Surface-epitope masking and expression cloning identifies the human prostate carcinoma tumor antigen gene PCTA-1 a member of the galectin gene family

(DNA cotransfection-nude mouse tumors/reverse transcription–PCR/moNoclonal antibodies/Immunohistochemistry/human tumor galectin-8)

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ABSTRACT The selective production of monoclonal antibodies (mAbs) reacting with defined cell surface-expressed molecules is now readily accomplished with an immunological subtraction approach, surface-epitope masking (SEM). Using SEM, prostate carcinoma (Pro 1.5) mAbs have been developed that react with tumor-associated antigens expressed on human prostate cancer cell lines and patient-derived carcinomas. Screening a human LNCaP prostate cancer cDNA expression library with the Pro 1.5 mAb identifies a gene, prostate carcinoma tumor antigen-1 (PCTA-1). PCTA-1 encodes a secreted protein of ~35 kDa that shares ~40% sequence homology with the N-terminal region of members of the S-type galectose-binding lectin (galectin) gene family. Specific galectins are found on the surface of human and murine neoplastic cells and have been implicated in tumorigenesis and metastasis. Primer pairs within the unique untranslated region of PCTA-1 and reverse transcription–PCR demonstrate selective expression of PCTA-1 by prostate carcinomas versus normal prostate and benign prostatic hypertrophy. These findings document the use of the SEM procedure for generating mAbs reacting with tumor-associated antigens expressed on human prostate cancers. The SEM-derived mAbs have been used for expression cloning the gene encoding this human tumor antigen. The approaches described in this paper, SEM combined with expression cloning, should prove of wide utility for developing immunological reagents specific for and identifying genes relevant to human cancer.

The early detection of prostate cancer and the accurate prediction of its clinical course is not possible by using current methodologies. Contemporary approaches, including physical examination, tissue biopsy, monitoring serum prostate-specific antigen (PSA) levels, and ultrasound and bone scans do not ensure early prostate cancer detection and are of only limited value in predicting disease progression. Of immense value for the accurate diagnosis and potentially for the therapy of human prostate cancer is the identification of immunological and genetic reagents displaying the appropriate specificity that will permit a clear distinction between prostate carcinoma versus normal prostate and benign prostatic hypertrophy (BPH). Immunological reagents that can detect and bind to tumor-associated antigens on the surface of prostate carcinoma cells, and that display limited expression on normal prostate and BPH tissue, may prove of particular benefit for the detection and ultimately for the therapy of prostate cancer.

Production of monoclonal antibodies (mAbs) reacting with antigens present on the surface of tumor cells, but displaying restricted expression on normal cells, is often a difficult and inefficient process (1–3). A procedure based on immunological subtraction, surface-epitope masking (SEM), has been developed that in principle can obviate many of the limitations preventing efficient mAb development toward molecules expressed on the cell surface (3, 4). SEM is based on the selective blocking of antigens on a genetically modified target cell (i.e., tester) with polyclonal antibodies produced against the same unmodified cell line (i.e., driver). Antigen-blocked cells are injected into BALB/c mice, and sensitized spleen cells are removed and fused to NS1 myeloma cells, and hybridomas secreting reactive mAbs are isolated (3). The SEM approach has been successfully used for a number of applications, resulting in the production of mAbs specific for surface expressed molecules with known and unknown functions (3, 4).

An improved procedure has been developed for identifying and cloning dominant acting oncosgenes, termed rapid expression cloning (5, 6). This approach involves transfecting high molecular weight human tumor DNA into a new acceptor cell line, CREF-Trans 6, selecting cells expressing genes inducing tumors in nude mice, and using molecular biological approaches, such as differential RNA display, to clone the putative oncogene (5, 6). Tumor-derived CREF-Trans 6 cells have also proven useful in combination with the SEM procedure for identifying tumor-associated antigens expressed on the cell surface of cancer cells serving as the initial source for transforming DNA (5, 6). Expression cloning of cDNAs using antibodies reacting with their encoded proteins represents a direct means of identifying and cloning functional genes (2). This approach has now been used to identify and clone a gene, prostate carcinoma tumor antigen 1 (PCTA-1), encoding tumor-associated antigens (TAA) recognized by the prostate carcinoma mAb Pro 1.5. Sequence analysis indicates that PCTA-1 consists of a cDNA of 3.85 kb with 83% nucleotide (nt) and 81% amino acid (aa) sequence homology to an 1.25-kb cDNA, rat galectin-8, cloned from a rat liver cDNA expression library (7). Some overlapping homologies are also

Abbreviations: PSA, prostate-specific antigen; BPH, benign prostatic hypertrophy; SEM, surface-epitope masking; PCTA-1, prostate carcinoma tumor antigen 1; TAA, tumor-associated antigen; RT–PCR, reverse transcription–PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, FACS, fluorescence-activated cell sorter; PIN, prostatic intraepithelial neoplasia.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. L78132).

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detected with several small noncontiguous partial cDNA sequences (of less than 500 nt) previously identified as expressed human sequence tags (8). Analysis of protein structure indicates that PCTA-1 has ~40% homology to specific structural domains of the S-type lectin family of galactose-binding proteins, the galectins (9–11). These highly homologous proteins include a carbohydrate-binding 35-kDa protein (CBP35) expressed on NIH 3T3 fibroblasts, a 34-kDa surface antigen present on metastatic murine tumors, a 31-kDa surface protein on metastatic human tumors, a 30-kDa carbohydrate-binding protein (CBP30) found on baby hamster kidney cells, rat and human lung 29-kDa galactose-binding lectins, an IgE-binding protein of rat basophilic cells, and a 32-kDa surface antigen Mac-2 found on thioglycollate-elicited murine macrophages (9–11). The roles of the galectin proteins are diverse and impinge upon important biological processes, including cell signaling, proliferative control, cell adhesion, and cell migration (9–11). In this context, the demonstration that PCTA-1 encodes a protein with specific homology to the galectins suggests that this molecule, a human tumor analogue of rat galectin-8 (i.e., galectin-RHT), may contribute to human prostate cancer development and evolution.

MATERIALS AND METHODS

Cell Lines. The LNCaP cell line was derived from metastatic deposits from a patient with advanced prostate cancer (12). CREF-Trans 6 and LNCaP DNA-transfected nude mouse tumor-derived CREF-Trans 6 cells, CREF-Trans 6:4 NMT, were isolated as described (5). The hormone independent prostatic carcinoma cell lines DU-145 and PC-3 were obtained from the American Type Culture Collection. Conditions for growing the various cell types were as described (5, 6, 12).

cDNA Library Construction, Expression Cloning, and Sequencing. An LNCaP cDNA library was constructed in the Uni-ZAP XR vector (Stratagene) (6, 13, 14). The cDNA library was screened by using the Pro 1.5 mAb following the protocol in the picoblot Immunoscreening Kit (Stratagene). Host bacterial cells (SURE) were grown on 150 × 15-mm NZY plates (Stratagene) to yield ~20,000 plaques/plate. After 3.5 hr incubation at 42°C, nitrocellulose filters soaked in 10 mM isopropl β-D-thigalactoside solution were added to the top of colonies for plaque lifts. The filters were washed three to four times with TBST buffer (20 mM Tris-HCl, pH 7.5/150 mM NaCl/0.05% Tween-20) and soaked in blocking solution [1% bovine serum albumin in TBS buffer (20 mM Tris-HCl, pH 7.5/150 mM NaCl)] and incubated for 1 hr at room temperature. The filters were then transferred into fresh blocking solution containing Pro 1.5 ascites (1:500 dilution) and incubated for 3 hr at room temperature with gentle agitation. After washing four times with TBST buffer, the filters were transferred into fresh blocking solution containing Ab–AP conjugate (1:2000 dilution) and incubated for 1 hr at room temperature. Positive colonies were identified by developing the filters in a solution containing 0.3 mg of nitroblue tetrazolium per ml, 0.15 mg of 5-bromo-4-chloro-3-indolylphosphate per ml, 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM MgCl₂. The reaction was terminated by adding stop solution (20 mM Tris-HCl, pH 2.9/1 mM EDTA). PCTA-1 positive plaques were isolated and the complete nt sequence of PCTA-1 was obtained by using the Sanger method (15). A series of oligonucleotides synthesized from both sides of the PCTA-1 insert in the pBluescript vector were used as primers.

The final nucleotide sequence was verified by using an Applied Biosystems (model 373A, version 1.2.1) sequencer.

In Vitro Translation of PCTA-1. The plasmid DNA containing PCTA-1 was linearized by digestion with XmaIII and used as a template to synthesize mRNA using the mCAP mRNA capping kit (Stratagene). In vitro translation of PCTA-1 was performed by using a rabbit reticulocyte lysate translation kit with conditions as described by GibCO/BRL (6).

Preparation of Mouse Polyclonal Antibodies and SEM. CREF-Trans 6 polyclonal antibodies were prepared as described (3). The CREF-Trans 6 polyclonal antibodies were used to coat CREF-Trans 6:4 NMT cells by the SEM approach, resulting in the production of hybridomas secreting Pro 1.5 mAbs (3).

Fluorescence Cell Staining and Immunostaining of Tissue Sections with Pro 1.5 and PSA. Fluorescence staining with Pro 1.5 mAB was as described (16). Tissue sections were prepared from fresh tissues frozen in liquid nitrogen. Serial sections for several tissues were used to prepare RNA for reverse transcription (RT)–PCR (6). Tissue sections were stained by using standard protocols [Super Sensitive Detection System (Biogenex Laboratories, San Ramon, CA)]. Briefly, sections were fixed in acetone, blocked with 3% H₂O₂ for 7 min at room temperature, and incubated for 20 min at room temperature in phosphate-buffered saline (PBS) containing 3% lamb serum. Sections were then incubated with Pro 1.5 (1:100) or PSA (1:200) (Dako) for 45 min at room temperature. Samples were incubated with biotinylated secondary antibody and horseradish peroxidase conjugated streptavidin in PBS. Prior to each incubation step, sections were washed three to five with PBS. Reactivity was detected by adding diaminobenzidine and counterstaining with Hematoxylin.

Immunoprecipitation Analysis. Cells were labeled with [35S]methionine and cell lysates were analyzed for PCTA-1 protein levels by immunoprecipitation analysis using Pro 1.5 mAbs as described (3, 17). Secreted PCTA-1 was detected by labeling cells for 4 hr with [35S]methionine, growing cells for an additional 24 hr in the absence of label, collecting the medium, concentrating the medium, and performing immunoprecipitation analysis with Pro 1.5 mAbs.

RNA Preparation and RT–PCR. Total cytoplasmic RNA was isolated from logarithmically growing cell cultures as described (6, 14). Tissue samples from normal prostates and patients with prostatic carcinomas or BPH were frozen in liquid nitrogen, and RNA was isolated using the TRIzol reagent as described by GibCO/BRL. Tissue samples were supplied by the Cooperative Human Tumor Network. Three samples of normal prostate were obtained from autopsies of males <40 years of age. All tissues were histologically confirmed as normal, BPH, or carcinoma of the prostate (6). RT–PCR using primer pairs for PCTA-1, PSA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as described (6, 17).

RESULTS

Production of Pro 1.5 mAbs by SEM and Reactivity of Pro 1.5 and PSA with Normal Prostate, BPH, and Prostate Carcinomas. The SEM approach of blocking antigenic epitopes on nude mouse tumor-derived LNCaP DNA-transfected CREF-Trans 6 cells with CREF-Trans 6 polyclonal antibody before injection into mice was used to produce hybridomas secreting the Pro (prostate carcinoma) series of mAbs (3). The Pro mAbs can detect by in situ fluorescence microscopy and fluorescence-activated cell sorter (FACS) analysis the surface expression of tumor-associated antigens on LNCaP-transfected primary (CREF-Trans 6:4 NMT) and secondary tumor-derived CREF-Trans 6 cells and LNCaP, DU-145, and PC-3 human prostate cancer cell lines (3) (Fig. 1A–C and data not shown). In contrast, Pro 1.5 does not react using fluorescence microscopy or FACS with CREF-Trans 6 cells (3). The staining pattern with Pro 1.5 in human prostate carcinoma cells is irregular with microclusters, as previously observed with mAbs reacting with specific galectins (10, 17, 18) (Fig. 1A–C). Immunoprecipitation analysis identifies an ~55- to 42-kDa protein in lysates from LNCaP-transfected primary

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epithelial cells (Fig. 2). As expected, PSA also stains prostate cells in tissue sections of BPH and prostate carcinomas. mAb Pro 1.5 reacts strongly with prostate carcinoma cells present in frozen tissue sections, but not with adjacent benign glands or tissue sections containing normal prostate or BPH epithelium. However, some reactivity with Pro 1.5 is found in PIN. These studies indicate that the Pro 1.5 mAb can distinguish between prostate carcinoma and PIN versus normal prostate epithelial cells and BPH. In this context, Pro 1.5 provides a discriminatory capacity for detection of cancer of the prostate that exceeds that of the nonspecific prostate epithelial cell marker PSA.

Expression Cloning of PCTA-1 Using SEM-Derived Pro 1.5 mAbs. To identify the gene encoding the tumor-associated antigens identified on human prostate cancer cells by mAb Pro 1.5, an antibody expression cloning strategy was used. An LNCaP cDNA library was constructed in the Uni-ZAP XR vector (Stratagene) and screened with the Pro 1.5 mAb (12, 13). This approach resulted in the identification of a 3.85-kb cDNA clone referred to as PCTA-1. In vitro protein translation of the PCTA-1 cDNA results in a 317-aa protein of ~35 kDa (data not shown). The DNA sequence of PCTA-1 displays 83% homology, and the protein sequence of PCTA-1 displays 81% sequence homology with rat galectin-8 (7), a member of the S-lectin family of galactose-binding lectin proteins. The full-length rat galectin-8 cDNA is 1247 bp, whereas the full-length PCTA-1 cDNA is 3850 bp. This result suggests that PCTA-1 encodes a human tumor homologue of galectin-8, galectin-8HT. Amino acid comparison of PCTA-1 with other members of the galectin gene family indicates that this protein is ~40% homologous to specific regions of these galectins (Fig. 3). Several amino acid sequence homologies are found between PCTA-1, rat galectin-8, and the other galectins. Stretches of identical amino acids are found in PCTA-1, rat galectin-8, and both the small ~14-kDa and larger ~29- to 34-kDa galectins, e.g., HFNPREF, IVCN, and WG. These amino acids are well conserved and are found in galectins isolated from diverse species, including eel, chicken, mouse, rat, bovine, and human (Fig. 3B) (11, 19, 20). They are important structural components of the galectins and may mediate galectin binding to its putative ligands (12). Of potential interest is the replacement in PCTA-1 of two amino acids normally present in the conserved regions of most of the galectins—i.e., the substitu-
The amino acid sequence of PCTA-1 with other galactose-binding lectin (galectin) proteins. The predicted amino acid sequence is shown below the nucleotide sequence. Comparison of PCTA-1 with sequences of the Mr, 14,000 and Mr, 29,000-35,000 galectins from eel, chicken, mouse, rat, bovine, and human galectins. * Amino acids unique to PCTA-1 and rat galectin-8; ** a, amino acid differences between PCTA-1 and rat galectin-8; & delta; amino acids shared by PCTA-1, rat galectin-8, mouse-L34, human-galectin-3-L29, and human-L31; & delta; a, amino acid difference between PCTA-1, human-galectin-3-L29, and human-L31 versus rat galectin-8; & delta; a, amino acids shared by PCTA-1, rat galectin-8, and the Mr, 14,000 and Mr, 29,000-31,000 galectins from different species.

Expression of PCTA-1 in Cell Lines and Normal Prostate, BPH, and Prostate Carcinomas. After obtaining the sequence of PCTA-1, studies were performed to determine if primers for specific regions of this gene could be identified that would permit detection of RNA expression by RT-PCR. Using primers located between bp 3010 and 3423 in the 3′ untranslated region, PCTA-1 expression is apparent in LNCaP DNA-transfected tumor-derived CREF-Trans 6, LNCaP, and DU-145 cells but not in untransfected CREF-Trans 6 cells (Fig. 4). PCTA-1 expression also occurs in seven of seven patient-derived prostate carcinomas, one of four BPH, and one of four putative normal prostate tissue samples (Fig. 4). In the one BPH sample displaying PCTA-1 expression, histological anal-
circulation might prove beneficial as a diagnostic marker for prostate cancer.

**DISCUSSION**

We describe the cloning of PCTA-1, a gene encoding TAAs that are present on the surface of invasive human prostate carcinoma and early prostate cancer, PIN, but not on histologically confirmed normal prostate or BPH tissue. The PCTA-1-encoded TAAs are detected on the surface of prostate cancers using the SEM-derived Pro series of mAbs, and these TAAs are shed by prostate carcinoma cells. Using a pair of primers hybridizing with sequences in the 3'-untranslated region of PCTA-1, RT-PCR detects PCTA-1 expression in prostatic carcinomas and PIN, but not in histologically confirmed normal prostate or BPH. These attributes should allow the direct use of the Pro series of mAbs and the PCTA-1 gene for cancer diagnostic applications. If the PCTA-1 gene displays appropriate specificity using a larger tissue sampling, this gene may also prove of value for designing gene-based strategies for the therapy of prostate cancer. Moreover, the innovative procedures used, including rapid expression cloning, SEM, and antibody expression cloning, represent integrated approaches for the efficient identification and cloning of molecules (including TAAs) of potential clinical interest that are expressed on the surface of diverse human cancer cells.

The PCTA-1 protein retains a number of conserved structural motifs that are found in most members of the galectin gene family (7, 11, 19, 22). These conserved regions are present in species as diverse as eel, mouse, rat, and human (11). On the basis of amino acid sequence, PCTA-1 is a human tumor homologue of rat galectin-8 (7), galectin-8HT, that may contribute to the cancer phenotype of human prostate carcinomas. The galectins display wide tissue distribution, clear developmental regulation, and differential levels in specific tissues, supporting the hypothesis that they contribute to many physiologically important processes in mammalian cells (11). Of direct relevance to cancer is the finding that the galectins, as well as the selectin subgroup of C-type lectins (20, 23), can mediate both cell-cell and cell-matrix interactions (11, 18, 24). These associations are critical elements in mediating the
metastatic spread of tumor cells (25, 26). Moreover, experimental evidence has accumulated indicating that galectin-3 may play an important role in the metastatic process (18, 27–31). Galectin-3 is overexpressed in human colon and gastric carcinomas versus normal and benign tissue, and elevated expression of recombinant L-34 in a weakly metastatic UV-2237-cl-15 mouse fibrosarcoma cell line increases lung metastases in syngeneic nude mice (18, 27–31). Moreover, anti-galectin mAbs inhibit homotypic aggregation, anchorage-independent growth, and experimental metastases in UV-2237 subclones (32–34). Studies are in progress to determine if the PCTA-1 gene and the Pro mAbs display similar properties as the cloned galectin-3 gene and the anti-galectin mAbs, respectively. In preliminary studies, the effect of mAb Pro 1.5 on tumor growth in athymic nude mice containing established DU-145 tumors (~200 mm³) has been determined. In this model system, injection of Pro 1.5 mAbs (200 μg/injection; 11 injections) reduced tumor size in seven of nine animals (unpublished studies). In contrast, 10 of 12 animals receiving an irrelevant mAb developed actively growing tumors (unpublished data). These preliminary findings demonstrate a direct effect of Pro 1.5 mAbs on the growth and progression of human prostate cancers in vivo. It will also be important to ascertain if inhibition in PCTA-1 expression, using antisense oligonucleotides, antisense expression vectors, or ribozyme approaches, alters the tumorigenic or metastatic properties of human prostate cancers.

Prior to SEM, no simple and direct procedure was available for efficiently generating mAbs reacting with differentially expressed surface molecules. Conceptually, SEM involves immunological subtraction that induces the immune system of mice to target antibody production toward surface molecules expressed on a genetically modified tester cell line, but not expressed or expressed in lower abundance on its cognate unmodified driver cell line (3, 4). By using polyclonal antibodies produced against the driver cell line to coat (mask) epitopes on the tester cell line, enriched production of mAbs targeted toward epitopes expressed on the surface of the tester cell type is achieved (3, 4). Confirmation of the SEM approach has come from studies in which this process was used to target the development of mAbs reacting with the M₇, 170,000 human multidrug resistance protein (P-glycoprotein) and the human interferon γ receptor (3, 4). The SEM approach has also been used to produce mAbs that react with unknown TAA’s, including those presently identified as the gene product of PCTA-1 and undefined TAA’s enriched by rapid expression cloning from the T47D human breast carcinoma cell line to CREF-Trans 6 (data not shown). These results indicate that rapid expression cloning combined with SEM may represent efficient strategies for identifying antigenic epitopes on human cancers. Additional applications of SEM may also result in the targeted production of mAbs and the identification of genes associated with important physiological processes, including cellular growth and differentiation, immunological recognition, tumorigenesis, metastasis, cellular senescence, atypical multiple drug resistance, and autoimmune disease.

In summary, we presently demonstrate direct applications of the rapid expression cloning and SEM technologies for the development of mAbs and the cloning of the PCTA-1 gene associated with human prostate cancer. The PCTA-1 gene and the recently identified prostate tumor associated gene PTI-1 (6) are genetic elements that can distinguish prostate cancer from normal prostate and BPH. Although further studies are required, it appears that PCTA-1 may represent an earlier genetic change in human prostate cancer development than PTI-1. In this context, both the Pro mAbs and the PCTA-1 gene should find direct applications for prostate cancer diagnosis and staging, and they may also represent important therapeutic reagents for intervention in this pervasive and often fatal neoplastic disease.

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