344. Of these rat AR sequences, only 6 amino acid sequences were different from that in the equivalent region of human AR and the homology between the two ARs at this region was 98%. Fusion protein-C should have a segment representing 117 amino acid sequences of rat AR at the C-terminal end of rat AR which was completely identical with that of human AR.

The three fusion proteins had significant sequence and domain overlaps (Fig. 1). Fusion protein-A contained about 40% of the N-terminal domain and 25% of the DNA-binding domain of human AR. Fusion protein-B contained 94% of the DNA-binding domain and about 70% of androgen-binding domain of rat AR or human AR. Fusion protein-C contained 49% of the androgen-binding domain of ARs.

Of the three fusion proteins, fusion protein-A proved to be most antigenic. Serum samples from 2 of 3 immunized rabbits and 4 of 10 rats exhibited the presence of high titer anti-AR antibodies. Ten microliters of serum samples from these animals were able to immunoprecipitate more than 10 fmol of the [3H]DMNT-receptor complexes. With fusion protein-B, we also detected the presence of the anti-AR antibodies in the serum samples of 1 of the 2 immunized rabbits and 1 of 4 immunized rats. The anti-AR antibody activities in the serum samples of 3 rabbits and 4 rats immunized with fusion protein-C were insignificant.

Using spleen cells from rats immunized with fusion protein-A and the hybridoma technique described above, we have succeeded in cloning three cell lines (designated as H-AN1-6, H-
TABLE 1. STEROID RECEPTOR SPECIFICITY OF MONOCLONAL ANTIBODIES TO ANDROGEN RECEPTOR

<table>
<thead>
<tr>
<th>Radioactive steroid receptor</th>
<th>Purified monoclonal antibody (IgG)</th>
<th>Immunoprecipitated receptor complex (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANI-6</td>
<td>ANI-7</td>
</tr>
<tr>
<td>4 µg</td>
<td>10.1</td>
<td>18.5</td>
</tr>
<tr>
<td>8 µg</td>
<td>0.0</td>
<td>0.1</td>
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<tr>
<td>9 µg</td>
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<td>0.0</td>
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</tbody>
</table>

A double immunoprecipitation method described elsewhere (4) was used to analyze steroid receptor specificity of monoclonal antibodies. A purified monoclonal antibody (IgG) preparation was incubated with a radioactive steroid-receptor complex (52.8 fmol) for 15 h at 4 °C. The mixture was incubated again with a goat anti-rat IgG and incubated for 6 h at 4 °C. After incubation, the mixture was centrifuged and radioactivity associated with the precipitate was estimated. No significant precipitation of the radioactive receptor complex was observed in the absence of the poly- or monoclonal antibody preparation or the second anti-IgG antibody. Radioactive steroids used in the preparation of 3H-steroid-receptor complexes were [3H]DMNT for AR, [17β]-[3H]estradiol for ER, [3H]testosterone for GR, and [3H]ORG2058 for PR.

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AN1-7 and H-AN1-15) which can produce anti-AR antibodies (designated as ANI-6, ANI-7 and ANI-15). These cell-lines have been maintained in culture for 15 months without losing the antibody producing capability. Immuno-precipitation assay showed that these antibodies were specific to androgen-receptor complexes either prepared from rat ventral prostate or synthesized in cell-free systems by transcription of rat and human AR-cDNAs and translation of the AR-mRNA product. Immuno-precipitation of glucocorticoid receptor complexes of rat liver, estrogen receptor complexes of human breast tumor MCF-7 cells, or progesterone receptor complexes of human breast tumor T-47D by these antibodies were not significant (Table 1). Gradient centrifugation analysis (Fig. 3) also showed that these monoclonal antibodies were able to shift the sedimentation coefficient of [3H]DMNT-receptor complexes from 4 S to about 9 S in 0.4 M KCl. Such a shift in the sedimentation pattern was not observed with other radioactive steroid-receptor complexes shown in Table 1.

Using spleen cells of rat immunized with fusion protein-B, we also cloned a hybridoma cell line (H-AN1-21) that produced a monoclonal anti-AR antibody (ANI-21). By immunoprecipitation assay, the anti-AR antibody was also receptor-specific (Table 1). The antibody titers of other cloned cell lines were much lower than that exhibited by the four cell lines described above. The low titers might be, in part, due to the fact that the immunogenic sites in the specific domains of steroid-receptor complexes were masked and not available for recognition by monoclonal antibodies. A similar observation was reported earlier with polyclonal antibodies that recognized the DNA-binding domain of glucocorticoid receptors (12). Since the DNA- and androgen-binding domains of AR in the fusion protein-B had regions with high homology with binding domains of other steroid receptors (6,7), some of the monoclonal antibodies produced may not exhibit a clear steroid-receptor specificity. Such an antibody, however, may be useful in the study of activation and transformation of steroid receptor.

Various monoclonal anti-AR antibodies will be useful in the immunocytolocalization and quantitation of different forms of androgen receptor in the target organs of androgens. In agreement with recent immunocytochemical studies using polyclonal anti-AR antibodies (13), our preliminary studies with monoclonal anti-AR antibodies have shown that androgen receptors are mainly localized in the epithelial cell nuclei of rat and human prostates.

Fig. 3. Sedimentation profiles of androgen-receptor complexes in the absence and presence of a monoclonal anti-androgen receptor antibody (ANI-15). [3H]DMNT-R (16,000 dpm) alone (open circle) or with 40 µg ANI-15 antibody (closed circle) in 0.2 ml was incubated at 4 °C for 1.5 h. The incubated mixtures were treated with dextran-coated charcoal to remove free steroid and then layered on the top of sucrose gradients (5-20%) containing 1.5 mM EDTA, 2 mM diethiothreitol, 0.4 M KCl, and 20 mM Tris-HCl, pH 7.5. Centrifugation was performed at 50,000 rpm for 18 h at 2 °C in a Beckman SW 60 rotor. Fractions (0.2 ml each) were collected and numbered from the bottom of each tube. Similar results were obtained with monoclonal antibodies ANI-6 and ANI-7.