Molecular Characterization of the Epitope in Prostate and Breast Tumor-associated PR92 Antigen

Yung D. Kim, Deborah Y. Robinson, George L. Manderino, Ilse I. E. Tribby, and Joseph T. Tomita

ABSTRACT

In our previous report, monoclonal antibody PR92 has defined prostate- and breast tumor-associated PR92 antigen. The molecular nature of PR92 antigen, especially the epitope involved in specific interaction with PR92 monoclonal antibody, is described. PR92 antigen was purified from the cell extract or tissue culture medium of prostate cancer cell line DU145 by means of monoclonal antibody-coupled Sepharose 4B affinity chromatography, followed by a Sephacryl S-500 chromatography. Physical and chemical characterization, coupled with high-performance liquid chromatography, determined that PR92 antigen is a glycoprotein with a molecular weight of about 470,000, comprising repeating subunits of about 44,000. Sialic acid was found to form a critical part, while galactose and A'-acetylgalactosamine were also involved, in the epitope structure. PR92 antigen is rich in serine, threonine, proline, glycine, and alanine and poor in aromatic amino acid residues. The carbohydrate moieties may be predominantly O-linked to polypeptide chains which contribute directly or indirectly to maintain the integrity of the epitope. Elucidation of the molecular nature of PR92 antigen may help understand the mechanism of shedding into the body fluids during tumor progression.

INTRODUCTION

We have previously identified and characterized murine monoclonal antibody PR92, produced against a prostate cancer cell line DU145 (1). A homologous, solid-phase sandwich radioimmunoassay, based on PR92 MAbs, recognized a new glycoprotein, PR92 antigen. The PR92-RIA, however, did not detect 16 other known tumor-associated markers including carcinoembryonic antigen, prostatic acid phosphatase, prostate-specific antigen, α-fetoprotein, ferritin, and β2-microglobulin. Preliminary studies showed that PR92 Ag levels in the serum specimens from patients with progressive prostate and breast carcinoma were elevated compared to those in age- and sex-matched normal and benign controls. The results further suggested a significant clinical correlation between the elevation of the antigen level and the advance of tumor state (1).

A number of MAb-defined tumor-associated antigens have been reported (2–6). Many of the antigens are glycoproteins, lipoproteins, or mucins with molecular weights in excess of $10^6$. Molecular characterization of these antigens was often difficult due to the inherent molecular heterogeneity of the antigens and the complexity of carbohydrate moieties involved in the structure.

The present study was aimed at determining the molecular nature of the epitope in PR92 Ag, involved in the immunochromatographic reaction with PR92 MAb. In some cases, PR92 Ag was examined both in purified form and in clinical specimens in order to verify that the molecular properties of the antigen have not been altered during the purification procedure.

MATERIALS AND METHODS

Clinical Specimens. Serum and urine specimens from patients diagnosed to have breast or prostate carcinoma were provided by Dr. H. Fritsche at M. D. Anderson Hospital (Houston, TX), Dr. A. Malkin at Sunnybrook Medical Center (Toronto, Ontario, Canada) and Dr. R. Vessella at the VA Hospital (Minneapolis, MN). Pleural effusions obtained from patients with benign or malignant disease were kindly provided by the Cytology Department, Highland Park Hospital (Highland Park, IL). Informed consent was obtained from each specimen donor at the respective institutions. The urine and pleural effusions were centrifuged for 30 min at 1400 × g on a Beckman J6-B centrifuge (Palo Alto, CA), aliquoted and stored at −70°C and −20°C, respectively, until tested.

 Lectins and Carbohydrates. A lectin kit was obtained from E. Y. Laboratories (San Mateo, CA), which comprised Arachis hypogaea (A. hypogaea agglutinin), Dolichos biflorus (D. biflorus agglutinin), Bauhinia purpurea (BPA), Ulex europaeus (U. europaeus agglutinin I), Canavalia ensiformis (concanavalin A), Glycine max (soybean agglutinin), Limax flavus aggl. (LFA), Triticum vulgaris (WGA), and Maclura pomifera (M. pomifera agglutinin). In addition, lectins from Helix pomatia (H. pomatia agglutinin), Sophora japonica (S. japonica agglutinin), and Wisteria floribunda (W. floribunda agglutinin) and carbohydrate compounds, D-galactose, lactose, sialic acid, N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine were supplied by Sigma Chemical Co. (St. Louis, MO). Blood group antigens M and N, and Thomson-Friedenrich antigen were prepared as described previously (7).

DU145 Cell Soluble Extract and Culture Medium. Propagation of DU145 cells and preparation of soluble cell extract were carried out as described (1). DU145 cells were also cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum and the medium was harvested once a week for 3 weeks. The pooled culture medium was concentrated about 50-fold using Amicon Concentrator with PM10 membrane (Amicon, Lexington, MA), and dialyzed against PBS overnight at 4°C.

PR92 Monoclonal Antibody and PR92-radioimmunoassay. Hybridoma PR92 clone was obtained by injection of prostate carcinoma cell line DU145 into BALB/c mice and fusion of the spleen cells with mouse SP2/0 melanoma cells (1). The PR92 hybridoma cells were cloned and passed repeatedly in BALB/c mice to produce PR92 MAb in ascitic fluid. The mouse ascitic fluids were pooled and processed by protein A-Sepharose (Pharmacia, Piscataway, NJ) column chromatography to isolate the PR92 MAb. A solid-phase sandwich radioimmunoassay was developed (1) utilizing a PR92 MAb-coated microtitration plate (Removewell strips; Dynatech, Alexandria, VA) and 125I-labeled PR92 MAb as probe antibody. The PR92 Ag assays and inhibition tests were performed as described previously (1).

Preparation of PR92 Antigen. DU145 cell extract and cell culture medium were the primary sources of PR92 Ag. An immunoaflinity column was prepared by coupling PR92 MAb to Sepharose 4B (Pharmacia) according to the procedure recommended by the supplier, and packing a 30-ml column, equilibrated with PBS. After loading DU145 cell extract or concentrated cell culture medium, the column was washed with PBS. The proteins bound to the column were eluted with 0.1 M citrate-Tris buffer (pH 3) and immediately neutralized with 1 M Tris solution. The PR92 Ag level in each fraction of the eluant was monitored by the PR92-RIA. The profile indicated that PR92 Ag eluted as one peak following application of the citrate-Tris buffer. This crude PR92 Ag preparation was further purified on a Sephacryl S-500 (Pharmacia) column (56 × 3 cm), which was equilibrated and eluted with PBS. Elution of the proteins from the column was monitored by absorbance measurement at 280 nm and also by measuring PR92 Ag...
activity of each fraction. PR92 Ag was found to elute as a single peak, following other contaminating proteins that had larger molecular size than PR92 Ag and exhibited significant absorbance at 280 nm. The fractions containing PR92 Ag were concentrated, dialyzed for 2 days against deionized and distilled water at 4°C, and finally lyophilized.

Absorption Spectroscopy and Amino Acid Analysis. Lyophilized PR92 Ag was dissolved in PBS to make 1 mg/ml concentration. UV absorbance was scanned between 300 and 200 nm on a DMS 100 UV-visible spectrophotometer (Varian Associates, Sunnyvale, CA). Amino acid analysis of PR92 Ag was performed using a Beckman 6300 amino acid analyzer (Palo Alto, CA). Samples in duplicate were hydrolyzed in 6 N HCl-0.3% phenol for a period of 24, 51, and 72 h, respectively, at 110°C, and the intrinsic value of each amino acid residue was determined based on the three sets of amino acid analysis data.

High-Performance Liquid Chromatography. A HPLC unit by Perkin-Elmer (Norwalk, CT) with a gel filtration column, Bio-Sil TSK 250 (300 × 7.5 mm) (Bio-Rad, Richmond, CA), was equilibrated with 0.1 M sodium sulfate-0.02 M monosodium phosphate, pH 6.8. Bio-Rad Gel Filtration Standard (Bio-Rad) or the samples were injected in 200-μl volumes. The column eluant was monitored in the same manner as mentioned previously for the Sephacryl S-500 column chromatography.

Enzyme Treatment of PR92 Antigen. Action of some glycosidases and proteolytic enzymes on PR92 Ag was investigated with the enzymes (neuraminidase, β-galactosidase, β-glucosidase, and trypsin) obtained from Sigma and endo-α-N-acetylgalactosaminidase purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Each enzyme reaction was carried out at the optimal conditions recommended by the suppliers. A control sample was included in each experiment so that any change of PR92 Ag activity resulting from the reaction could be quantitatively assessed by PR92-RIA.

**RESULTS**

Isolation and Purification of PR92 Antigen. PR92 Ag was isolated from DU145 culture medium, soluble cell extract, or pleural effusion of some adenocarcinoma patients (primary sites unknown). The immunoaffinity column specifically bound PR92 Ag and allowed elution of the antigen at acidic pH condition. The eluted proteins were further purified by Sephacryl S-500 column chromatography to remove some proteins coeluted with the PR92 Ag. Characteristically, purified PR92 Ag did not exhibit significant absorbance in the 300–250 nm range. The HPLC elution profiles of DU145 cell extract and purified PR92 Ag, monitored in absorbance at 280 nm and PR92 Ag activity, are shown in Fig. 1. The presence of PR92 Ag activity, coupled with the absence of A280, was considered as a criterion for purity in the purification of PR92 Ag.

Molecular Weight Determination. The PR92 Ag activity profile of HPLC eluant yielded a single band, corresponding to an apparent molecular weight of 450,000–500,000. Some clinical specimens of serum and urine from the patients with progressive prostate or breast carcinoma and pleural effusions from adenocarcinoma patients were also subjected to HPLC analysis, and the results are summarized in Table 1. In all cases, PR92 Ag eluted as a single peak corresponding to an apparent molecular weight of the same range as the purified antigen.

To elucidate the molecular structure, PR92 Ag was pretreated by incubating for 1 h at 80°C in 1.3% SDS solution, in the presence or absence of 3% 2-mercaptoethanol, prior to HPLC. As shown in Fig. 2 the elution profiles in PR92 Ag activity exhibited a single peak (M, 470,000) for the SDS-treated sample and a single peak (M, 44,000) for the SDS plus 2-mercaptoethanol-treated sample, respectively.

Inhibition of PR92 Antigen Binding to PR92 MAb by Carbohydrates and Lectins. The carbohydrate or lectin was allowed to inhibit the interaction between PR92 MAb (coated on the solid-phase well) and PR92 Ag. The PR92 Ag bound by the unknown MAb was subsequently recognized by 125I-PR92 MAb. Among 5 carbohydrate moieties examined only sialic acid effectively inhibited the PR92 Ag-antibody reaction, whereas none of the blood group substances tested affected the reaction. While PNA, BPA, LFA, and WGA caused significant inhibition, 10 other lectins did not. The results of these findings are summarized in Table 2.

Physical, Chemical, and Enzymatic Treatment of PR92 Antigen. Heating PR92 Ag for 15 min at 70°C in 0.2 M citrate solution at pH 5 or for 1 min at boiling temperature in PBS did not alter its immunochemical properties as evaluated by PR92-RIA. PR92 Ag was found soluble in 0.6 M perchloric acid solution at pH 5 or for 1 min at boiling temperature in PBS did not alter its immunochemical properties as evaluated by PR92-RIA. PR92 Ag was found soluble in 0.6 M perchloric acid solution at pH 5 or for 1 min at boiling temperature in PBS did not alter its immunochemical properties as evaluated by PR92-RIA.
chloric acid or heat treatment (8, 9). As judged from the HPLC (8) and retaining immunochemical properties following per

digestion of PR92 Ag reduced the immunochemical binding

typical glycoproteins by being soluble in 0.6 M perchloric acid

DISCUSSION

Amino Acid Composition of PR92 Antigen. Four amino acid
analyses were performed with two preparations of purified
PR92 Ag. The arithmetic mean molar concentration for each
amino acid residue was computed and normalized with respect

to that of alanine. The results presented in Table 4 show that

results, PR92 Ag is a relatively homogeneous molecule with a
molecular weight of approximately 470,000. Comparison of
the observed molecular weight of PR92 Ag, in purified form
and in various clinical specimens (serum, urine, or pleural
effusion), asserts that the PR92-RIA specifically measures the
same intact molecules in all clinical specimens and that the
antigen has not been altered during the purification procedure
(Table 1). The HPLC profiles in Fig. 2 indicate that 2-mercap-
toethanol reduced the intramolecular disulfide bonds and dis-
sociated PR92 Ag (M, 470,000) into a subunit structure (M/r

Table 2 Inhibition of carbohydrates, lectins, and human blood group substances
in the interaction between PR92 antigen and PR92 MAb

Each substance was tested by PR92-RIA, in 10 units/ml PR92 antigen
concentration, and at two levels of inhibitor concentrations. An appropriate control
sample was included in each experiment so that the percentage inhibition by a
substance could be calculated. The carbohydrate specificity of lectin was taken from
the E. Y. Laboratories catalogue (pp. 4 and 5, 1987).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Carbohydrate specificity</th>
<th>Concentration</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Galactose</td>
<td>d-Galactose</td>
<td>0.1 M</td>
<td>0</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>GalNAc*</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>GalNAc</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Lactose</td>
<td>Lactose</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>Sialic acid</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>Lectin</td>
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<td></td>
<td></td>
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<td>A. hypogaea (PNA)</td>
<td>d-Galactose, β-d-GalNAc</td>
<td>50 µg/ml</td>
<td>99</td>
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<tr>
<td>B. purpurea (BPA)</td>
<td>d-GalNAc</td>
<td></td>
<td>99</td>
</tr>
<tr>
<td>C. ensiformis (concanavalin A)</td>
<td>α-d-Mannose, glucose</td>
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<td>0</td>
</tr>
<tr>
<td>D. biflorus (D. biflorus antigen)</td>
<td>α-d-GalNAc</td>
<td>50 µg/ml</td>
<td>95</td>
</tr>
<tr>
<td>G. max (soybean agglutinin)</td>
<td>α,β-GalNAc</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>H. pomatia (H. pome-tia agglutinin)</td>
<td>α-d-GalNAc</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>L. flavus agglutinin (LFA)</td>
<td>Sialic acid</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>M. pomifera (M. pomifera agglutinin)</td>
<td>d-Galactose</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>S. japonica (S. japonica agglutinin)</td>
<td>α-β-GalNAc</td>
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<td>95</td>
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<tr>
<td>T. vulgaris (WGA)</td>
<td>β-d-GalNAc, sialic acid</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>U. europaeus (U. europaeus agglutinin I)</td>
<td>α-L-Fucose</td>
<td></td>
<td>95</td>
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<tr>
<td>W. floribunda (W. floribunda agglutinin)</td>
<td>d-GalNAc</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>Blood group substance</td>
<td></td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>M, N, T antigen</td>
<td></td>
<td>50 µg/ml</td>
<td>95</td>
</tr>
</tbody>
</table>

* GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylgalactosamine.

Table 3 Effect of some enzymes on binding property of PR92 antigen to PR92 MAb

Each enzyme reaction with PR92 antigen was carried out under the optimal
conditions suggested by the supplier of the enzyme except the length of reaction
time, which is listed in Column 2. An appropriate control sample was treated
under the same conditions as the enzymatic reaction without the enzyme present.
Following the enzyme reaction, the remaining PR92 antigen activity was assessed
by PR92-RIA. The results of two experiments using duplicate samples are
presented as mean ± SD.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction time (h)</th>
<th>PR92 antigen remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidase</td>
<td>16</td>
<td>5 ± 5</td>
</tr>
<tr>
<td>D-Galactosidase</td>
<td>16</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>16</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>Endo-α-N-acetylgalactosaminidase</td>
<td>16</td>
<td>92 ± 9</td>
</tr>
<tr>
<td>Trypsin</td>
<td>52</td>
<td>15 ± 5</td>
</tr>
</tbody>
</table>

Fig. 2. HPLC elution profiles of pretreated PR92 antigen. PR92 Ag was
incubated in 1.3% SDS (4) or in 1.3% SDS plus 3.0% 2-mercaptoethanol (8),
for 1 h at 80°C prior to HPLC. The eluted fractions were analyzed in PR92 Ag
activity by PR92 RIA. KD, molecular weight in thousands.

acid solution and retained its antigenicity after removing the
acid by dialysis against PBS. The effect of a combination of an
acidic pH value and heating was tested by incubating the antigen for
0.5 h in 0.05 M sulfuric acid at 80°C.

Neuraminidase treatment of PR92 Ag abolished its capacity
to bind PR92 MAb, whereas β-glucosidase and endo-α-N-ac-
ylgalactosaminidase treatments had virtually no effect. Action
of D-galactosidase on PR92 Ag reduced its binding capacity to
PR92 MAb by about 50%. As indicated in Table 3, trypsin
digestion of PR92 Ag reduced the immunochemical binding
property of the antigen to PR92 MAb as much as 85%.

Amino Acid Composition of PR92 Antigen. Four amino acid
analyses were performed with two preparations of purified
PR92 Ag. The arithmetic mean molar concentration for each
amino acid residue was computed and normalized with respect
to that of alanine. The results presented in Table 4 show that
serine, threonine, proline, glycine, and alanine are the major
amino acids present in the polypeptide backbone of PR92 Ag.

PR92 Ag exhibited physical and chemical characteristics of
typical glycoproteins by being soluble in 0.6 M perchloric acid
(8) and retaining immunochemical properties following per-
chloic acid or heat treatment (8, 9). As judged from the HPLC

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they are composed of subunits cross-linked through disulfide bonds.

Involvement of sialic acid moiety in the epitope of PR92 Ag was demonstrated by the abolition of its binding property to PR92 MAb after neuraminidase or acid-heat treatment desialylated PR92 Ag. Additionally, sialic acid and sialic acid-specific lectins (LFA and WGA) were found to inhibit effectively the reaction between PR92 Ag and PR92 MAb. Cleavage of D-galactose moieties on PR92 Ag by galactosidase resulted in loss of 50% of PR92 Ag binding to PR92 MAb, indicating a significant contribution of D-galactose to its antigenicity within the epitope structure. Although D-galactose-specific lectins, PNA and M. pomifera agglutinin, could inhibit the antigen-antibody reaction, D-galactose alone did not. Similarly, N-acetyl-D-galactosamine alone had not affected the antigen-antibody interaction while the N-acetyl-D-galactosamine-specific lectin (BPA) effectively inhibited the reaction. It is evident, therefore, that sialic acid is the essential constituent and D-galactose and N-acetyl-D-galactosamine are also involved in maintaining the carbohydrate structure for the epitope recognized by PR92 MAb.

Some tumor-associated markers have been reported to have close structural relationship with human blood group substances. For example, CA 19-9 MAb recognizes sialylated Lewis A antigen (2), and blood group A, B, and H antigens were found to be absent in the tissue specimens of transitional cell carcinoma of bladder (11). The inhibition test results, summarized in Table 2 and also in another report (1), revealed that PR92 Ag does not share the antigenic determinant with blood groups A, B, H, M, N, and T as well as mucins CA 19-9 and Ca 125, and human colonic mucins.

The observation that tryptic digestion of PR92 Ag could reduce the ability of the antigen to bind PR92 MAb by 80–90% indicates a strong contribution of the polypeptide structure in maintaining the integrity of the epitope. Relatively high content of serine and threonine, compared with that of aspartic acid, in PR92 Ag suggest that the oligosaccharide chains are predominantly O-linked to the polypeptide backbone (12). The amino acid composition of PR92 Ag, rich in proline, glycine, and alanine but poor in aromatic amino acids (tryptophan, tyrosine, and phenylalanine), resembles the compositions of some human glycoproteins previously reported (13). The notable absence of absorbance in 300–250 nm region of UV absorption spectrum corroborates the amino acid analysis results.

The results presented in this report do not allow us to determine the relative contributions made by carbohydrate moieties and polypeptide structure, separately. It seems that both groups participate directly or indirectly to provide specific epitope structure recognized by PR92 MAb. The amino acid sequence and oligosaccharide structure of the epitope on PR92 Ag have yet to be elucidated. While immunohistochemical studies with the PR92 MAb would reveal the intracellular distribution and etiology of PR92 Ag, the information about the overall molecular structure would enable us to understand its relationship with other known glycoproteins and its possible physiological role. This information would also assist us in understanding the mechanism of how PR92 Ag is shed into the body fluids during the development of progressive carcinomas. Current investigation is directed toward these goals.

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