

Mapping Tumor Epitope Space by Direct Selection of Single-Chain Fv Antibody Libraries on Prostate Cancer Cells

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ABSTRACT

The identification of tumor-specific cell surface antigens is a critical step toward the development of targeted therapeutics for cancer. The epitope space at the tumor cell surface is highly complex, composed of proteins, carbohydrates, and other membrane-associated determinants including post-translational modification products, which are difficult to probe by approaches based on gene expression. This epitope space can be efficiently mapped by complementary monoclonal antibodies. By selecting human antibody gene diversity libraries directly on the surface of prostate cancer cells, we have taken a functional approach to identifying fully human, tumor-specific monoclonal antibodies without prior knowledge of their target antigens. Selection conditions have been optimized to favor tumor-specific antibody binding and internalization. To date, we have discovered >90 monoclonal antibodies that specifically bind and enter prostate cancer cells, with little or no binding to control cells. These antibodies are able to efficiently deliver intracellular payloads when attached to nanoparticles such as liposomes. In addition, a subset of the antibodies displayed intrinsic antiproliferative activity. These tumor-specific internalizing antibodies are likely to be useful for targeted therapeutics either alone or in combination with effector molecules. The antigens they bind constitute a tumor-specific internalizing epitope space that is likely to play a significant role in cancer cell homeostasis. Targeting components of this epitope space may facilitate development of immunotherapeutic and small molecule-based strategies as well as the use of other therapeutic agents that rely upon delivery to the interior of the tumor cell.

INTRODUCTION

Cancer cells differ from normal cells in a variety of ways, one of which is the molecular composition of the cell surface. These differences may be exploited in the development of targeted therapeutics. In principle, a variety of antineoplastic agents can be attached to affinity molecules, such as monoclonal antibodies (mAbs), which recognize tumor-specific cell surface molecules to achieve targeted killing (1). The epitope space⁵ at the tumor cell surface, however, is highly complex, including, in addition to proteins, carbohydrate determinants and other post-translational modification products that are difficult to probe by gene expression-based approaches (2, 3). For carcinoma of the prostate (CaP), there are few known specific cell surface markers and even fewer specific markers for hormone-refractory CaP (4–9). Moreover, despite recent advances in early diagnosis and treatment, prostate cancer remains the most common and second most lethal

tumor in American men (10), and no curative treatment currently exists for metastatic disease (11).

Tumor-specific epitope space may be efficiently mapped by complementary mAbs. Phage display of nonimmune, single-chain Fv (scFv) or Fab antibody repertoires has proven to be an important tool for generating highly specific antibody-combining sites that may be readily converted into mAbs (12–15), if needed. Nonimmune phage libraries are derived from naïve human lymphocytes and thus recapitulate the primary immune response (12) and overcome difficulties with generating antibodies to evolutionarily conserved, or “self”, antigens that may make up a large portion of tumor antigens (16, 17). This broader repertoire of specificities allows a less biased and more thorough mapping of epitope space (18). mAbs to tumor antigens have been isolated by directly selecting phage libraries on native and modified tumor antigens (19). The success of direct cell selections in generating tumor-targeting mAbs has been limited, however, by high nonspecific binding of phage to cell surfaces and by high representation of phage antibody binding to common cell surface molecules (19, 20).

Recently, we reported that phage antibody selections on cells could be significantly improved by selecting for mAbs that trigger receptor-mediated endocytosis, because endocytosed phage may be recovered from within the tumor cell after stripping nonspecific binders from the cell surface (21). Besides increasing selection efficiency, this approach generates mAbs that have desirable biological properties: receptor-mediated intracellular drug delivery, induction of apoptosis, or inhibition of proliferation (22–24). We applied this approach to breast tumor cells and generated mAbs to a number of known internalizing receptors, including epidermal growth factor receptor and ErbB2 (23, 24). To broaden applicability, libraries of phage displaying multiple copies of scFv were engineered (25–27), which unlike existing phage libraries can cross-link receptors, allowing more efficient phage endocytosis (21).

Here we report, for the first time, the application of this library to generate prostate cancer-targeting mAbs that bind to components of the tumor-specific internalizing epitope space. By using a library designed to trigger receptor endocytosis and by devising selections to eliminate cross-reactive mAbs, a panel of 93 CaP-specific mAbs were generated without prior knowledge of their target antigens. Over 70 of those mAbs recognize hormone-refractory prostate cancer cells. All mAbs examined were efficiently endocytosed by CaP cell lines and thus can be used for efficient delivery of antitumor drugs to the cytosol. In addition, a subset of the mAbs possesses intrinsic antiproliferative activity and may have therapeutic utility as “naked” mAbs.

MATERIALS AND METHODS

Phage Display Library Construction and Preparation. A multivalent fd phage display library consisting of 5×10^8 members was derived from a 7×10^9 -member phagemid library (16) by subcloning the *SfiI/NotI* scFv insert from pHEN1 into bacterial vector fd-*SfiI/NotI* (25–27). Phage were produced via growth in culture of *Escherichia coli* TG1, concentrated by precipitation with polyethylene glycol 8000, and purified by CsCl gradient centrifugation, as reported previously (25–27).

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⁵ *Epitope space* is the complete set of antigenic determinants (epitopes) recognizable by an ideal, naïve phage antibody library. Operationally, the size of this space is a function of experimental details, such as the size and quality of the library, selection methods, and the cell biology involved in handling cells of interest. *Tumor epitope space* is the complete set of tumor-specific or tumor-associated epitopes on a particular tumor specimen or tumor cell lines, recognizable by an ideal, naïve phage antibody library. Operationally, the size of this space is a function of experimental details, such as the size and quality of the library, the success of preadsorption against a corresponding normal cell line, the selection methods, and the cell biology involved in handling tumor cells or cell lines of interest.

Selection for Prostate Cancer-specific Internalizing Antibody. Normal human fibroblasts and noncancerous epithelial lines RWPE-1, BPH-1, MCF10A, and human mammary epithelial cell (HMEC) were used to deplete the phage library of nonspecific binders by incubating 10^{12} phage particles with 10^8 cells for 4 h at 4°C. Supernatant containing the depleted phage library was then incubated with 10^6 prostate cancer cells for 1 h at 4°C. Cells were washed with cold PBS and incubated with prewarmed (37°C) medium/10% FCS at 37°C for 30 min to allow receptor-mediated internalization. Non-internalized phage were removed by washing cells with glycine buffer (50 mM glycine, 150 mM NaCl, 200 mM urea, and 2 mg/ml polyvinylpyrrolidone, pH 2.8) and by digesting cells with trypsin at 37°C for 10 min. Cells were collected by centrifugation and lysed with 1 ml of 100 mM triethylamine. Lysate was neutralized with 0.5 ml of 1 M Tris-HCl (pH 6.8) and was used to infect exponentially growing *E. coli* TG1 as described previously (25–27). The number of unique phage antibodies was determined by patterns of *Bst*NI digestion of scFv genes amplified by PCR from phage-infected bacteria (28). When restriction digestion patterns were ambiguous, scFv genes were sequenced to determine uniqueness.

Analysis of Phage Antibody Binding by Flow Cytometry. Cells (10^6) were incubated with phage antibody (5×10^{11} colony-forming units/ml) for 1 h at 4°C. Bound phage were detected by using biotinylated anti-M13 antibody (Amersham Pharmacia) and streptavidin-R-phycoerythrin or streptavidin-FITC (Molecular Probes). Cells were analyzed using a FACSort (Becton Dickinson). Mean fluorescence intensity was calculated using CellQuest software (Becton Dickinson). For analysis of mixed cell populations by two-color flow cytometry, the relevant normal cell line was prelabeled with FITC labeling reagent, 6-(fluorescein-5-[and-6]-carboxamido)hexanoic acid, succinimidyl ester (Molecular Probes) according to the manufacturer's instructions. Labeled and unlabeled cells were mixed and incubated with phage antibody at 4°C for 1 h. Bound phage were detected by biotinylated anti-fd rabbit polyclonal antibody (Sigma) and streptavidin-phycoerythrin. For cytometry, the prelabeled cell population was identified on the FL1 (FITC) channel, and the bound phage were detected on the FL2 (PE) channel, appropriately compensated. Experiments were performed with either cell population (normal *versus* prostate cancer) labeled with similar results.

Expression and Purification of Prostate Cancer-specific, Single-Chain Fv Antibody. The scFv gene was subcloned from the phage vector into the secretion vector pUC119mycHis, resulting in the addition of a c-myc epitope tag and hexahistidine tag at the COOH terminus of the scFv. To create the (scFv)₂ dimer (29) for immunohistochemistry, the c-myc epitope tag was genetically removed from pUC119mycHis, and a free cysteine was introduced at the COOH terminus of the scFv preceding the hexahistidine tag. scFv or (scFv)₂ dimer protein was harvested from the bacterial periplasm and purified by immobilized metal affinity chromatography and gel filtration (22). After purification of the (scFv)₂ dimer protein, ~50% of scFv was in dimeric form as determined by nonreducing SDS-PAGE.

Immunohistochemistry. Tissue sections from frozen and paraffin-embedded blocks were obtained from the Tissue Core of UCSF Comprehensive Cancer Center. For immunohistochemical analysis, tissue sections were incubated with purified dimeric scFv (50 µg/ml in 2% milk/PBS) at 4°C for 4 h, washed with PBS, incubated with a rabbit polyclonal anti-(His)₆ antibody diluted 1:400 (Santa Cruz Biotechnology), followed by biotinylated anti-rabbit antibody diluted 1:400 (Vector Lab) and horseradish peroxidase-conjugated streptavidin diluted 1:400 (Sigma). Binding was detected using diaminobenzidine as the substrate (Sigma).

Growth Inhibition and Internalization Assays. PC3 cells at 30% confluence were incubated with various concentrations of affinity-purified scFv at 37°C for 72 h in medium containing 1% FCS. Growth status was assessed using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Promega), and the IC₅₀ was calculated using Kaleidagraph 3.5 (Synergy Software). For internalization assays, CaP-specific phage antibodies were biotinylated with sulfo-NHS-LC-biotin (Pierce), mixed with unlabeled helper phage M13K07 at the molar ratio of 1:100, and incubated with target cells at 37°C for various amounts of time. Cells were washed with 100 mM glycine buffer (pH 2.8), fixed with 2% formaldehyde, permeabilized with ice-cold 100% methanol, and incubated with streptavidin-FITC. The stained cells were first examined with an AxioPhot fluorescence microscope (Zeiss) and further studied with a Leica TCS NT confocal laser fluorescence microscope (Leica).

Immunoliposome Preparation and Assay of Intracellular Delivery. Liposomes were prepared from 1-palmitoyl-2-oleoyl-phosphatidylcholine, cholesterol, and methoxypoly(ethylene glycol) (molecular weight 2000)-distearoylphosphatidylethanolamine (3:2:0.3 molar ratio; Avanti Polar Lipids) by lipid film hydration in a solution containing the pH-sensitive fluorophore 1-hydroxypyrene-3,6,8-trisulfonic acid, pyranine (HPTS), followed by extrusion through track-etched polycarbonate membranes with 100-nm pore size (22, 30). A lipophilic derivative of nitrilotriacetic acid-nickel (Avanti Polar Lipids) was further inserted into the liposome to create a surface capable of capturing (His)₆-tagged scFv. To assess intracellular liposome delivery, HPTS liposomes (0.2 mM phospholipid) were added to cells along with 1 µg/ml of purified (His)₆-tagged scFv, incubated at 37°C for 30 min, and washed three times with saline containing 1 mM EDTA to remove cell surface-bound liposomes that failed to internalize. Uptake of scFv-HPTS immunoliposomes was determined by microfluorimetry with a Gemini microfluorometer (Molecular Devices) and by an inverted fluorescence microscope (Nikon).

RESULTS

Subtractive Selection for CaP-specific Internalizing Phage Antibody. A nonimmune, multivalent phage display library that contains >100 million different antibody variable fragments was used for subtractive cell selection. The phage display library was pre-absorbed against a panel of normal cell lines, including a normal, immortalized prostate epithelium line (RWPE-1), epithelial cells derived from benign prostatic hyperplasia glands (BPH-1; Ref. 31), normal human fibroblasts, and normal breast epithelial lines (MCF10A and HMEC), to remove those antibodies that bind to common cell surface molecules. The depleted antibody library was incubated with either one of two hormone-refractory CaP lines (PC3 and DU-145) and one hormone-sensitive line (LNCaP) under conditions that allowed receptor-mediated endocytosis (Fig. 1A). Surface-bound phage that failed to internalize were removed by low-pH glycine buffer washes, and internalized phage were recovered by lysing the cells, followed by amplifications in *E. coli*.

After two rounds of subtractive selection, 22–43% of the phage output bound the selecting cell line (Table 1), as determined by cell ELISA (Fig. 1B). Using comparative cell ELISA and flow cytometry, it was determined that 90 of 1320 (6.8%) phage antibodies selected on CaP cell line PC3 bound PC3 cells but not normal cells, including BPH-1, RWPE-1, MCF10A, and HMEC (Fig. 1, B–D; and Table 1). These phage antibodies were designated as “highly” specific binders. By the same criteria, 30 of 360 (8.3%) and 25 of 270 (9.3%) phage antibodies were highly specific for DU-145 and LNCaP prostate tumor cell lines, respectively. The number of unique antibodies was determined by DNA fingerprinting and sequencing. Ninety unique antibodies were identified that were highly specific for CaP cell lines; 72 of these antibodies bound specifically to hormone-refractory metastatic cell lines PC3 and DU-145. Most of these antibodies (total, 70 of 72) bound to both the hormone-refractory cell lines (Fig. 1C). Fifty of 51 Du-145 binders cross-reacted with PC3 cells, whereas 20 of 21 PC3 binders cross-reacted with Du-145 cells. These results indicate that a large number of unique antibodies recognizing membrane antigens specific to prostate cancer cells were identified.

To further confirm antibody specificity, the ability of phage antibodies to identify tumor cells in mixed cell populations was assessed using two-color fluorescence-activated cell sorter. FITC-labeled normal cells were mixed with unlabeled prostate cancer cells and coin-cubated with CaP-specific phage antibody. CaP-specific phage antibodies specifically stained prostate cancer cells but not normal cells in the mixed populations (Fig. 1D).

Selected Phage Antibodies Recognize Different Antigens Than Existing Antibodies. Cell profiling experiments were performed using flow cytometry with CaP-specific phage antibodies in comparison

Fig. 1. Subtractive selection of CaP-specific internalizing antibody. **A**, selection scheme. A naïve human phage antibody library was depleted on normal cells and subsequently incubated with prostate cancer cells at 37°C to induce receptor-mediated endocytosis. **B**, comparative cell ELISA. Supernatants containing monoclonal phage antibody were incubated with PC3 and BPH-1 cells in parallel to reveal differential binding. **C**, binding specificity determined by flow cytometry. Phage antibody A12 was incubated with prostate cancer cells (PC3 and DU-145) and control cells, and bound phage were detected by FITC-conjugated anti-M13 antibodies. *Filled peak*, control, an irrelevant, hapten-binding phage mAb. *Unfilled peak*, CaP-specific phage mAb. **D**, binding specificity determined by two-color FACS analysis of mixed cell populations. RWPE-1 (control) cells were labeled with FITC, mixed with unlabeled PC3 cells, and incubated with phage antibodies that bind to prostate cancer [*M9E4*, *M10A12*, and *M11G12* (*G12*)]. *Control*, helper phage only. *H3*, pan cell binding antibody. Binding of erbB2 phage antibody *F5* is shown as a reference.

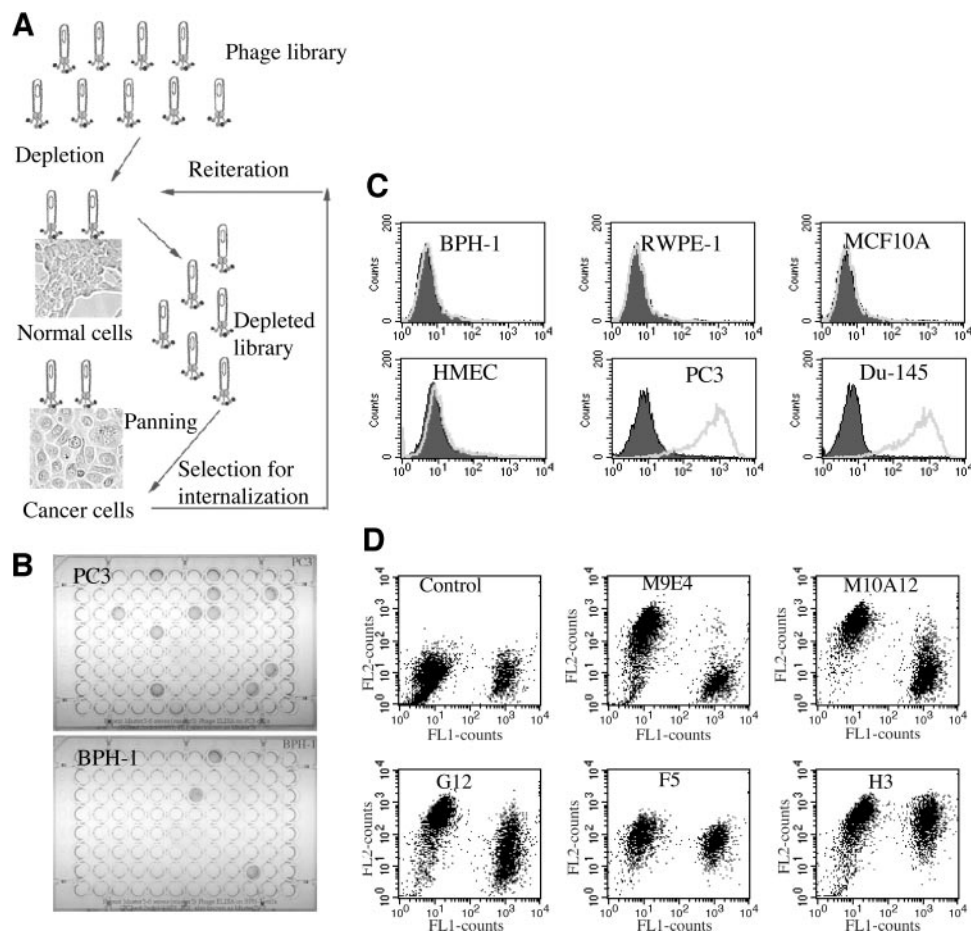


Table 1 Summary of selection results on PC3, DU-145 (hormone-refractory), and LNCaP (hormone-sensitive) prostate cancer cell lines

Binding specificities were determined by whole-cell ELISA and flow cytometry. DNA fingerprinting and sequencing were used to identify the number of unique antibodies.

	PC3	DU-145	LNCaP
Total output (cfu ^a)	4 × 10 ⁶	3 × 10 ⁶	2 × 10 ⁶
Positive/clones screened	255/1320 = 19.3%	116/360 = 32.2%	95/270 = 35.2%
Nonspecific binder	67/1320 = 5.1%	35/360 = 9.7%	24/270 = 8.8%
Binding to overexpressed marker	137/1320 = 10.4%	60/360 = 16.7%	68/270 = 25.2%
Highly specific binder	90/1320 = 6.8%	30/360 = 8.3%	25/270 = 9.3%
Unique clones	51/1320 = 3.9%	21/360 = 5.8%	21/270 = 7.8%

^a cfu, colony-forming units.

with known antibodies that have been described as prostate- or prostate cancer-specific in the literature. The results are summarized in Table 2. The binding patterns of the selected CaP-specific phage antibodies are very different from those of known CaP antibodies, including anti-prostate-specific membrane antigen (PSMA), anti-prostate stem cell antigen, anti-STEAP, and anti-hepsin, and therefore likely recognize novel antigens or epitopes on the prostate cancer cell surface (Table 2). Phage antibodies that exhibited the most specific binding patterns to both hormone-refractory lines (PC3 and Du-145) were selected for further analysis.

Selected Phage Antibodies Are Rapidly Internalized and Can Be Used to Construct a Targeted Drug Delivery Vehicle. Phage antibodies were isolated using a functional selection for triggering receptor endocytosis. To confirm that the selected antibodies possessed this phenotype and were endocytosed by CaP cells, the intracellular uptake of six unique phage antibodies was measured. All six phage antibodies were efficiently internalized by PC3 cells (Fig. 2A).

This property of tumor cell-specific internalization can be exploited to create a generic approach for efficient tumor-specific drug delivery. When attached to drug-encapsulated nanoparticles such as liposomes, CaP-specific scFv are expected to deliver the liposomes to the tumor cytosol. To determine the utility of the CaP antibodies for intracellular drug delivery, we determined the quantitative uptake of immunoliposomes. Liposomes with surface-bound CaP scFv were loaded with a pH-sensitive fluorophore, pyranine (HPTS; Ref. 22), and intracellular uptake was determined by measurement of the pH-dependent fluorescence of HPTS, allowing quantification of scFv-HPTS-liposome in

Table 2 Summary of flow cytometry profiling of selected phage antibodies and known antibodies to prostate tumor antigens

Data were compiled from current studies and published data. Mean fluorescent intensity (MFI) per cell is indicated as: ++, strongly positive (sample/control > 100); +, positive (10 < sample/control < 100); +/-, weakly positive (5 < sample/control < 10); -/+ , slightly positive (2 < sample/control < 5). PSMA, prostate specific membrane antigen; PSCA, prostate stem cell antigen; STEAP, six-transmembrane epithelial antigen of the prostate; PSGR, prostate-specific G protein coupled receptor.

	BPH-1	RWPE-1	LNCaP	PC3	DU-145	Ref.
PSMA	+	+/-	+	-	-	This study + Ref. 4
PSCA	ND ^a	ND	-	-	-	Ref. 9
STEAP	+	+	+/-	+	+	This study + Ref. 7
PSGR	-	-	+/-	-	-	Ref. 6
Hepsin	ND	ND	+/-	-	+/-	This study + Ref. 5
A33	-	-	-	++	++	This study
M10A12	-	-	-	++	++	# ^b
M9E4	-	-	++	++	++	#
OAI2	-	-	-	++	++	#
M11G12	-	-/+	++	++	++	#
M11F12	-	-	++	-	++	#
C10	-	++	++	++	++	#

^a ND, not done.

^b #, this study.

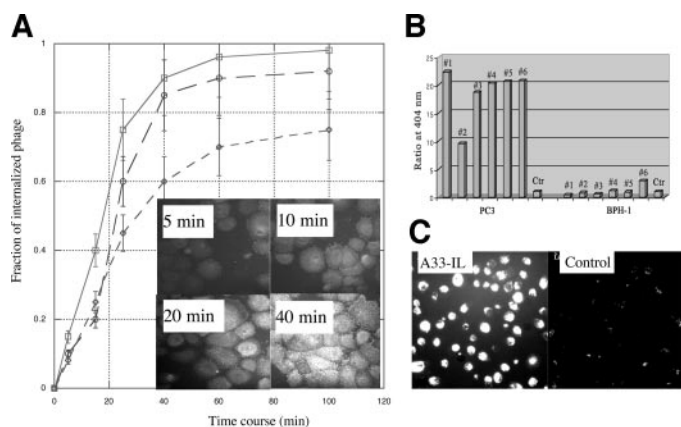


Fig. 2. A, time course of phage antibody internalization by PC3 cells. The percentage of internalized phage is calculated as a fraction of the total phage bound and plotted as a function of time. Circle, M9E4 phage antibody; square, A33 phage antibody; diamond, M10A12 phage antibody. Insert, internalized phage visualized by fluorescent microscopy. Bars, SD. B, ScFv-directed liposome endocytosis by prostate cancer cells. Fluorescent dye containing immunoliposomes (ILs) were constructed from six scFv that bound PC3 cells but not noncancerous BPH-1 cells. After removal of surface bound ILs, internalized liposomes were quantified by fluorescence at 404 nm. Ctr, control, nitrilotriacetic acid-nickel liposome containing HPTS but without scFv coating. The ratio of sample reading over control was shown for comparison between PC3 and BPH-1 cells. C, direct observation of liposome HPTS uptake by PC3 cells using fluorescent microscopy. The result is shown for PC3 with A33 scFv (clone 1 in A). Control, liposome containing HPTS but without scFv coating.

acidic endosomal compartments. Such immunoliposomes prepared from six different CaP-specific scFv were efficiently endocytosed by PC3, with minimal uptake into BPH-1 cells (Fig. 2B). Without scFv, untargeted liposomes were not efficiently taken up by prostate cancer cells (Fig. 2C). These experiments demonstrate that scFv antibodies obtained via selection for internalization are capable of mediating targeted payload delivery. Those antibodies are candidates for the development of immunoliposome-based targeted prostate cancer therapeutics.

Intrinsic Antiproliferative Activity of CaP Binding scFv. It is likely that some of the surface molecules bound by internalizing CaP scFv are receptors, transporters, or adhesion molecules that mediate important physiological processes of tumor cells. We thus hypothesized that a subset of the scFv might have intrinsic antiproliferative activity. To test this hypothesis, the ability of CaP-specific internalizing scFv to inhibit CaP cell proliferation *in vitro* was assessed. PC3 and DU-145 cells were incubated with various concentrations of highly purified, soluble native A33 or M9E4 scFv, and cell proliferation was assessed by the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Both scFv showed dose-dependent growth inhibition, with IC_{50} s between 0.5 and 1.8 μ M, with a control scFv showing no inhibitory effect (Fig. 3). No inhibitory effect was observed on BPH-1 cells. These inhibitory scFv are candidates for the development of naked antibody-based therapeutics.

Immunohistochemistry. There is some controversy in the literature as to how well cell lines actually represent patient tumors, because cell lines may have undergone genetic and physiological changes during *in vitro* culture. To further address the relevance of the CaP-specific antibodies to human prostate cancer, immunohistochemical studies were performed on tissue sections from primary prostate tumor of high Gleason grades. Fig. 4 shows the staining results of the A33 (scFv)₂ dimer antibody on two different tissue specimens obtained from Gleason 3 + 4 patients (Fig. 4, A and B). There is intense staining of tumor epithelium, with minimal staining of normal adjacent prostate epithelium (Fig. 4C), normal breast epithelium (data not shown), or normal colon epithelium (Fig. 4D). A total of 20 high-grade prostate cancer patient samples have been examined, and pos-

itive A33 staining patterns were observed in 18 of 20 cases. A total of eight CaP-specific scFv antibodies have been subjected to immunohistochemistry studies on frozen tissue slides, six of which showed specific reactivity to prostate cancer epithelium. These experiments indicate that antibodies obtained from selection on tumor cell lines bind antigens that exist in patient samples and thus are clinically relevant to human prostate cancer. The corresponding antigens are overexpressed in prostate cancer and are likely targets for therapeutic intervention.

DISCUSSION

Mapping Tumor Cell Surface Epitope Space by a Direct, Antibody Library-based Approach. Tumor-specific cell surface markers are invaluable for the development of targeted oncologic therapeutics because of their relatively easy accessibility to targeting molecules. Differential gene expression-based approaches have been used widely for discovery and identification of these markers, but these approaches have significant limitations: (a) the level of mRNA transcript production does not always correlate with that of protein expression (32); and (b) neither cDNA microarray nor other gene expression-based approaches can profile neoplastic changes in glycosylation or other post-translational modifications. Such changes have been shown to play an important role in tumor metastasis (33) and may be critical determinants in modulating active antitumor immunity. Thus, to analyze the entire epitope space on the tumor cell surface, alternative methodologies must be explored and developed.

For this work, a functional approach was taken to tumor-cell epitope mapping; specifically, we sought to identify tumor-specific epitopes not readily predicted or identified from microarray or other gene expression-based analyses. A large nonimmune phage antibody library was constructed and selected directly on the tumor cell surface to generate CaP-specific antibodies. The nonimmune antibody library functions as an unbiased random shape repertoire potentially capable of recognizing any shape of antigen on the tumor cell surface. Unbiased repertoires should provide more complete coverage of the tumor

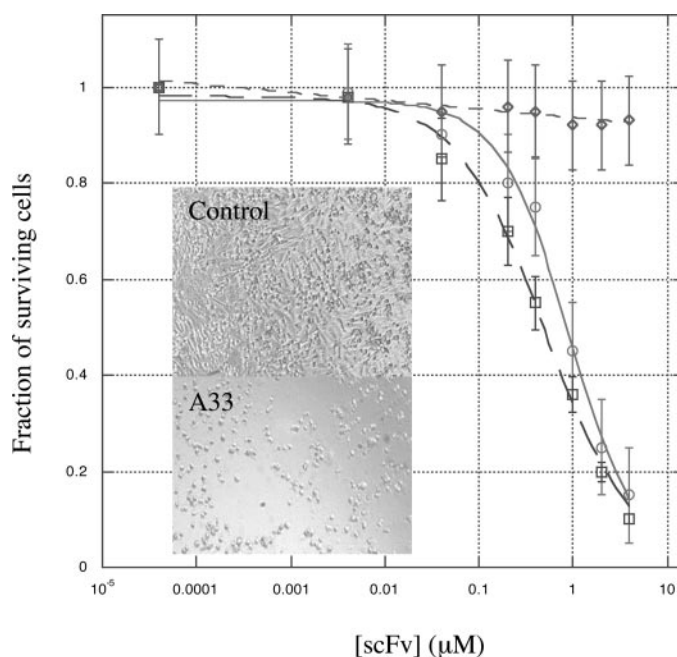


Fig. 3. Cell proliferation assay. Purified scFv were incubated with PC3 cells. Circle, M9E4 scFv; square, A33 scFv; diamond, irrelevant, anti-hapten scFv. Insert, PC3 treated with PBS (Control) and 1.8 μ M A33. Bars, SD.

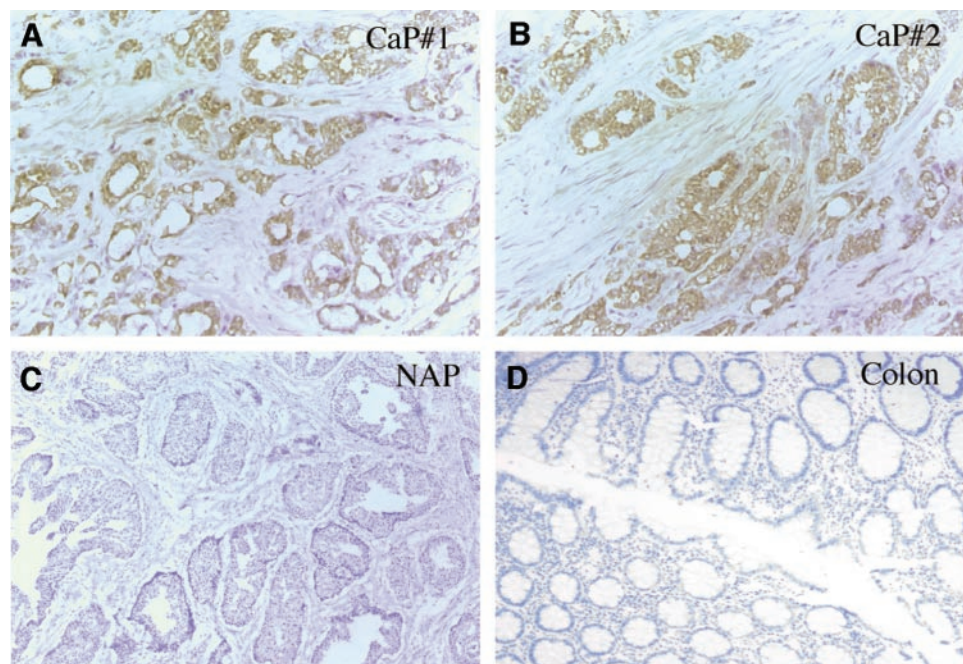


Fig. 4. Immunohistochemistry studies of scFv binding to prostate cancer tissue. Frozen tissue sections were stained with A33 (scFv)₂ using horseradish peroxidase-diaminobenzidine, and the tissues were counterstained with hematoxylin. A and B, staining on sections of prostate cancer, Gleason scale 4 + 3, from two different patients (*CaP#1* and *CaP#2*). C, staining on *NAP*, normal prostate tissue adjacent to prostate cancer. D, staining on normal colon epithelium. Magnification, 12.5 × 10.

epitope space, compared with immunization and hybridoma technology, which typically yield multiple antibodies against a few dominant epitopes.

Although phage libraries have been used previously to identify tumor-specific antigens, the number of specific mAbs isolated has typically been small (20, 23, 34, 35). Previously used antibody libraries were constructed in phagemid vectors that on average have only a single scFv molecule displayed on each phagemid particle. For this work, a multivalent antibody library was used where three to five identical scFv molecules are displayed per phage. Multivalent display increases both the efficiency of depletion of nonspecific antibodies and the positive selection of specific antibodies attributable to the increased binding from avidity effects of the multivalent phage antibody to the target cell surface (27, 28). Moreover, the selection methodology was devised to isolate antibodies that trigger receptor-mediated endocytosis (23) and are thereby delivered directly into the tumor cell. This not only selects for antibodies with desirable biological effects that can be exploited for intracellular drug delivery (22) but also leads to more efficient selection of phage libraries (21). Because many receptors require dimerization or cross-linking for efficient internalization, the use of a multivalent phage display library increases the efficiency of phage internalization through receptor cross-linking (21). This allows more efficient recovery of phage antibody binding to more epitopes on more receptors and probably accounts for the much larger number of antibodies generated in this study, compared with the same antibody repertoire displayed monovalently and selected on breast tumor cells (23).

A Panel of Novel CaP-specific Monoclonal mAbs. Direct cell selection yielded >90 phage antibodies that bind specifically to prostate cancer cells, including hormone-refractory ones. Profiling by flow cytometry on a panel of normal and tumor cell lines revealed that the majority of CaP-specific antibodies display binding patterns different from those of known tumor antibodies and therefore likely recognize novel cell surface antigens. Because only a few thousand clones were screened from the second-round output of 5×10^5 antibodies, it is likely that a much larger number of tumor-specific cell surface epitopes and antibodies remain to be identified. It is possible that a phage antibody that is unique in sequences may nevertheless

bind to the same epitope, effectively reducing the size of the tumor epitope space in our estimation. We have performed competition experiments with 15 unique phage antibodies by examining phage binding to target cells in the presence of soluble scFv of a different sequence. In all cases examined, there were no significant, dose-dependent competitions except when the phage antibody was coincubated with soluble scFv of the same sequence (data not shown). Although this type of analysis has not been performed on all 93 CaP-specific phage antibodies, we conclude, based on the available data, that the majority of those antibodies recognize different epitopes. We have therefore not grossly overestimated the size and complexity of the tumor-specific epitope space.

Our study points to a significant up-regulation of antigenic determinants on the surface of prostate cancer cells. This contrasts with previous studies using cDNA microarrays that suggest that a global transcriptional repression mediated by the polycomb gene *EZH2* is the principal force driving prostate cancer development (36). Our results indicate that functional, proteome-based approaches provide complementary information on the immunochemical features characteristic of cancer cells that may not be revealed by gene expression-based studies.

Phage Antibodies Are Efficiently Endocytosed. All phage antibodies studied possessed the phenotype that the selections were designed to capture, the ability to trigger receptor-mediated endocytosis. Purified native CaP-specific scFv were rapidly internalized into tumor cells and were capable of delivering nanoparticles (liposomes) specifically into prostate cancer cells. Drug-loaded immunoliposomes must be endocytosed for antitumor activity to provide both direct killing of tumor cells and bystander killing by diffusion of small-molecule drugs to neighboring tumor cells (37). Immunoliposomes can be constructed from scFv (22) and can be designed to have a long circulating half-life and to be nonimmunogenic (30). Other strategies can be designed to use internalizing antibody fragments for targeted tumor therapeutics. These applications include intracellular delivery of small molecule drugs via direct conjugation, cytotoxic gene fusions, immunotoxins, viral and nonviral gene delivery vehicles, and radionuclides (1).

Phage Antibodies Have Intrinsic Antitumor Activities. A subset of CaP-specific scFv had intrinsic antitumor activity, inhibiting proliferation of prostate cancer cells *in vitro* at submicromolar concentrations. This antitumor activity has not been typically observed with scFv isolated previously from cell selections of phage antibody libraries (19). This direct antitumor activity may have resulted from selecting for antibodies that trigger internalization and that are likely to bind to biologically active receptors, transporters, or adhesion molecules that may play significant roles in tumor physiology. Such antigens, in effect, may not be merely byproducts of tumor transformation but rather may play an integral role in this process. On the basis of this investigation, it appears that antibody endocytosis can be used as a surrogate marker for selection of antibodies with direct, intrinsic antitumor activity.

Clinical Relevance to Human Prostate Cancer. A panel of >90 internalizing antibodies was generated that can be used therapeutically, either for intracellular drug delivery or as naked antibodies with direct antiproliferative effects. These phage antibodies may also be useful diagnostically, and they may provide the means to identify novel tumor antigens that might be vaccine candidates or important new targets for the treatment of prostate cancer. Currently, few monoclonal antibodies exist that specifically recognize CaP cells, and fewer still are specific for hormone-refractory cells. These include antibodies to a number of cell surface molecules including PSMA (4), prostate stem cell antigen (PSCA; Ref. 9), PSGR (6), STEAP (7), and hepsin (5). PSMA was isolated via murine immunization with membrane preparations of LNCaP cells. The other markers were identified during various studies of differential mRNA expression. Although originally considered to be prostate or prostate cancer specific, follow-up studies have found that expression of these markers in many cases is actually less restricted (5, 9).

Phage selections were performed on established CaP cell lines. PC3 and DU-145 cells do not express PSA or androgen receptor and exhibit androgen-independent growth. LNCaP cells, on the other hand, retain PSA and androgen receptor expression and remain dependent on androgens. These diverse phenotypes were used because they may reflect distinct stages of prostate cancer development. It is possible that such cell lines cultured *in vitro* have undergone physiological and genetic changes and do not truly represent human prostate cancer *in vivo*. Tissue staining with CaP-specific single-chain antibodies on frozen tissue was performed to observe binding patterns *in situ* within tumors and identify those antibodies that are relevant to human prostate cancer. The results show that antibody obtained from cell line studies may be highly relevant clinically, consistent with previous observations (38).

Several challenges remain to be addressed for the development of these antibodies in therapeutic applications. Foremost is the identification of the antigen bound by the antibodies. Although we have been able to immunoprecipitate small amounts of antigen with some of the scFv, immunoprecipitation with high-avidity IgG based on these scFv is the method of choice. Toward this objective, full-length IgG are being constructed from the V genes of six of the scFv with the most specific staining patterns by immunohistochemistry. IgG will be used for immunoprecipitation, and the antigen bound can be readily identified by mass spectrometry. Construction of IgG will overcome two other scFv limitations: their use for *in vivo* studies of antitumor activity, which are precluded by the small size and rapid clearance of scFv, and their limited use in immunohistochemistry. In the future, we anticipate that use of the IgG will allow antigen identification, determination of its temporal and spatial pattern of expression, and determination of direct *in vivo* antitumor activity. Such data combined with *in vivo* studies of the drug delivery ability of the antibodies (22) should result in the identification of new antigens and targets for

metastatic prostate cancer and the development of therapeutically useful antibodies for improved clinical strategies.

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