Disulfide-constrained peptides that bind to the extracellular portion of the prostate-specific membrane antigen

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Abstract

The prostate-specific membrane antigen (PSMA) is a well-characterized surface antigen, overexpressed in the most advanced, androgen-resistant human prostate cancer cells. We sought to exploit PSMA cell surface properties as a target for short peptides that will potentially guide protein-based therapeutics, such as viral vectors, to prostate cancer cells. Two separate phage display peptide strategies were applied, in parallel, to purified PSMA protein bound to two separate substrates. We reasoned that peptide sequences common to both substrate selections would be specific binders of PSMA. Additionally, the design allowed for stringent cross-selections, where phage populations from one selection condition could be applied to the alternative substrate. These strategies resulted in a series of phage displayed peptides able to bind to PSMA by ELISA and direct binding assays, both with purified protein and in prostate cancer cells. Cell binding is competitively inhibited by purified PSMA. The synthesized peptides are capable of enhancing PSMA carboxypeptidase enzymatic activity, suggesting protein folding stabilization. The discovery of these peptides provides the foundation for subsequent development of peptide targeted therapeutics against prostate cancer. [Mol Cancer Ther 2004;3(5):597–603]

Introduction

Tumor antigens provide a promising means to target therapeutic agents, theoretically resulting in lower dose treatments, enhanced therapeutic efficacy, and minimized side effects. For prostate cancer, the prostate-specific membrane antigen (PSMA) is considered among the most promising cell surface markers. We describe a phage display strategy applied to isolate small, cysteine-constrained, PSMA binding peptides that will potentially target viral gene therapy vectors to prostate tumor cells.

PSMA is a prostate cell surface glycoprotein with markedly enhanced expression in prostate cancer cells (1). PSMA expression is ubiquitous, with expression in nearly all tumor sites (2). Unlike most prostate markers, PSMA expression is increased in the absence of androgens, with the highest levels seen in the most advanced androgen-resistant prostate cancers (3). Additionally, in normal prostate tissue, alternative splicing localizes the majority of PSMA protein in the cytoplasm, where it is known as PSM′, where in prostate cancers the majority is displayed on the cell surface (4). This ratio of cell surface PSMA to cytoplasmic PSM′ increases with Gleason grade (5). These properties have made PSMA an ideal target for developmental prostate cancer imaging agents and therapeutics, especially in advanced disease.

Here, we applied a stringent phage display strategy to identify potential PSMA binding peptides. A fusion protein containing only the extracellular portion of PSMA was immobilized to two separate resins to carry out two independent, parallel phage display strategies. Selected peptide libraries from each resin background were then applied to the complementary PSMA resin, termed a cross-selection, to stringently select only those peptides that bound PSMA. The resulting peptides bind purified PSMA, stabilize the protein to enhance enzymatic activity, and target phage to prostate cancer cells.

Materials and Methods

Materials

The M13 phage display library, containing the insert CX_7C, and ER2738 bacteria were acquired from New England Biolabs (Beverly, MA). S-tag agarose beads, CBind cellulose resin, recombinant enterokinase, recombinant enterokinase capture beads, and S-tag assay components were acquired from Novagen, Inc. (Madison, WI). LNCaP and PC-3 cells were obtained from the American Type Culture Collection (Rockville, MD). Ready plaque Sf-9 cells were obtained from Novagen. Anti-M13 monoclonal antibody, horseradish peroxidase/anti-M13 monoclonal antibody, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were acquired from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). 7E11-C5 antibody was produced from the media of cultured hybridomas (American Type Culture Collection). Peptides were synthesized without additional flanking amino acids, oxidized, and high-performance liquid chromatography purified (SynPep Co., Dublin, CA). Peptide compositions were as follows: R5-XC1: CQKHHNYLC, C_1-C_9 disulfide; R4-C9: CTLVPHTRC, C_1-C_9 disulfide; and Arg-Gly-Asp (RGD): GRGDTP. Peptide stock solutions were 1 mg/ml in distilled water.

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PSMA Immobilization and Purification

The PSMA fusion proteins, tag-xPSM and xPSM, and their purification have been described previously in detail (6). Tag-xPSM is a secreted baculoviral fusion protein consisting of the entire extracellular portion of PSMA with two amino-terminal affinity tags, a cellulose binding domain (CBD) and an S-tag. Enterokinase digestion releases both affinity tags, producing xPSM. For purification, ready plaque Sf-9 cells were plated as monolayers and infected with recombinant Tag-xPSM baculovirus at a multiplicity of infection of 5 plaque-forming units (pfu)/cell. Infected cell media was harvested 72–80 h postinfection and recombinant protein levels were quantified by S-tag assay. Prior to purification, S-protein agarose beads were washed several times in bind/wash buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl] to remove all EDTA. Fusion protein was bound to S-protein agarose using a ratio of 1 ml S-protein beads/500 μg fusion protein for 12–18 h at room temperature. For cellulose immobilization, CBind cellulose resin was resuspended in distilled water and incubated with fusion protein at a ratio of 40 μg fusion protein/1 mg resin and allowed to bind for 12–18 h at room temperature. CBind resin and S-tag beads were then washed thrice with bind/wash buffer, resuspended in 1 ml aliquots, and quantified by S-tag assay and Bradford assay. These beads were then stored at 4°C until use in binding or phage selection experiments. To release soluble xPSM, S-tag agarose immobilized Tag-xPSM was treated with 10–20 units of recombinant enterokinase/500 μg fusion protein at 37°C for 4–16 h. Recombinant enterokinase was then captured for 10 min at room temperature by EKapture agarose beads using a ratio of 1:1 (μl beads/μg fusion protein). The resulting protein, xPSM, contains no affinity tags and is over 95% pure by evidence of silver staining.

Tag-xPSM Phage Display

S-protein agarose [100 μl; 2× in RPMI 1640 + 1% fetal bovine serum (FBS)] and CBind resin (100 μl; 10 mg/ml in RPMI 1640 + 1% FBS), without fusion protein, were used to negatively select the original phage display library; 2×10^{11} pfu of 1.29×10^{9} different sequence phages were incubated with each aliquot of resin for 1 h at 37°C while rotating. The S-tag agarose was pelleted and supernatant was saved as the agarose counterselected pool. The cellulose resin was transferred onto 200 μl of 9:1 dibutyl phthalate/cyclohexane and the resin was pelleted through the organic phase. The aqueous supernatant was saved as the cellulose counterselected pool. Each counterselected pool was then added to tag-xPSM agarose or cellulose resin and incubated for 1 h at 37°C while rotating. The tag-xPSM agarose was pelleted and washed thrice in RPMI 1640 + 1% FBS. The agarose pellet was then saved for phage isolation. The tag-xPSM cellulose was transferred onto 200 μl of 9:1 dibutyl phthalate/cyclohexane and the resin was pelleted through the organic phase. The tube was then snap frozen in liquid nitrogen to isolate the resin pellet. Over 75% of tag-xPSM remained bound to the cellulose resin through these conditions (data not shown). The resulting phage-bound tag-xPSM agarose and tag-xPSM cellulose were then resuspended in 200 μl of log-phase ER2738 cells and phages were allowed to infect 1 h at room temperature. A small aliquot was taken at this time to quantify the number of phage bound per round of selection. The remaining PSMA-bound phages were then amplified, titered, and applied to the next round of phage display. There was only one counterselection round followed by a total of five positive selection rounds, applying between 10^{10} and 10^{11} pfu/round. The final round of selection included cross-selections, where selected phage pools from one PSMA background were applied to the other. Individual phages were isolated and sequenced from rounds 3 to 5.

Sequencing and Analysis

Individual phage clones were amplified for 5 h in ER2738 cells at 37°C. Amplified supernatant was incubated 1:1 with polyethylene glycol/NaCl buffer (20% polyethylene glycol 8000, 2.5 M NaCl) for 10 min at room temperature. Phages were pelleted 10 min at 20,000 × *g* at 4°C. The phage pellet was suspended in 100 μl iodide buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 M NaI]. Single-stranded DNA (ssDNA) was precipitated with 250 μl of 100% ethanol, pelleted, and washed in 70% ethanol. Each ssDNA pellet was suspended in 30 μl 10 mM Tris (pH 7.4) and sequenced using the Amersham DYE ET mix by incubating 6 μl ssDNA, 1 μl of 5 μM −96 pIII sequencing primer (CCCTCATAGTTAGCGTAACG), and 8 μl DYE ET mix in a 96-well PCR plate and amplified by 30 cycles of 95°C for 20 s, 50°C for 15 s, and 60°C for 1 min. Sequencing reaction products were spun through G50 fine-Sephadex column plates prepared on Millipore (Billerica, MA) multiscreen plates. Products were sequenced and analyzed on the MegaBACE capillary sequencer. All sequences, minus the flanking cysteines, were aligned using the Baylor College of Medicine MAP Global Progressive in Linear Space (http://searchlauncher. bcm.tmc.edu/multi-align/multi-align.html) with alignment by Boxshade 3.2 (http://www.ch.embnet.org/software/BOX_form.html).

ELISA

Tag-xPSM protein was immobilized on 96-well tissue culture plates by the incubation of 200 μl baculovirus infected Sf-9 cell supernatant/well overnight at 4°C. The supernatants were discarded and the plate was then blocked for 30 min in PBS (pH 7.4) + 1% BSA at room temperature. The blocking solution was then discarded. Phages (1×10^{10}) diluted in PBS + 1% BSA were then added to each well and incubated for 1 h at room temperature in a total volume of 200 μl. Each well was washed five times in PBS + 0.05% Tween 20 followed by incubation with 200 μl 1:5000 horseradish peroxidase/anti-M13 monoclonal antibody (in PBS) for 1 h. Wells were again washed five times in PBS + 0.05% Tween 20 and signal was detected by incubation in 1× ABTS stock solution [100 mg ABTS in 450 ml 0.05 M citric acid (pH 4.0)] and absorbance reading at 410 nm. Binding assays were executed in triplicate and have been repeated. Data represent the average result with error bars as SE. Significance was calculated by two-tailed, two-sample, equal variance *t* test.

N-Acetylated α-Linked Acidic Dipeptidase Assays

N-acetyl-aspartyl-glutamate (NAAG) hydrolysis was performed essentially as described previously (7). In short, 65 ng purified xPSM was resuspended in *N*-acetylated α-linked acidic dipeptidase (NAALADase) buffer [50 mM Tris (pH 7.4), 0.5% Triton X-100] and incubated in the presence of peptides or inhibitors (1, 10, and 100 μM) along with the radiolabeled substrate *N*-acetyl-L-aspartyl-L-[3,4-^{3}H]glutamate (NEN Life Science Products, Boston, MA) at 30 nM in a total volume of 50 μl. Reactions were incubated at 37°C for 10 min. For whole cell assays, 10,000 cells/well were plated in 96-well culture plates and allowed to adhere overnight. Cells were washed and then incubated in the presence of 100 μM peptides and 30 nM tritiated NAAG in NAALADase buffer for 10 min at 37°C. The reactions were stopped by the addition of an equal volume of ice-cold 100 mM sodium phosphate, 2 mM EDTA. Products were separated from intact substrate using AG 1-X8 formate resin (Bio-Rad Laboratories, Hercules, CA) anion exchange chromatography. The reaction product, tritiated glutamate, was eluted with 2 ml 1 M sodium formate and quantified by scintillation counting. Intact NAAG remained in the column resin. Experiments were executed in triplicate and have been repeated. Experiments were designed to allow 20% or less of the total substrate to be cleaved.

Cell Binding Analysis

Cell monolayers were washed several times in PBS and incubated 5 min in PBS at 37°C. Cells were knocked free, pelleted, and suspended in RPMI 1640 + 1% FBS at a concentration of 10^6 cells/ml; 10^8 pfu from each clone were incubated with 100 μl cells at 37°C for 20 min while rotating. For competitive inhibition, purified xPSM, BSA, or peptides were added to the cells along with 10^9 to 10^{10} pfu of phages. The cells were then centrifuged through 200 μl of 9:1 dibutyl phthalate/cyclohexane, described previously as biopanning and rapid analysis of selective interacting ligands (BRASIL) partitioning (8). The tube was then snap frozen in liquid nitrogen to isolate the cell pellet. The cell pellets were then suspended in 200 μl log-phase ER2738 cells and phages were allowed to infect 1 h at room temperature. The number of cell-bound phages was quantified by serial dilution titer. Competitive binding experiments were executed in triplicate.

Results

PSMA Binding Increased with Selection Rounds

We have described previously a highly pure, NAALADase-active, extracellular PSMA fusion protein termed tag-xPSM, which contains two affinity tags for immobilization or purification (6). Here, tag-xPSM was immobilized on two separate solid substrates: S-protein agarose beads through the S-Tag and cellulose resin through the CBD tag. Two independent phage display strategies were then carried out on these substrates, in parallel, both applying the same pIII-displayed disulfide-constrained heptamer peptide library (Fig. 1). Traditional phage

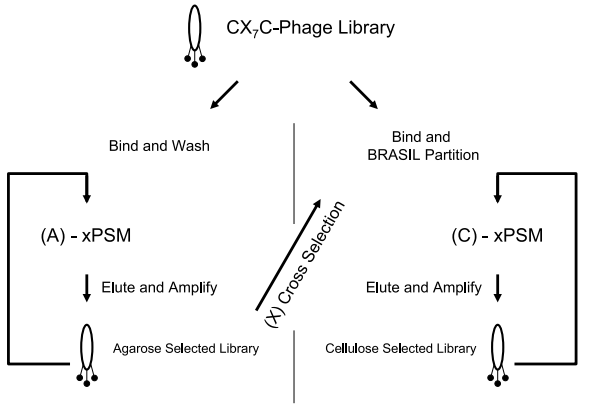

Figure 1. Schematic of phage display strategy. A single disulfide-constrained heptamer peptide phage display library was selected, in parallel, to bind a PSMA fusion protein immobilized to two separate substrates. *Left,* tag-xPSM was immobilized to S-protein-conjugated agarose beads via the S-tag. *Right,* tag-xPSM was immobilized to cellulose resin via the CBD tag. Four rounds of binding, washing, and partitioning by centrifugation were applied to the agarose-bound tag-xPSM. Four rounds of binding and BRASIL partitioning were applied to cellulose-bound tag-xPSM. The fifth and final round was a cross-selection, where the agarose-xPSM selected library was applied to cellulose-xPSM and vice versa.

display techniques were applied to agarose-bound tag-xPSM, and a modified BRASIL technique (8), originally designed for isolating cell-binding phages, was applied to cellulose-bound tag-xPSM. The BRASIL technique has been shown to reduce nonspecific background binding and result in higher sensitivity for cell surface targeted phage display selections. We found that tag-xPSM immobilized to cellulose, but not agarose beads, remained resin bound after partitioning through the organic solvents and could therefore be targeted in a BRASIL-like strategy. Thus, two separate selection resins and partitioning strategies were applied to the same target. Four rounds of binding, partitioning, and amplification were applied to each system. The fifth and final round included a cross-selection, where selected pools from agarose were applied to cellulose and vice versa.

The stringent selection conditions produced increased phage pool binding with each round of selection on the agarose-bound tag-xPSM (Fig. 2). The final cross-selection round onto cellulose-bound tag-xPSM resulted in a significant increase in the number of phages bound. The inverse selection, starting on cellulose resin and ending on agarose, resulted in considerably less phage binding after the cross-selection and as a result was not pursued any further.

Sequences from Selected Phage Pools

Twelve phages from rounds 3–5 of each selection condition were cloned and the peptide coding regions were sequenced. The most stringent selection condition, the cross-selection of the agarose selected pool onto cellulose immobilized tag-xPSM in round 5, resulted in 12 of 12 peptides with the same sequence (Fig. 3A). Similar sequences were seen in previous rounds; however, the peptide sequence CQKHHNYLC, termed R5-XC1, was

unique to this final round. A second sequence, CTITSKRTC, was found in both agarose and cellulose selected pools. This sequence, termed R5-C6, was found a total of 12 times with increasing representation in advanced rounds (Fig. 3B). Finally, a third sequence termed R4-C9, CTLVPHTRC, was identified in five clones from late rounds of both agarose and cellulose pools. Alignment reveals some weakly similar sequences, providing the consensus VPHTR (Fig. 3C). The collective alignment of all sequences results in three groupings, each headed by a multicopy peptide R5-XC1, R5-C6, or R4-C9 (Fig. 3). The presence of these three sequences in numerous selection rounds and alternate substrate backgrounds suggests that they bind the PSMA protein and not the background resin.

Individual Selected Phages Bind PSMA Protein

Individual phage clones were tested for the ability to bind the tag-xPSM fusion protein bound to polystyrene microtiter plates in an ELISA. Clones R5-XC1 and R4-C9 bound significantly better than insertless phages with $P = 0.009$ and 0.005, respectively (Fig. 4). Clone R5-C6 did not bind tag-xPSM in this assay; however, a similar clone from its homology grouping, R3-A4 (CFPQSSARC), bound with significance ($P = 0.0001$).

Purified R5-XC1 and R4-C9 Peptides Bind and Stabilize PSMA Enzyme Activity

Enzyme assays often provide a sensitive means to analyze protein-ligand interactions. The enzymatic activity of PSMA has been well studied and used previously to demonstrate PSMA-ligand interactions with high sensitivity (6). To investigate whether the purified peptides retained PSMA binding ability apart from the phages, they were synthesized and analyzed for their effect on PSMA NAALADase activity. Quisqualic acid, a potent NAALADase inhibitor, significantly inhibited xPSM NAALADase activity (Fig. 5). However, peptides R5-XC1 and R4-C9 enhanced, rather than inhibited, PSMA enzyme activity in a dose-dependent manner. These data suggest that peptides bind and stabilize PSMA protein. Peptide enhancement is significant with $P = 0.0001$ (100 μM R5-XC1), $P = 0.0001$ (100 μM R4-C9), $P = 0.015$ (10 μM R5-XC1), and $P = 0.024$ (10 μM R4-C9). A negative control RGD peptide had no effect on enzyme activity. The PSMA specific peptides enhanced enzymatic activity on both purified xPSM protein (Fig. 5) and whole cell LNCaP NAALADase assays (Supplemental Fig. 1).[2] The

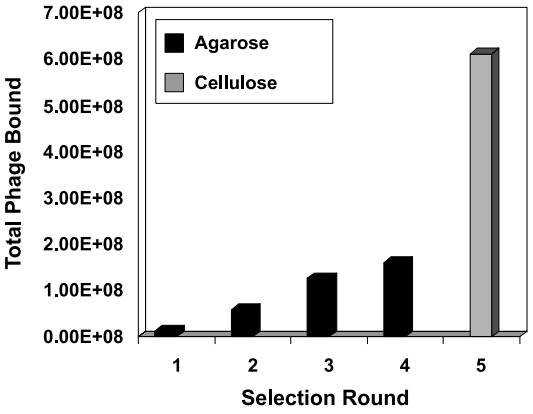

Figure 2. Increased phage binding per round of tag-xPSM selection. 2×10^{11} pfu (*rounds 1*, *2*, and *3*) or 2×10^{10} pfu (*rounds 4* and *5*) of phages were incubated with tag-xPSM protein bound to agarose beads (*rounds 1*, *2*, *3*, and *4*) followed by a cross-selection onto tag-xPSM bound to a cellulose (*round 5*) substrate. After partitioning, bound phages were quantified by serial dilution titer. Each round of selection resulted in an increased number of phages bound.

A

Clone	Copies	Sequence
R5-XC1	12	---QKHHNYL----
R3-A9	1	----QRHDYPA---
R4-C10	1	------HKFQSKM-
R4-C18	1	-------DAVRYPV
R4-C6	1	LKSHSHQ-------
consensus		---qkhhnyl----

B

Clone	Copies	Sequence
R5-C6	12	---TITSKRT
R4-C16	1	---TPLSPRY
R5-C2	1	--QTPYDLR-
R3-A4	1	-FPQSSAR--
R3-A6	1	-PPDRSAN--
R4-C8	1	GLPTRTA---
R3-C5	1	---PIPGLRQ
R4-C17	1	-KPTNQHK--
R3-A1	1	--PIMLSER-
R5-C10	1	-KPSMMSY-
R3-C8	1	-KPNSQPW--
consensus		--ptitskrt

C

Clone	Copies	Sequence
R4-C9	5	-----TLVPHTR----
R5-C7	1	----STRAPHL-----
R4-C3	1	----------HTSLKTH
R5-C8	1	----------HTKHASH
R3-A5	1	------QFRHSAQ---
R3-A2	1	------KLQHSST---
R3-C2	1	------HRLHSTS---
R3-A12	1	----------HTTTDVY
R5-C11	1	----------PSVNTKQ
R4-C1	1	---------KHSVSPS-
R5-C9	1	---SSHSTVH------
R3-A7	1	----TNSNMHH-----
R4-A2	1	---APNKYKH------
R3-A3	1	-----NKTTHYA----
R5-C3	1	---SHNDTRH------
R3-C1	1	--LSSNSSL-------
R3-C12	1	--TSNNSRI-------
R3-A10	1	--SSTNSKL-------
R3-C10	1	LTSSVNF---------
R4-C4	1	LSTTISY---------
consensus		---s-n-vphtr----

Figure 3. Sequence alignments of PSMA binding peptides. The collective alignment of all sequences from rounds 3 – 5 resulted in three separate groups, each one headed by a multicopy number peptide. Clones are named by round (*R#*), background [agarose (*A*) or cellulose (*C*)], and clone number. Multicopy peptides are named by a single sequence.

[2] Supplemental material for this article can be found at MCT online (http://mct.aacrjournals.org).

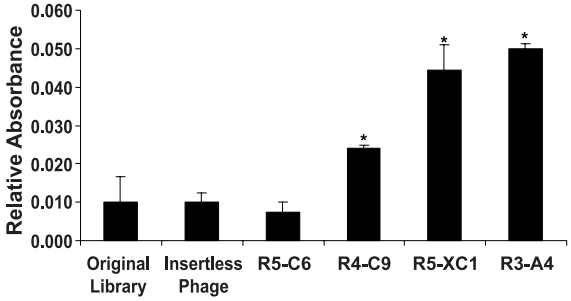

Figure 4. Selected phage clones bind purified extracellular PSMA. Tag-xPSM was immobilized on microtiter plates and targeted for binding with 10^{10} pfu of each individual phage. Bound phages were quantified by ELISA absorbance at 410 nm. R4-C9, R5-XC1, and R3-A4 bound with the following significance compared with insertless phage: $P = 0.005$, 0.009, and 0.0001, respectively.

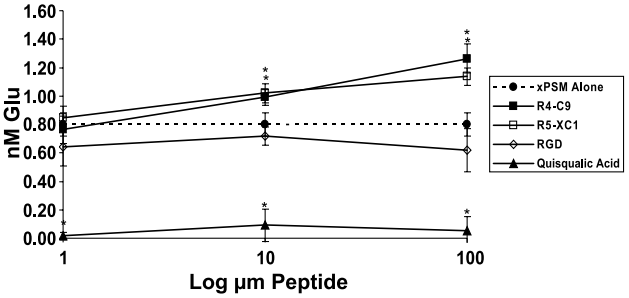

Figure 5. PSMA peptides dose-dependently enhance NAALADase activity. Purified xPSM, lacking the affinity tags, was assayed for NAALADase activity in the presence of 1, 10, and 100 μM concentrations of the selected peptides, a control RGD peptide, or quisqualic acid. The known NAALADase inhibitor, quisqualic acid, strongly inhibited NAALADase activity at low micromolar concentrations. The selected PSMA binding peptides R5-XC1 and R4-C9 enhanced xPSM NAALADase activity in a dose-dependent manner ($R^2 = 0.997$ and 0.988 respectively). Peptide enhancement is significant with $P = 0.0001$ (100 μM R5-XC1), $P = 0.0001$ (100 μM R4-C9), $P = 0.015$ (10 μM R5-XC1), and $P = 0.024$ (10 μM R4-C9).The control RGD peptide had no significant effect on xPSM activity.

enhanced carboxypeptidase activity could not be confirmed in phage backbone as phage concentrations similar to the applied peptide levels are not achievable in these volumes.

Individual Phages Bind PSMA Expressing Cells

Cloned phages were then tested for the ability to bind a prostate cancer cell line known to express high levels of the PSMA protein, LNCaP. Severalfold more R5-XC1 and R4-C9 phages bind when compared with earlier selection rounds 1 and 4 (Fig. 6). R5-C6 phage, which bound poorly in the ELISA assay, showed little improvement over the earlier rounds. Clone R3-A4 phage, which bound purified protein as well as R5-XC1, showed only moderate binding of LNCaP cells.

To demonstrate that cell binding is through the PSMA protein, the best binding phage, R5-XC1, was subjected to competition experiments with soluble xPSM (Fig. 7). Soluble xPSM significantly blocked R5-XC1 from binding LNCaP cells ($P = 0.014$) but had no effect on a control phage. A nonspecific protein, BSA, was unable to inhibit phage cell binding.

Discussion

Random peptide libraries displayed on the surface of filamentous phages can be successfully selected to identify ligand-binding sequences through repeated cycles of binding and amplification (9). *In vitro* selection systems, such as these, are very powerful and can potentially identify ligands to almost any target, including polystyrene resins (10). One must therefore carefully design a strategy to guide the library toward binding the target protein and not unwanted affinity tags or resins. Using a recombinant PSMA protein encoding the extracellular portion of PSMA and two removable affinity tags, we developed a phage display strategy to maximize the possibility of selecting target binding phages (Fig. 1). By carrying two simultaneous phage display selections, one applying traditional phage display techniques on tag-xPSM immobilized to agarose beads and another applying a modified BRASIL phage display technique on tag-xPSM immobilized to cellulose resin, we were able to later bind preselected libraries to the same target in two different backgrounds. This stringent cross-selection in round 5 resulted in a single PSMA binding sequence from the round 4 agarose pool (Fig. 3A). Additional peptides CTITSKRTC and CTLVPHTRC were found in multiple copies of round 4 for both agarose and cellulose selections, suggesting their specificity for PSMA. No other sequences were found in more than a single copy.

The alignment of all sequences did not indicate any consistent or significant consensus motifs but did result in three groupings of peptides, each headed by a multicopy peptide. The lack of consensus may be due to the high stringency of the cross-selection. Similar results were found in an aptamer *in vitro* selection strategy against PSMA (6). Potent NAALADase inhibitors have been designed previously by mimicking known substrates, such as NAAG (11–13). One may therefore expect aspartate- or glutamate-rich sequences to be preferentially selected in this report; however, polyglutamates were noticeably absent from

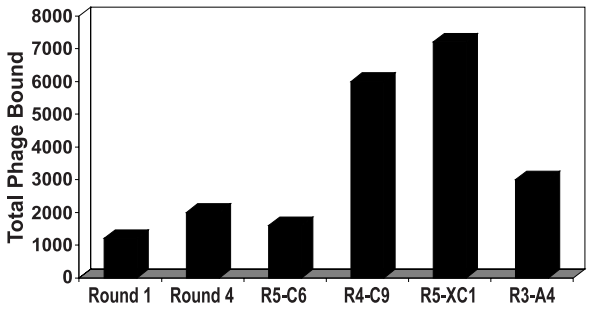

Figure 6. Selected phage clones bind PSMA expressing cells. 1.3×10^5 harvested LNCaP cells were incubated with 10^8 pfu of each individual phage for 20 min. Cell-bound phages were isolated by BRASIL partitioning and titered by serial dilution.

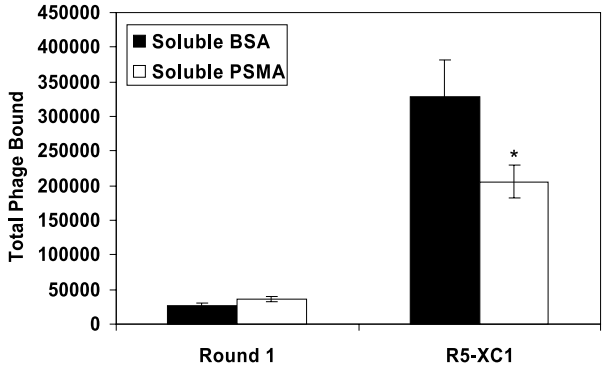

Figure 7. LNCaP binding by phage is specifically blocked with soluble PSMA. 10^{10} R5-XC1 phages or the *round 1* phage pool were allowed to bind LNCaP cells in the presence of 380 nM PSMA or 500 nM BSA. PSMA significantly blocks R5-XC1 phage binding ($P = 0.014$) but not the control phage. R5-XC1 bound LNCaP significantly better than the *round 1* phage pool in the presence of PSMA or BSA ($P = 0.000001$ and 0.000013, respectively).

selected sequences. For unknown reasons, the preferential binding site in this selection was that other than the active site as evidenced by the enzymatic activity in the presence of binding peptides (Fig. 5).

ELISA assays confirmed that clones of promising sequences were able to bind purified PSMA (Fig. 4). PSMA enzymatic assays demonstrated that two of these peptides were able to bind PSMA as free synthetic peptides, resulting in stabilized PSMA protein and therefore dose-dependent enhancement in enzymatic activity (Fig. 5). This was a surprising result as *in vitro* selection strategies often identify enzyme inhibitors by preferential binding to the rigid active site or ligand binding site. It is not unique as others have reported phage display ligand enhancement of the serine protease prostate-specific antigen (14). The enhanced NAALADase activity is not specific to purified protein as similar results were demonstrated on whole LNCaP cells (Supplemental Fig. 1).[2] While interesting, it is unlikely that the enhanced enzymatic activity of these peptides will have any therapeutic value for prostate cancer.

The most important criteria for a successful peptide is the ability to target phages to bind PSMA expressing cells. The two most promising phages, clones R4-C9 and R5-XC1, repeatedly targeted phage to the PSMA expressing cell line, LNCaP (Figs. 6 and 7). Further, soluble PSMA protein was able to block clone R5-XC1 from binding LNCaP cells, demonstrating that phage binding was through the cell surface PSMA protein (Fig. 7). Soluble peptides were unable to specifically inhibit phage cell binding at similar concentrations (data not shown). This could be due to peptide internalization, serum inactivation, or inability to saturate PSMA on the cell surface. Neither soluble PSMA nor soluble peptides were able to block phage binding to non-PSMA expressing cells (data not shown), confirming that the cell-phage interaction occurs at the peptide portion of the phage coat.

Because the peptides resulting from this selection will eventually be presented in a backbone other than the phage pIII protein, it was important to use a conformationally constrained peptide library. It is known that constrained or cyclic phage peptide libraries, engineered with flanking cysteines, present peptides with a stable backbone that are capable of binding their ligands outside of the phage coat protein (15), either in a different protein backbone (16) or while coupled to a therapeutic (17). Furthermore, cyclic peptide libraries have been found to identify higher affinity ligands (18). The estimated affinities of these peptides, based on the concentration of soluble PSMA required to inhibit cell binding by the multivalent phage and by the concentration of peptide required to enhance PSMA enzymatic activity, are in the micromolar range. If these affinities are too low to target therapeutics, the peptides will be further improved by affinity maturation, a technique known to significantly improve peptide affinities (19).

The concept of targeting prostate markers, rather than cancer markers, is attractive as the prostate is a nonessential organ and many cancer markers are found in normal tissues. While PSMA is not entirely prostate specific, with some expression in the brain, kidney, and small intestine, its levels are much lower in these tissues when compared with prostate cancer (20). Additionally, kidney and small intestinal PSMA protein is likely compartmentalized in the lumen and is therefore unavailable to circulating therapeutics. This is consistent with the lack of side effects seen in clinical trials with the PSMA targeted monoclonal antibody HuJ591 and its conjugates (21). Moreover, recent imaging results with the same antibody show specific targeting to tumor sites without apparent localization to noncancer sites (22). PSMA has also been shown to be expressed in the neovasculature of many solid tumors other than prostate cancer, furthering its value as a target for cancer therapeutics (23–25).

We have presented a stringent phage display strategy, which resulted in the identification of several disulfide-constrained PSMA binding peptides. These peptides bind and stabilize purified PSMA protein and are capable of targeting bacteriophage particles to prostate cancer cells. R5-XC1 and R4-C9 can be easily incorporated into existing protein-based therapeutic systems for targeting. The combination of targeting peptides with systems already specific for prostate cells, such as conditionally replicating oncolytic viruses, would theoretically further improve safety and specificity.

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