Phage Display Selection of Peptides That Affect Prostate Carcinoma Cells Attachment and Invasion

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BACKGROUND. Prostate cancer-specific proteins must be identified to serve as diagnostic and prognostic markers. Cell surface proteins are especially important, because they have potential utility as diagnostic markers and therapeutic targets. Identification of ligands for these proteins will allow use of these ligands as diagnostic and therapeutic tools and permit the investigation of receptor function. We performed a search for peptide ligands to prostate cancer cell-specific receptors.

METHODS. Peptide phage display library was used to isolate specific ligands to LNCaP prostate carcinoma cells receptors. Selected phage and cognate peptides were investigated for their cancer-related functions, such as the ability to interfere with cell adhesion, spreading, motility, and invasion.

RESULTS. Phage designated pg35, blocked spreading of LNCaP cells and their derivatives C4–2 and C4–2b. Cognate peptide did not inhibit spreading, but incubation of C4–2 and C4–2b cells with cognate peptide increased their affinity for endothelial cells and invasiveness. In addition, the peptide activates matrix metalloproteinase (MMP)-2 and 9 in C4–2 and C4–2b cells.

CONCLUSIONS. These results indicate that identified ligands may play a role in tumorigenicity and metastatic transformation of prostate cancer. To our knowledge, this is the first identification of a functional cancer-specific peptide ligand using the phage display approach. Prostate 47:239–251, 2001. © 2001 Wiley-Liss, Inc.

KEY WORDS: phage display; peptide ligands; metastasis

INTRODUCTION

Prostate cancer is a significant health problem, recorded as the leading cancer diagnosed and the second most common cause of death from cancer in North American men [1]. Unfortunately, current methods of predicting how rapidly a given prostate cancer will acquire metastatic potential and progress from an androgen-dependent to an androgen-independent state are unreliable. Consequently, prostate cancer-specific proteins must be identified in order to serve as reliable diagnostic and prognostic markers as well as targets for effective therapy. Many proteins playing a substantial role in other cancer types are functional in prostate cancer [2]. There is also a group of proteins whose expression and function are specifically associated with prostate cancer progression [3–6]. Some of them can be used for diagnostic purposes and some have prognostic value, while others can be used as targets for drug delivery. Since ideal prostate cancer markers and targets for drug delivery have not yet been identified, it is important to continue the search for proteins specifically associated with prostate cancer cells. Secreted and cell surface proteins are especially important among protein

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targets, because they have potential utility as both diagnostic markers and targets for drug delivery.

A variety of methods are currently employed for the isolation of genes and their products specifically expressed in particular cells or tissues; however, only a small fraction of differentially expressed genes code membrane-associated or secreted proteins. Therefore, direct identification of these proteins is preferable.

The selection of membrane-associated proteins specific for the cells of interest is often based on the initial identification of ligands for these receptors. Peptide-based compounds are the most likely ligand candidates. Selected ligands can be used to identify their receptors and as diagnostic reagents. In addition, these peptides may be conjugated with drugs and liposomes or be expressed on a viral surface for use in gene therapy [7].

The method exploited in this study, biopanning using peptide phage display, combines many useful features of other ligand searching techniques and also offers unique opportunities. Phage displayed random peptide libraries are generated by shot-gun cloning of random oligonucleotide segments at the 5’ end of the pIII or pVIII genes of filamentous phage. The encoded fusion protein is displayed on the surface of the phage. Combinations of such phage with random peptide sequence can be used to search for potential ligands. The sequence of the binding peptide is determined by sequencing the DNA insert in the fusion construct [8]. Sequences of selected peptides are then aligned for the identification of consensus sequences. Screening of protein databases may identify matches with consensus sequences [9]. The most direct and successful use of the peptide phage technology is the search for ligands of homogeneous target molecules [10,11]. In an extension of phage display technology, several groups have selected phage peptides for targets presented by heterogeneous molecular population like whole serum samples from clinically well-defined patients, with the goal of identifying specific epitopes without the need of previous knowledge of the nature of the antigen [8,9].

The peptide phage display approach has been used to search for ligands that bind epitopes on cell surfaces in vitro and in vivo. Phage with binding sequences specific for several cultured cell types have been selected from peptide phage libraries [12,13].

Biopanning with peptide phage display libraries has also been used to identify receptors exposed on the surface of endothelial cells in vivo. In these experiments, phage peptide libraries have been directly injected intravenously into mice with subsequent phage rescue from individual organs. Peptides capable of mediating selective localization of phage to brain and kidney blood vessels were identified by this approach [14]. Peptides capable of homing to tumor vasculature were also recognized by the use of this strategy. Furthermore, coupling of these peptides to doxorubicin enhanced its efficiency against human breast cancer xenografts [15].

Therefore, although targets in the examples described have been a mixture of cell surface receptors with different affinities and conformations, specific ligands for these targets were discovered by biopanning with peptide phage libraries. Identification of such specific peptide ligands allowed purification and characterization of their receptors on the cell surface and investigation of their possible role in cancer progression. For example, peptides able to bind selectively to mouse lung vasculature were used for the isolation of receptors by affinity chromatography [16]. In summary, selection of cancer-specific ligands and their epitopes with peptide phage display libraries is a powerful method that allows direct identification of peptide ligands, that in turn can be used for the discovery of cancer cell-specific surface proteins and for elucidation of their functions.

In this study, the human prostate carcinoma cell line LNCaP was used as a target for the selection of prostate carcinoma-specific ligands. The selection process was focused on the discovery of biologically active ligands. Selected phage were able to block spreading of LNCaP cells and their more aggressive derivatives on tissue culture plastic. Cognate peptide of the selected phage amplified attachment of C4-2 cells to HUVEC monolayers, induced heightened invasiveness of C4-2 and C4-2b cells through matrigel, and increased activity of matrix metalloproteinases (MMP)-2 and 9.

**MATERIALS AND METHODS**

**Cell lines, antibodies, and peptides.** Human prostate cancer cell lines LNCaP, PC-3, and DU145 were obtained from American Type Culture Collection (Rockville, MD). BPH-1, prostate epithelial cells (derived from the prostate with benign hyperplasia and immortalized with SV-40) were a kind gift of Dr. Simon Hayward, University of California at San Francisco. Cells were maintained in RPMI medium containing 10% FBS, 2 mM L-glutamine and antibiotics (Life Technologies, Gaithersburg, MD) 95% air, 5% CO2 at 37°C. C4-2 and C4-2b more aggressive derivatives of LNCaP cells were obtained from UroCor (Oklahoma City, OK) and were maintained in T-medium according to the recommendations of the contributor Dr. L.W.K. Chung. The C4 subline was derived by the co-inoculation of LNCaP and a nontumorigenic human osteosarcoma cell line into a
male athymic nude mouse. The host was castrated at 8 weeks and a tumor specimen was excised at 12 weeks (4 weeks after castration). This specimen was used for production of a second generation line, C4-2, maintained in a castrated male host. When these cells were injected into a castrated nude mouse, a primary tumor and metastases to lymph node and bone were produced. Cells derived from a bone metastasis were isolated and maintained as the cell line designated C4-2b [17]. Normal prostate bone were produced. Cells derived from a bone primary tumor and metastases to lymph node and which was designed by the insertion of random eight vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and maintained in the media provided by the company. Monoclonal anti-β1 antibodies C27 were a generous gift of Dr. WT Chen (SUNY at Stony Brook). The 4b4 anti-β1 antibodies were purchased from Coulter (Hialeah, FL). Other anti-integrin antibodies were obtained from Chemicon (Temecula, CA). Peptides were synthesized in the protein core facility of the University of Virginia.

Libraries. In this study we used a landscape library, which was designed by the insertion of random eight amino acids in N-terminal of pVIII coat protein as described in detail previously [18].

Biopanning. Biopanning was started with the cell line used for the depletion, followed by 3–4 rounds with cells of interest (tester). Briefly: 10 μl of the primary library (10^{11} colony forming units (cfu)/ml) in 5 ml of PBS was added to the confluent cell monolayer (depletor- normal prostate epithelial cells), grown in a 60-mm dish, and incubated for 2 hr on a rocker platform in a cold room. Culture medium containing unbound phage was transferred to another cell monolayer (tester-LNCaP) and cells were incubated for another 2 hr at 4°C. After four intensive washes of cells with PBS-0.2% Tween 20-bound phage were eluted from cells by incubation with 1 ml of elution buffer- 0.2 M Glycine-HCL pH 2.2 for 10 min at 4°C. The eluate was immediately neutralized by the addition of 150 μl of 1 M Tris-HCL buffer at pH 9.0. An aliquot of eluted phage was used for the microtitration, and the rest of the phage was amplified for next rounds of biopanning according to Smith et al. [19]. The ratio of output to input (number of cell-associated phage divided by the numbers of total phage applied to the cells) was calculated for each round. The final round of biopanning was defined after this parameter reached the value of 1%. That is a convenient and effective way to monitor the performance of the selection process. The sequences of DNA coding inserts were determined for 30 of the selected phage from the output of the last round of biopanning as described before [18]. Translated peptide sequences were aligned for further analysis.

Databases search. Matches of peptide consensus sequences with proteins in databases were examined using the BLAST and PMOTIF programs. Matched proteins were considered for further investigation dependently of the level of their homology and biological relevancy. Eukaryotic proteins with homologies of higher than 60%, which were able theoretically to serve as ligands for receptors on the surface of target cells or be expressed on the surface of potential partners for cell–cell interactions, were considered as candidates for further investigation.

Binding Assays

Immunofluorescence labeling of bound phage was performed according to the procedure described by Barry et al. [12] with some modifications. We used anti-M13 biotin conjugated polyclonal antibodies and streptavidin-FITC as secondary antibodies. For binding experiments, cells were incubated with phage for 2 hr in a cold room and were fixed immediately by 4% paraformaldehyde for 15 min at 4°C. When it was necessary to examine the ability of selected phage to be internalized, cells were incubated at 37°C for 3 hr, and immunomicroscopy was performed for permeabilized and non-permeabilized cells. To distinguish between surface bound and internalized phage, cells were examined under a confocal fluorescent microscope. Nuclei of cells in experiments with permeabilized cells were labeled with DAPI to help define position of internalized phage inside the cell relative to the nuclei. DAPI labeling was performed by the incubation of cells for 20 min at room temperature with PBS containing 5 μg/ml of DAPI.

For the second type of binding assays, phage attached to the cell surface were directly used for the infection of competent bacteria as described by Arap and Pasqualini [15]. This assay is quantitative and very useful in searching for potential phage binding competitors (relative peptides and antibodies). Target cells (10^{5}) were incubated with the selected phage (10^{8–9} cfu) and, if necessary, with a potential binding competitor (corresponding peptides or antibodies to candidate proteins) at 4°C. After three washes in PBS-Tween 20, cells were resuspended in PBS and 100 μl of starved cells were added. Starved cells were prepared as described by Smith et al. [19]. After 15 min of incubation, 400 μl of Luria broth (LB) with a low concentration of tetracycline (0.2 μg/ml) was added to the cells for the activation of tetracycline resistance gene. Cells were incubated in this solution for 45 min on a shaking incubator. After dilution in LB, 20 μl
 aliquots were plated on tetracycline containing Agar dishes. The next day colonies were counted. The number of colonies is proportional to the number of phage bound to cells.

**Cell adhesion.** Ability of selected phage and peptides to interfere with attachment of cells to different extracellular matrix substrates was examined by a cell adhesion assay as described previously [20]. Cells were preincubated with phage or peptides at concentrations $10^3$–$10^5$ cfu/cell and 1–100 μM, respectively, for 10 min at RT before plating.

**Cell spreading.** Ability of the phage and peptides to affect cell spreading was examined microscopically by counting the number of spread cells, defined as cells with decreased refractivity and formation of projections around the cell periphery as described [21] and total cells in microscopic field. Cells were preincubated with phage or peptides at concentrations of $10^4$–$10^5$ cfu/cell and 1–100 μM, respectively, for 10 min at RT before plating.

**Cell survival assay.** LNCaP cells plated on the tissue culture plastic with pg35 were assayed for the survival by the trypan blue exclusion assay and by the DAPI staining for the identification of necrotic and/or apoptotic cells. Briefly: 5000 cells per well were plated in 24-well clusters with $10^9$ phage particles added into 1 ml of growth media. Twenty-four hours later, cells were assayed by the trypan blue exclusion test. DAPI staining was performed as described above for the immunofluorescent microscopy of bound phage.

**Endothelium adhesion assay.** The ability of C4–2 cells to adhere to HUVEC monolayer was determined as follows. C4–2 cells ($1 \times 10^6$ cells) were harvested from tissue culture and fluorescently labeled by incubation for 15 min in 1 ml DMEM with 1% BSA and 3 mM calcein-AM (Molecular Probes, Eugene, OR). The cells were washed three times and $1 \times 10^5$ cells were layered in quadruplicate over monolayers of HUVEC growing in 96-well tissue culture plates and incubated for 30 min in a CO$_2$ incubator. The plates were gently washed three times with PBS and fluorescence of attached C4–2 cells was measured by scanning the plate with fluorescence multi-well plate reader CytoFluor2 (PerSeptive Biosystems, Farmingham, MA). Inhibition of C4–2 cell adhesion to HUVEC was assayed by incubation of $10^5$ C4–2 cells with $10^7$ phage or 40 μM of peptides for 20 min at RT prior to the addition to the HUVEC monolayer.

**Invasion and motility (chemotaxis) assays.** For the motility (chemomigration) assay, uncoated Transwell inserts were used. For the invasion assay, the upper surface of 8-μm Transwell insert (Becton Dickinson, Franklin Lakes, NJ) was coated with 30 μg of matrigel per filter for 3 hr at 45°C. Matrigel was reconstituted in DMEM before plating the cells. The lower compartments were filled with 0.8 ml of RPMI 1640 supplemented with 5% FCS. Cells (5×10^4 per well) were suspended in 200 μl of serum-free RPMI 1640 containing 10 μg/ml of BSA (no treatment), 5×10^9 cfu/ml of phage or 0.1–100 μM of synthetic peptide. Cells were incubated for 30 min before they were applied to the upper compartment. After incubation in a CO$_2$ incubator for 6 hr for the motility assay and 15 hr at 37°C for the invasion assay, the filters were fixed and stained with the Diff-Quick stain kit (Baxter, Miami, FL). Non-invading cells from the upper part of filter were wiped off, and the migrated cells were counted in three randomly selected fields for each Transwell insert. Three Transwell inserts were used for each experimental condition.

**Gelatin zymography.** Expression level and activity of metalloproteinases produced by C4–2 and C4–2b cells were analyzed by gelatin zymogram as described [22]. Cells were preincubated in complete growth medium with and without phage or peptide in concentrations noted in the figure legends for 30 min before plating for 4 hr. After three washes in serum-free media, cells were incubated in RPMI 1640 without serum for 48 hr. For some experiments, phage and peptides were added to the media. The conditioned media was collected and concentrated with Centricon 30 concentrators. The samples were supplemented with 2× loading buffer, and the loading volumes of the samples were adjusted according to cell number. The samples were resolved in 10% gelatin gel (Invitrogen, Carlsbad, CA). The gel was developed according to the manufacturer’s instructions and stained with Coomassie brilliant blue R250.

**Statistical analysis.** Results are expressed as mean ± SE of three or more observations (as indicated). The data shown are representative results of consistent qualitative responses observed in triplicate or more experiments. Data were analyzed for significant differences at the $P < 0.001$ level by the use of the two-sided Student $t$ or ANOVA tests using SigmaStat program (Jandel Scientific, San Rafael, CA).

**RESULTS**

**Selection of Phage That Bind Specifically to the Surface of LNCaP Cells**

The choice of LNCaP cells as a target for the biopanning was dictated by the special features of this
cell line making it invaluable among existing in vitro models of prostate cancer. This cell line, derived from a lymph node metastasis of prostate cancer, is androgen sensitive and expresses both androgen receptors and prostate specific antigen (PSA). Several proteins important to prostate cancer biology were first identified in LNCaP. Prostate membrane specific antigen (PMSA) [23], PAGE and GAGE [24] were discovered using LNCaP cells or their derivates. In in vitro and in vivo assays, these cells demonstrate low tumorigenicity and invasiveness, but form a tumor in nude mice when co-injected with matrigel [25].

The starting library was initially depleted by one round of panning with a primary prostate epithelial cell monolayer followed by three rounds of biopanning with LNCaP cell monolayers. The depletion step was necessary to enhance the selection of phage specific for prostate cancer cells. Because the aim of the study was the identification of ligands specific for prostate cancer cells, we used a depletion strategy (subtraction) to enhance the selection of cancer-specific ligands from the starting phage library by panning primary prostate epithelial cells before biopanning of target LNCaP cells. Depletion of the primary library enriched the starting pool with phage specific for prostate cancer cells, since some ligands specific for benign prostate epithelial cells were eliminated. A similar technology called the “subtraction method” was used to search for differentially expressed proteins using an antibody phage library [26].

Another important modification of this search is that a different type of phage display library was chosen for the screening. In these experiments, we used a landscape library that was designed by the insertion of random eight amino acids at the N-terminus of pVIII coat protein [18]. The major property exploited with this library is the multiplicity of inserts expressed on the phage surface. Additionally, inserts are fused with pVIII in a manner allowing the most natural conformation. This provides multivalent binding of phage to target and, as a result, stronger interactions with target. Many ligands have been found by using libraries where inserts were presented as many protein copies on the phage surface by creating a pVIII fusion protein [27]. This is especially applicable for complicated targets with unknown geometry and distribution of binding epitopes. Therefore, the chosen selection conditions allowed the identification of phage with high binding affinity and biological activity.

The input/output phage ratio was calculated for each round of LNCaP cells biopanning. Measuring this parameter allowed for the monitoring of the selection process. This value was increased with every round of biopanning. As soon as the input/output ratio reached 1%, the selection process was considered complete. Thirty individual phage from the output of the last (4th) round were propagated and the DNA encoding insert in the fusion protein (pVIII) was sequenced. DNA sequences were translated into amino acid sequences; 22 of these sequences are presented in the Table 1. Similar sequences within the peptides have been aligned to show the consensus.

Only a few of the selected sequences are identical, but most of the sequences reveal a consensus sequence at their amino terminal end. Databases were searched for proteins matching with consensus motif. Some biologically relevant matches were discovered. For instance, a 62% match with the extracellular domain of the discoidin domain receptor (DDR) was found. Recently several collagens, or tightly associated proteins, have been proposed as ligands for DDR1 and DDR2; however, the function of this protein kinase has not been completely elucidated [28,29]. One of the possible roles of this receptor is its participation in intercellular interactions. Therefore, this match satisfies our definition of biological relevancy. Still, the major goal of this project was to determine the biological activity of selected ligands and their receptors.

To further characterize the selected phage, they were examined for binding specificity and affinity. The 15 individual bacteriophage with unique insert sequences were tested for binding to cells using a colony counting assay (Material and Methods). Each

<table>
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<th>Clone</th>
<th>Peptide sequence</th>
<th>Binding efficiency %</th>
<th>Incidence</th>
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<td>QXSAAEGE</td>
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<tr>
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<td>DPRATAMS</td>
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<td>DPRATTST</td>
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of the selected phage showed up to 25-fold greater binding yields than an irrelevant bacteriophage (Table 1). Based on the results of this screening, pg35 phage with the DPRATPGS sequence was chosen as the best binder to the LNCaP cell surface, and the corresponding peptide with the sequence (ADPRATPGSDPAKAC) of the insert including flanking amino acids of pVIII (pd35) was synthesized. Phage without insert, phage with an irrelevant insert sequence, and synthetic peptide DPRIATMCS were used as negative controls. Control peptide was also synthesized based on the sequence of phage pg27.

Fig. 1. Binding of the selected phage to cell surfaces. Assay was performed as described in the Materials and Methods section. A. Immunofluorescent staining of pg35 on the surface of LNCaP cells. Non-permeabilized cells were incubated with pg35. After fixation phage were labeled as described in the Materials and Methods section. Left plate—phage contrast, right—immunofluorescence. B and C. Phage were incubated with cells and after washing out unbound phage competent bacteria were added directly to cells with bound phage. After plating bacteria, the number of colonies was counted. This number is proportional to the number of bound phage. Three spots were plated for each experimental condition. B. Binding of pg35 and control phage to LNCaP cells. Second bar, inhibition of pg35 binding with cognate peptide pd35. Third bar, binding of pg35 to LNCaP cells when control peptide was used as a competitor. Cognate peptide decreases pg35 binding to LNCaP cells. Control phage adhere less to the LNCaP cells as compared with pg35; *P < 0.001. C. Specificity of pg35 binding to different prostate derived cells. Values represent the mean ± SE of the mean of three observations from 1 of 3 representative experiments. Phage pg35 preferentially adhered to the LNCaP, C4-2, and C4-2b cells as compared with BPH-1, PrE, PC-3, and DU-145, *P < 0.001.
without any flanking amino acids of pVIII. This phage reveals relatively low binding affinity (37%) and lacks spreading inhibition activity.

Localization of the pg35 bound to the surface of LNCaP was examined by immunofluorescent microscopy (Fig. 1A). Bound phage were localized on the surface of LNCaP cells and were not found inside the cells under conditions that allowed its internalization (37°C 2 hr incubation). The phage pg35, but not control phage, adhered to the surface of LNCaP cells. The ADPRATPGSDPAKAC, but not the control peptide decreased the binding of pg35 to the LNCaP cell surface by 49% (Fig. 1B). The ability of pg35 to bind the cell surface was also examined for C4–2 and C4–2b, two derivatives of LNCaP cells, showing higher tumorigenicity than parental LNCaP, for non-cancerous prostate cell lines PrE and BPH-1 and for two metastasis-derived prostate carcinoma cell lines PC-3 and DU-145 (Fig. 1C). Pg35 phage were able to bind specifically to the LNCaP cells used for biopanning and to their derivates C4–2 and C4–2b and demonstrated lower binding affinity to the surface of other examined cells (Fig. 1C).

We next tested the influence of pg35 and cognate peptide pd35 on the behavior of prostate epithelial cells. The ability of these ligands to affect cell attachment and spreading was examined first. Phage pg35 but not control phage were able to block spreading of LNCaP (Fig. 2A–C), C4–2 and C4–2b (Fig. 2C) cells on tissue culture plastic. This effect was cell-specific: spreading of normal prostate epithelial cells, two other prostate carcinoma cell lines, and cells not of prostate origin (not shown) were not affected (Fig. 2C). Adhesion of all tested cells to tissue culture plastic under these experimental conditions was not affected.

Adhesion and spreading of LNCaP, C4–2, and C4–2b cells plated on tissue culture plastic was not affected by the preincubation with cognate peptide (Fig. 2C). The concentration of phage used in these experiments was 10⁹ cfu/ml, which is equivalent to 1 μM of peptide when accounting for the number of peptide copies expressed on the phage surface. Peptide was applied at concentration 0.1–100 μM.

We then examined cell survival under conditions that block cell spreading. LNCaP cells remained non-spread but attached to the plastic for 24 hr after plating with pg35 phage. An increased cell death rate was not observed under these experimental conditions as shown by the trypan exclusion assay and DAPI

![Fig. 2. Inhibition of cell spreading on tissue culture plastic. A. LNCaP cells spread on tissue culture plastic. B. LNCaP cells as on panel A, but plated with 10⁹ cfu/ml of pg35. C. Inhibition of cell spreading by the selected phage. Cells were plated on plastic with 10⁹ cfu/ml of phage or 50 μM of peptide. After 24 hr the number of spread cells was calculated. Bar value is a percentage of spread cells compared with control (related cells without phage or peptide added). Two cell counts in three wells were used for every experimental condition. Values represent the mean ± SE of the mean from 1 of 3 representative experiments. Phage pg35 inhibit spreading of LNCaP, C4–2, and C4–2b cells as compared to control phage; *P < 0.001.](image-url)
staining; however, cells plated with pg35 and transferred after 4 hr into serum-free media started to detach after 48 hr of incubation, and many of the suspended cells became necrotic (data not shown).

To test the hypothesis that phage may block cell surface receptors responsible for the spreading of cells on particular extracellular matrix (ECM) components, we examined the ability of pg35 to block spreading of LNCaP on extracellular matrix substrates known to promote LNCaP cell attachment and spreading [30]. Since anti-β1 induced an effect similar to that of pg35 when LNCaP cells were plated on tissue culture plastic with 1 μg/ml of either 4B4 or C27-anti-β1 blocking antibodies (data not shown), these antibodies were also used as an attachment substrate to examine the ability of pg35 to prevent interaction of β1-containing integrins on the LNCaP cell surface with these antibodies (Fig. 3). Phage pg35, but not control phage or cognate peptide were able to block spreading of LNCaP cells and their derivatives plated on anti-β1 substrate but not on matrigel, fibronectin, or laminin (Fig. 3). Blocking of the spreading on the anti-β1 blocking antibodies suggests either that pg35 phage directly interact with β1-containing integrins or that pg35 bound to the cell surface mask β1-containing integrins while interacting with other molecules located in proximity to those extracellular matrix receptors. To confirm the possibility that integrins may directly bind pg35 phage, we studied the ability of several anti-integrin antibodies recognizing integrins on the surface of LNCaP cells (α5, α6, and β1) to compete with pg35 phage for binding sites on the cell surface. None of the tested anti-integrin blocking antibodies, including two antibodies against the β1 integrin subunit, were able to block binding of pg35 to LNCaP cells (data not shown). Thus, direct interaction of pg35 with the examined integrins is improbable.

Cell motility and, as a part of this process, cell spreading, are directly related to the ability of cancer cells to invade through extracellular matrix and to metastasize. The influence of phage and peptide on cell motility was assayed in Transwell chambers without filter coating. The pg35, but not control phage, partly blocked C4–2 and C4–2b cell movement through membranes (not shown). Peptide pd35 (Fig. 4A and 4C), but not control peptide (Fig. 4B), at a concentration of 10 μM induced alterations in cell shape, but did not dramatically change transport of cells through the membrane.

During the process of metastasis, transformed cells detach from the primary tumor and interact with and penetrate through endothelium. To study the mechanism of these interactions, adhesion of prostate cancer to a model of endothelium was investigated in vitro. Since C4–2 cells revealed invasive activity, they were selected to elucidate phage and peptide inhibition ability in the adhesion to endothelium. HUVEC monolayer was used as a model of endothelium. These cells have been most intensively used for in vitro studies of interactions of leukocytes and cancer cells with endothelium. Attachment was assayed by the measuring fluorescence of labeled C4–2 cells.
attached to HUVEC. Adhesiveness of C4–2 cells to endothelial cells was increased by incubation with 50 μM of peptide 35, but not with pg35 or control peptide (Fig. 5).

To examine whether selected ligands can interfere with cell invasiveness, we performed an in vitro matrigel invasion assay. LNCaP cells, though used extensively in prostate cancer biology, have some limitations because of their low tumorigenicity. However, their derivatives, C4–2 and C4–2b, reveal higher aggressiveness in in vitro and in vivo assays. C4–2 and C4–2b demonstrated invasive ability in this assay without treatment (Fig. 6A and 6E). Invasiveness of neither cell line was significantly altered after pre-incubation with pg35 (Fig 6B and 6E). Cognate peptide in concentration 10 μM was able to increase invasiveness of C4–2 cells by four fold (Fig. 6C and 6E).

Tumor invasion and metastasis requires controlled degradation of extracellular matrix (ECM) and increased expression and activity of matrix metalloproteinases. Type IV matrix metalloproteinases 2 and 9 have been linked with tumor invasion and metastasis [31]. To assess the role of MMPs in the increased invasiveness of cells preincubated with pg35 and cognate peptide, we performed a gelatin zymography assay. Both examined cell lines, C4–2 and C4–2b, secreted 72-kDa MMP-2 and 92-kDa MMP-9, and gelatinolytic activity of 85–88 kDa was also observed. Increased amounts of both isoforms were observed after preincubation of C4–2 cells with peptide, but not with pg35 phage. The effect was the same whether cells were plated on tissue culture plastic or on matrigel (Fig. 7).

**DISCUSSION**

Phage pg35 was identified as a prostate cancer cellspecific ligand. To further elucidate its properties and functions, cognate peptide was synthesized. The cognate peptide was only partially able to block the binding of the selected phage to the cell surface. The difference between binding properties of synthetic peptide and selected phage has been described for other experimental systems and could be explained by the conformational difference between insert in proteins exposed on the phage surface and the corresponding synthetic peptide. Several investigators have noted that to compete with phage binding, a large molar excess of cognate peptide is necessary [14]. In the selection of the specific ligands for fibroblasts, synthetic peptide did not inhibit selected phage binding to target. This was first found for phage in which insert was fused with pIII, and the authors concluded that this might have occurred because peptide in this case is out of context of the “scaffold” of the pIII protein. However, when the same peptide was used for inhibition of phage expressed as a fusion protein with pVIII, surprisingly, phage binding affinity increased [12]. Therefore, the difference in the binding affinity and functionality between selected phage and cognate peptides is a recognized phenomenon.

The pg35 phage not only specifically bind receptors on the surface of LNCaP, C4–2, and C4–2b cells, but also affect target cell behavior. One of the possible modes of inhibition of LNCaP, C4–2, and C4–2b cell
spreading on plastic by pg35 could be direct blocking of receptors responsible for cell spreading. Some of those receptors are integrins [32]. To examine the possibility that pg35 directly interacts with integrins on the surface of LNCaP, we performed attachment-spreading assays using extracellular matrix proteins and antibodies to integrins that are expressed on the surface of LNCaP cells as a substrate. In these experiments, anti-integrin antibodies and phage or peptides were used as inhibition agents. The pg35 phage blocked spreading of LNCaP cells on plastic and on anti-β1 antibodies; however, the anti-β1 antibodies could not block binding of pg35 to the LNCaP cell surface, although anti-β1 antibodies were able to block spreading of LNCaP cells on culture plastic. These data suggest that direct interaction of phage with β1 integrins on the surface of LNCaP is unlikely. Another argument against this possibility is the identification of pg35 phage receptor as a protein with apparent molecular mass 34–38 kDa (data not shown). There are no known integrin subunits in this molecular weight range. More likely, the pg35 phage mask molecules activating spreading, which results in spreading inhibition, while other cell surface molecules are used for phage binding. Failure of peptide to block spreading may be explained not only by the lower binding affinity of peptide pd35 compared with phage pg35, but also by the peptide binding of receptors on the cell surface distinct from the molecules responsible for the activation of spreading. In summary, effectiveness of pg35, but not peptide in blocking cell spreading on plastic and on anti-β1 antibody, provides additional support for the masking hypothesis. Since inhibition was observed when cells were plated on plastic without substrate, an additional possibility is that the pg35 binding could inhibit the production of ECM proteins by the cells.

Spreading inhibition activity of pg35 encouraged us to examine other possible effects induced in LNCaP and their derivatives by this phage and cognate peptide. Since adhesion of metastatically potent cells to endothelium is an important step in metastasis, we next investigated the ability of pg35 and cognate peptide to interfere with this process. Peptide pd35 increased C4–2 binding to HUVEC monolayer used as a model for endothelium. Phage itself could not modulate this effect. Many proteins including cadherins, selectins, and integrins are recognized as important mediators of tumor cell/endothelium association [33]. Phage pg35 during the binding to specific molecules on the surface of C4–2 cells may block by masking some of the molecules important for these interactions, but other adhesion molecules remain available for the interaction with endothelial cells. Peptide binding to the same receptors may induce some signal transduction events leading to the up-regulation of endothelium binding molecules. Attachment of pg35 phage to the C4–2 cell surface may not induce these events, because of the cross-linking of cellular receptors by neighboring peptides on the...
phage and/or due to the necessity of peptide internalization for the initiation of signal transduction events.

Cell motility and invasion are major components in the multistage process of metastasis. For many systems, invasion has been shown to be directly dependent upon motility [34,35]. Since spreading is a part of the motility process, it is expected that blockade of spreading is related to the cell movement and invasiveness. Motility was measured in the Transwell system without a coating. As in the case of spreading on plastic, pg35 blocked the spreading of C4–2 and C4–2b and, therefore, prevented movement across filter. Peptide pd35 altered the morphology of cells passing through a non-coated filter possibly inducing reorganization of the cyto-skeleton, but this did not alter cell motility. Since, invasion of C4–2 cells through matrigel-coated filters was increased substantially when cells were preincubated with peptide pd35, it is likely that a peptide binding molecule on the cell

Fig. 6. Invasion of PC-3, LNCaP, C4–2, and C4–2b. A. Invaded C4–2 cells. B. C4–2 cells preincubated with pg35. C. Invaded C4–2 cells pretreated with 10 μM of peptide pd35. D. Invaded C4–2 cells pretreated with 10 μM of control peptide. E. Comparison of the invasiveness of prostate carcinoma cells preincubated with pg35 or peptide pd35. Numbers: cells that invaded through the Matrigel-coated filters (in one microscopic field). Cells in four fields on two inserts were counted. Values represent the means ± SE of the mean from one of three representative experiments. C4–2 and C4–2b cells pretreated with peptide pd35 are more invasive than non-treated cells; *P < 0.001.
The presence of 85–88 kDa forms of enzyme. MMP-9 activation was indicated by the Lanes 2, 6: cells plated with phage p35. Lanes 3, 7: cells plated with nate peptide pd35. An additional band probably relates to an acti-

Control phage. Lanes 4, 8: cells plated with peptide pd35. Condi-

tion media was collected and processed as described in Materials and Methods section. Seventy-two and 92-kDa gelatinase activity is elevated in conditioned medium from C4–2 cells plated with cognate peptide pd35. An additional band probably relates to an acti-

vated form of enzyme. MMP-9 activation was indicated by the presence of 85–88 kDa forms of enzyme.

surface is a key element in the activation of the production of proteolytic products important for cell invasion. Matrix metalloproteinases production is linked in cancer cells to elevated invasiveness. To examine whether increased invasiveness is related to the MMP activity, we performed zymographic analysis. Increased activity of MMP-2 and MMP-9 in cells treated with peptide pd35 suggests that binding of selected ligand to the surface can activate existing MMP or induce its production. Activity of MMP-9 has been associated with the acquisition of the metastatic phenotype in many cancers, including prostatic cancer [35]. Activation of prostate cancer-specific molecule(s) on the surface of C4–2 and C4–2b prostate cancer cells by the binding of peptide pd35 may play a role in metastasis via its ability to increase MMP activity.

The difference in the function of phage pg35 and peptide pd35 in this instance as well as in case of the binding to endothelium may relate to the mechanism of binding of each ligand to receptor. It may be that the binding and ligation of those receptors by phage on the cell surface prevents further processing necessary for the MMP activation. Peptide, in turn, does not ligate receptors and allows further intracellular signal transduction (possibly by internalization). Further understanding of the effects of the selected ligand will be possible after identification of the ligand-binding protein on the cell surface.

In summary, the phage peptide library approach provides a powerful method for the selection of specific functional ligands to the molecules exposed on the surface of cells of interest. Ligands selected in this study were specific to and altered the function of target prostate cancer cells and their derivatives. Binding of the peptide ligand to its putative receptor on the cell surface induced metastasis-associated functions in target cells. Identification of the receptor for the DPRATPGS peptide may yield new insights into the mechanism of metastatic transformation in prostate cancer.

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