Identification of Receptor Ligands by Screening Phage-Display Peptide Libraries Ex Vivo on Microdissected Kidney Tubules

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Abstract. A novel method to identify receptor ligands for defined renal tubular segments has been developed. Ex vivo screening of phage-display peptide libraries on isolated intact rat proximal convoluted tubules (PCT) and cortical collecting ducts (CCD) allowed the direct access of phage to the basolateral surface of tubular epithelial cells. Two distinct peptide motifs were selected for CCD and PCT, indicating differential expression of some membrane receptors on the basolateral surface of defined kidney tubule segments. Using the linear peptide motif ELRGD(R/M)AX(W/L), recovered from freshly isolated rat CCD, mediated 16-fold selectivity of phage binding to CCD compared with PCT. Binding to CCD was 39-fold higher than that of a random control phage. Binding and subsequent internalization of phage, most likely by an integrin-mediated endocytosis pathway, was abolished by the addition of the corresponding synthetic peptide. Furthermore, the results demonstrate that presentation and flanking amino acids determine the specific binding properties of RGD ligands to their putative integrin receptors. The results emphasize the need of a native cell system for the identification of renal epithelial cell surface ligands. Such ligands are of potential relevance for the analysis of interactions between extracellular matrix and kidney tubules or for the development of improved vectors for kidney-specific drug delivery or gene transfer.

The coordinated action of a variety of specialized cells is required to mediate the complex and highly regulated functions of the kidney. Each nephron segment contains one or several specific cell types, each expressing a unique set of cell surface receptors and membrane proteins, involved in functions such as regulation of salt retention, hormone action, and cell-adhesion and cell-matrix interactions (1–3). The identification and characterization of ligands and cell surface receptors that show differential expression in the nephron is a prerequisite for understanding the functional differences between defined nephron segments in both normal and pathologic processes.

Phage-display peptide libraries have been applied successfully to identify peptide-binding domains on purified proteins (4) or of peptide ligands with binding preference for selected tissues in vivo (5,6) or cells in vitro (7–9). For instance, in vivo screening of phage-display peptide libraries demonstrated the molecular heterogeneity of endothelial cells in different organs (6), suggesting that the vasculature of each organ could be targeted by specific ligands. Two peptide ligands preferentially homing to kidney endothelium in the glomerulus and mediating up to sevenfold increased binding compared with control were selected by this approach (5).

Knowledge about specific basolateral tubular epithelial markers is limited. The basolateral surface of cells grown on culture dishes is not directly accessible for analysis, and evidence exists for the loss of tissue-specific traits of isolated cells upon culture (10,11). To identify ligands that bind selectively to receptors with a differential expression on the basolateral cell surface of defined nephron segments, we incubated phage-display peptide libraries with microdissected intact cortical collecting ducts (CCD) or proximal convoluted tubules (PCT). By using the approach presented in this study, the identification of ligands specific for the basolateral membrane of kidney epithelium should increase knowledge about interactions between the membrane domain and extracellular matrix. It might also contribute to the future development of kidney cell–specific vectors for drug delivery or gene transfer, making a possibility the treatment for diseases including tubulointerstitial fibrosis, polycystic kidney disease, and genetic disorders of salt balance causing hypertension.

Materials and Methods

Phage-Display Libraries and Peptides

LL9, a library that displays linear random nonapeptides, and CL10, which expresses random decapeptides with a structural constraint imposed by a disulfide bond between two cysteine residues flanking the variable region, have been described previously (8,12). Synthetic peptides were purchased from Macromolecular Resources (Colorado State University, Fort Collins, CO).
Microdissection of Rat Kidney Tubules

Rat CCD and PCT were microdissected as described earlier with some modifications (13,14). Briefly, male Sprague-Dawley rats were anesthetized with nembutal (pentobarbitone, 50 mg/kg body wt, intraperitoneally). The left kidney was perfused in situ via the abdominal aorta with 10 ml of Hank’s balanced salt solution (HANKS; pH 7.4), followed by 5 ml of HANKS containing collagenase (2.6 mg/ml, 0.9 U/mg, clostridium histolyticum, Serva, Heidelberg, Germany). At the end of the perfusion, small pieces of cortex were removed and incubated in HANKS containing 0.65 mg/ml collagenase for approximately 30 min at 32°C. Neophron segments were dissected under a stereomicroscope in cold microdissection buffer (HANKS containing 1 mg/ml bovine serum albumin). The recovered neophron segments were measured individually with an ocular micrometer, and isolated segments (50 mm) were immediately subjected to incubation with phage as described below. Endothelial cells and basement membrane were removed by the collagenase treatment, allowing the direct access of phage to the basolateral tubular cell surface. Counting of cells revealed 360 cells/mm of isolated CCD and 300 cells/mm for isolated PCT (15).

Affinity Purification of Binding Phage

Isolated rat kidney tubules (50 mm) were preincubated in 200 µl of phage incubation buffer (HANKS [pH 7.4] containing 1% low-fat milk and 100 µM chloroquine) for 15 min at 37°C to adapt the cell metabolism and to reduce hypothermic effects on the cytoskeletal network and on membrane protein trafficking (16). Phage libraries (1010 transducing units (TU) of LL9 or CL10) were added, followed by further incubation for 40 min at 37°C, allowing internalization of ligand-activated receptors. Reactions were stopped with ice-cold phage incubation buffer, tubules centrifuged at 700 × g for 3 min, washed twice with 25 ml of phage incubation buffer, once with 25 ml HANKS, and transferred with a 75-ml Micro/pettor (SMI, Berkeley, CA) to 1.5-ml tubes. After incubation of tubules with 400 µl of 0.1 M glycine-HCl (pH 2.2) for 5 min, the acid-eluted fraction, containing phage that were attached to the cell surface with low affinity, was removed by centrifugation. The pH of the pellets tubules (cell-associated fraction) was neutralized by adding 200 µl of HANKS, supplemented by 10 mM Tris (pH 7.4). Trypan blue staining (4 µg/ml) revealed that more than 98% of tubular cells remained intact after exposure of the tubules with the phage. Phage were then recovered and quantitated from the cell-associated fraction by plaque assay.

Determination of Phage Titer by Plaque Assay

Phage were titered by infecting *E. coli* K91 cells in the logarithmic growth phase with serial dilutions of eluted phage. Upon incubation at 37°C for 15 min, melted soft-agar (55°C) was added and the mixture was plated onto LB-agar. The number of plaques, representing areas of reduced bacterial growth due to infection with individual phage, was determined. Typically, between 200 and 1000 plaques were counted, and the number of phage corresponding to 1 cm of tubule length was calculated.

Quantitation of Phage-Binding and Competition Assay

To quantify binding of a selected phage, we incubated tubules (25 mm) at 37°C for 40 min according to the selection procedure described above, except that the phage library equivalent was replaced by 1010 TU of an individual phage clone. In competition experiments, the selected phage and its corresponding synthetic peptide or irrelevant control peptides (MHNRHPMIKH or PSRHPQPL) were added simultaneously, with peptide concentrations ranging from 100 nM to 100 µM. To analyze the effect of the peptide on phage binding, we preincubated tubules and synthetic peptide for 15 min before adding phage. Alternatively, the peptide was added 15 min after exposure of the tubules with the phage. Phage were then recovered and quantitated from the cell-associated fraction by plaque assay. The binding specificity of the CCD motif was further assessed by incubation of 104 T84 cells (or other cell lines) with 1010 TU of a purified, individual phage clone in the presence or absence of the competing peptide or a control peptide at 37°C for 40 min. Because suitable rat cell lines were not available, epithelial cell lines from other species were used. The number of bound or internalized phage was determined from the cell-associated fraction, and the results expressed as a ratio to the binding of an irrelevant phage.

Detection of Phage Binding by Fluorescence Microscopy

Microdissected tubules (25 mm) were incubated in phage incubation buffer in the presence of 1010 TU of a purified, individual phage clone for 2 or 40 min at 37°C in the presence or absence of 10 µM synthetic peptide. Tubules were extensively washed, and phage attached to the cell surface were eluted by acidic buffer as described above. The pelleted tubules were taken up in 100 µl of HANKS (pH 7.4), subjected to cytospin centrifugation for 10 min at 600 × g onto glass cover slides, and fixed with 4% paraformaldehyde for 10 min at 25°C. When T84 cells were used, approximately 104 cells were subjected to cytospin centrifugation for 2 min at 600 × g, followed by paraformaldehyde fixation. Tubules or cells were washed three times with sodium/phosphate/sucrose/triton buffer (NAPST) (100 mM sodium phosphate, pH 7.4, 120 mM sucrose, 0.5% Triton X-100 to permeabilize cells) and blocked for 30 min with buffer NAPSTM (NAPST buffer containing 1% low-fat milk). Upon incubation with mouse monoclonal anti-M13 phage IgG (Pharmacia, Duendorf, Switzerland) for 1 h at 25°C, samples were washed three times with buffer NAPST, followed by incubation for 30 min with fluorescein-conjugated goat anti-mouse IgG (Molecular Probes, Leiden, The Netherlands). After extensive washing, cells were mounted on glass cover slips using a Slow-Fade-Antifade kit (Molecular Probes). Samples were analyzed by confocal microscopy (Zeiss LSM 410, Zeiss, Goettingen, Germany) using a laser for excitation of fluorescein at
Results

Selection of Phage with Preferential Binding to Microdissected Intact Kidney Tubules

Three independent experiments of screening the linear nonapeptide phage-display library LL9 on isolated intact CCD yielded the peptide motif ELRGD(R/M)AX(W/L), containing an RGD sequence and additional, highly conserved flanking residues (Table 1). The individual sequences ELRGDMAAL and ELRGDRAHW were selected predominantly in all three independent panning experiments conducted and were recovered eight and seven times, respectively. Different nucleotide triplets were observed for a given amino acid residue of the conserved motif, indicating a true selection for the amino acid triplet was observed for a given amino acid residue of the conserved motif, indicating a true selection for the amino acid rather than an overrepresentation of a certain nucleotide motif. ELRGD(R/M)AX(W/L) was absent in screenings of the circular decapeptide phage-display library CL10 and was re-covered eight and seven times, respectively. Different nucleotide consensus sequence. Phage bearing the consensus ELRGD(R/M)AX(W/L) were absent in screenings using PCT from which phage with the consensus K(X)3TNHP were selected (Table 1).

Binding Studies with Purified Phage

To assess the specificity of the selected phage, we incubated isolated intact CCD or PCT with purified phage expressing the peptides ELRGDMAAL or ELRGDRAHW, selected from CCD, the peptide KMGGTNHPE, selected from PCT, or a random linear nonapeptide (Figure 1). Analysis of the cell-associated fractions revealed 39-fold increased binding of either ELRGDMAAL- or ELRGDRAHW-displaying phage to CCD compared with control phage. Furthermore, phage that displayed peptides with the consensus sequence ELRGD(R/M)AX(W/L) bound at 16-fold higher to CCD than to PCT, indicating their clear preference for CCD. The KMGGTNHPE-displaying phage was less specific for a defined tubule type. Its binding to PCT was twofold higher than to CCD, but binding to PCT was 10-fold higher than that of control phage. Because this motif did not mediate highly tubule-specific binding, we did not analyze further the binding properties. Control phage showed similar binding to both tubule types. The resistance of phage binding to acidic elution suggested internalization of phage upon binding to its cognate receptor. Tubules were incubated with phage for 40 min at 37°C in the presence of 100 µM chloroquine, which prevents endosome/lysosome fusion (20). Incubation of tubules for a prolonged period of time at 37°C (2 h) in the absence of chloroquine led to a significant decrease of phage recovery (not shown), suggesting lysosomal degradation of the phage.

The cell specificity of the ELRGDMAAL-displaying phage was examined further by determining phage binding

Table 1. Peptides selected from microdissected intact kidney tubules*

<table>
<thead>
<tr>
<th>Tubule Type/Experiment</th>
<th>Selected Sequence</th>
<th>No. of Identical Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>ELRGDMAAL*</td>
<td>(3x)</td>
</tr>
<tr>
<td>(3 rounds)</td>
<td>SLRGDRAGW</td>
<td>(2x)</td>
</tr>
<tr>
<td></td>
<td>ELRGDRAHW</td>
<td>(1x)</td>
</tr>
<tr>
<td></td>
<td>YRDFRDWIA</td>
<td>(1x)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>ELRGDMAAL</td>
<td>(5x)</td>
</tr>
<tr>
<td>(4 rounds)</td>
<td>ELRGDMAAL</td>
<td>(2x)</td>
</tr>
<tr>
<td></td>
<td>LRGDASFLA</td>
<td>(1x)</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>ELRGDRAHW*</td>
<td>(6x)</td>
</tr>
<tr>
<td>(3 rounds)</td>
<td>ELRGDRAHW</td>
<td>(1x)</td>
</tr>
<tr>
<td></td>
<td>EMRGDLGAF</td>
<td>(1x)</td>
</tr>
<tr>
<td>Consensus sequence</td>
<td>ELRGD(R/M)AX(W/L)</td>
<td></td>
</tr>
<tr>
<td>PCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>KMGGTNHPE*</td>
<td>(4x)</td>
</tr>
<tr>
<td></td>
<td>MKTASVRHP</td>
<td>(1x)</td>
</tr>
<tr>
<td></td>
<td>FTNHSVSTRR</td>
<td>(1x)</td>
</tr>
<tr>
<td></td>
<td>VNLHEMSIM</td>
<td>(1x)</td>
</tr>
<tr>
<td></td>
<td>KMAGTNHPS</td>
<td>(1x)</td>
</tr>
<tr>
<td>Consensus sequence</td>
<td>KXXXTNHP</td>
<td>(1x)</td>
</tr>
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</table>

* Peptides displayed by the phage isolated from screening the linear nonapeptide phage-display library LL9 on microdissected CCD or PCT are aligned. The number of isolated nucleotide sequences encoding each peptide is indicated in parenthesis. The star indicates the phages used for analytic studies. Residues characteristic for the binding motif are shown in bold, the RGD motif is underlined. CCD, cortical collecting duct; PCT, proximal convoluted tubule.

Figure 1. Binding of purified phage to microdissected cortical collecting ducts (CCD) and proximal convoluted tubules (PCT). Isolated tubules were incubated with 10¹⁰ transducing units (TU) of purified ELRGDMAAL-displaying phage particles, selected from CCD, K(X)₃TNHP-displaying phage, selected from PCT, or with control phage expressing a random peptide. Recovered phage were titered from the cell-associated fractions (■) or from the acid-eluted fractions (○). Data represent mean ± SD of the number of phage plaques on lawns of bacterial cells, determined from seven independent experiments.
to various epithelial cell lines (Table 2). Whereas binding to cell lines or to microdissected PCT was low, ELRGDMAAL-displaying phage bound with a high preference to isolated CCD (39-fold over control phage). This phage showed relatively high binding (13.9-fold over control phage) to colon carcinoma epithelial T84 cells but not to colon carcinoma SW620 cells. Surprisingly, binding to MDCK cells, a cell line derived from canine kidney with some features of CCD, was not significantly increased, indicating low expression levels of the corresponding receptor in this transformed cell line.

**Inhibition of Phage Binding to Microdissected CCD by Coincubation with Synthetic Peptide**

Competition assays revealed significantly inhibited binding of ELRGDMAAL- or ELRGDRAHW-displaying phage to CCD after preincubation of tubules with synthetic peptide (not shown) or when the peptide was added simultaneously with phage (Figure 2). A random control peptide had no effect. The synthetic peptide did not affect phage binding and recovery after a 15-min preincubation of tubules with phage, suggesting receptor internalization upon ligand binding. The half-maximal inhibition (IC50) of either ELRGDMAAL- or ELRGDRAHW-displaying phage binding to CCD by the synthetic peptide ELRGDMAAL was approximately 500 nM (Figure 2B).

**Internalization of ELRGDMAAL-Displaying Phage upon Binding to Microdissected CCD**

To identify ligands for the receptor-mediated endocytosis pathway, we performed biopanning at 37°C for 40 min, and phage that were resistant to acidic buffer elution were selected from the cell-associated fraction. Isolated tubules were centrifuged onto glass coverslips by cytospin, and the localization of CCD-bound phage was visualized by confocal microscopy (Figure 3). The focus of the confocal microscope was set through the center of the two cell layers of the centrifuged tubule. After a 2-min incubation time, a multifocal staining pattern restricted to the CCD cell surface indicated binding of ELRGDMAAL-displaying phage to the basolateral membrane (Figure 3, D and J). A scattered staining of phages was observed after 40 min, typical for endosomal localization (Figure 3F). Incubation of CCD with a control phage (Figure 3, A and B) or with ELRGDMAAL-displaying phage in the presence of 10 µM synthetic peptide (Figure 3, G and H) resulted in background immunofluorescence. Incubation of PCT with ELRGDMAAL-displaying phage resulted in no detectable immu-

### Table 2. Relative binding of ELRGDMAAL-displaying phage to isolated tubules and cell lines

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Binding Relative to Control Phage</th>
<th>No. of Independent Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCD</td>
<td>39.0 ± 6.5</td>
<td>n = 7</td>
</tr>
<tr>
<td>PCT</td>
<td>2.5 ± 1.1</td>
<td>n = 7</td>
</tr>
<tr>
<td>MDCK (canine kidney)</td>
<td>2.5 ± 1.3</td>
<td>n = 5</td>
</tr>
<tr>
<td>MCF-7 (human breast)</td>
<td>3.6 ± 2.0</td>
<td>n = 7</td>
</tr>
<tr>
<td>SUT (human lung)</td>
<td>1.7 ± 1.0</td>
<td>n = 5</td>
</tr>
<tr>
<td>HeLa (human cervix)</td>
<td>2.0 ± 0.9</td>
<td>n = 5</td>
</tr>
<tr>
<td>SW620 (human colon)</td>
<td>1.6 ± 0.8</td>
<td>n = 7</td>
</tr>
<tr>
<td>T84 (human colon)</td>
<td>13.9 ± 4.7</td>
<td>n = 5</td>
</tr>
</tbody>
</table>

* Isolated tubules or epithelial cell lines were incubated with 10^{10} TU of purified ELRGDMAAL-displaying phage for 30 min at 37°C. Binding of specific phage was compared with that of control phage. Data represent mean ± SD of the multiple of control phage recovered from the cell-associated fraction. n, the number of independent experiments; TU, transducing units.
Selection of Phage with Preferential Binding to Colon Carcinoma T84 Cells

Because the CCD-binding motif ELRGDMAAL exhibited considerable binding to T84 cells, we tested the specificity of the ex vivo selection on kidney tubules by performing analogous experiments on T84 cells. We screened phage-display peptide libraries on colon carcinoma T84 cells and recovered phage expressing circular peptides with the consensus RGDLGXL(K/R) (Table 3). Like the CCD-binding motif, this peptide contains an RGD sequence but different, highly conserved flanking amino acids. Whereas the T84-binding peptide was recovered exclusively in a circular form, caused by a structural constraint imposed by two cysteine residues flanking the variable region, the CCD binding peptide was selected exclusively from the linear library and is not structurally constrained.

Incubation of T84 cells with library LL9 yielded predominantly phage with the consensus sequences NFYXGXRSL and VHXWD. These sequences, which were not analyzed further, may have higher affinity for their putative receptors on T84 cells than the linear sequence ELRGD(R/M)AX(W/L). This may explain the absence of ELRGD(R/M)AX(W/L)-displaying phage among phage recovered from T84 cells.

Analysis of the Binding Specificity of the Two Distinct RGD Ligands for Either CCD or Colon Carcinoma T84 Cells

Binding of purified phage expressing the linear CCD-motif ELRGDMAAL, the circular T84-motif CQARGDLGKIRC, or a control peptide sequence was determined for both microdissected CCD and T84 cells (Figure 4). Binding to CCD compared with control phage was 39-fold increased for the ELRGDMAAL-displaying phage and 5.4-fold for the CQARGDLGKIRC-dis-

Table 3. Peptides recovered from colon carcinoma T84 cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Selected Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1 (3 rounds)</td>
<td>CKRM&lt;sup&gt;1&lt;/sup&gt;RGDLAPLC</td>
</tr>
<tr>
<td></td>
<td>CRGDLGRGHWC</td>
</tr>
<tr>
<td></td>
<td>CQARGDLGKIRC&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CPLRGDLGTLLC</td>
</tr>
<tr>
<td></td>
<td>CRSBGDGTLLKC</td>
</tr>
<tr>
<td></td>
<td>CPHRRGDLGTLC</td>
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<tr>
<td></td>
<td>CRGDMARLRC</td>
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<td>CMDRGLGALKC</td>
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<td></td>
<td>CAGKRGDLKTLC</td>
</tr>
<tr>
<td></td>
<td>CLRGRDLGGMRC</td>
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<tr>
<td></td>
<td>CPRGRDLGSMFC</td>
</tr>
<tr>
<td>Consensus sequence</td>
<td>RGDLGXL (K/R)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Peptides selected from screening the circular decapptide phage-display library CL10 are aligned. The asterisk indicates the phage used in analytic studies. Identical residues and conservative substitutions are shown in bold; the RGD motif is underlined. The two flanking cysteines are shown in italic.

Figure 3. Localization of CCD-binding phage ELRGDMAAL by immunofluorescence. Microdissected CCD were incubated with 10<sup>10</sup> TU of purified ELRGDMAAL-displaying phage particles (C through J) or with control phage (A, B) either for 2 min (A through D, I and J) or for 40 min (E through H) at 37°C. CCD were simultaneously incubated with ELRGDMAAL-displaying phage and the corresponding synthetic peptide (G, H). Subsequently, tubules were centrifuged by cytospin onto glass coverslips followed by fixation with paraformaldehyde, permeabilization with Triton-X 100, and immunostaining using anti-M13 antibody and fluorescein-labeled secondary antibody. The left panel depicts Nomarski images and the right panel shows immunofluorescence images. Magnifications: ×200 in A through H, ×800 in I and J.)

nofluorescence and was not distinguishable from that of control phage (not shown).
playing phage, demonstrating a 7-fold preference of ELRGD-
MAAL over CQARGDLGKIRC-displaying phage for CCD. In 
addition, the peptide CQARGDLGKIRC inhibited binding of 
CQARGDLGKIRC-displaying phage to CCD with an IC50 of 1 
mM (not shown), compared with the IC50 of 500 nM of the 
ELRGDMAAL peptide (Figure 2B). Both phage showed approx-
imately twofold increased binding to PCT compared with control 
phage (not shown). These results indicate a clear preference of the 
putative CCD cell surface receptor to bind the linear motif 
ELRGDMAAL.

Binding studies on T84 cells revealed 42- and 14-fold in-
creased binding of CQARGDLGKIRC- and ELRGDMAAL-
displaying phage over control phage, respectively, demonstrat-
ing a 3-fold preference of the CQARGDLGKIRC over the 
ELRGDMAAL motif for T84 cells. Although the CQARGDL-
GKIRC-displaying phage bound at threefold higher levels to 
T84 cells than ELRGDMAAL-displaying phage, similar IC50 
values of approximately 100 nM were obtained for both motifs 
in competition assays with the corresponding peptides (not 
shown).

Internalization of CQARGDLGKIRC-Displaying Phage 
upon Binding to T84 Cells

T84 cells incubated with CQARGDLGKIRC-displaying phage 
were centrifuged by cytoospin onto glass coverslips. The 
localization of phage was analyzed by immunofluorescence 
staining and confocal microscopy. After 2 min of incubation at 
37°C, phage binding was restricted to the cell surface, whereas 
the distributed pattern of phage staining observed after 40 min 
indicated internalization of phage via the endocytotic pathway 
(Figure 5). Experiments using ELRGDMAAL-displaying 
phage resulted in a distribution pattern similar to that of 
CQARGDLGKIRC-displaying phage, but staining was much 
less intense and control phage did not result in a detectable 
immunofluorescence signal (not shown).

Discussion

We describe here a novel method of screening phage-display 
peptide libraries ex vivo on microdissected intact kidney tu-
bules. No alternative approach exists for identifying peptide 
ligands that bind selectively to receptors expressed on the 
basolateral membrane of the renal tubular epithelium. We 
applied phage libraries displaying either linear nonapeptides 
or circular decapeptides and identified a linear peptide ligand 
with the consensus ELRGD(R/M)AX(W/L) that binds preferen-
tially to the basolateral cell surface of CCD. The specificity 
of this motif for CCD was demonstrated by the low levels of 
binding to PCT and to a range of epithelial cell lines derived 
from different tissues, including cervix, breast, lung, colon, 
and kidney. Furthermore, this motif was absent in screenings using 
microdissected PCT. Screening isolated intact PCT yielded 
phage with the consensus peptide motif K(X)TENHP. Using 
this motif mediated a 10-fold increased binding to PCT com-
pared with control phage, but binding to PCT was only 2-fold 
higher than to CCD. In analogy to the observed molecular 
heterogeneity of the vascular endothelium of different organs 
(5,6), our results provide evidence for a kidney tubule segment 
specific individuality of the basolateral cell surface.

The CCD-binding motif ELRGD(R/M)AX(W/L) was exclu-
sively displayed in the linear form. In contrast, biopanning the
polarized epithelial T84 cells yielded the peptide motif RGDLGXL(K/R) exclusively in the circular form. Both the CCD- and the T84-binding phage showed high specificity for and affinity to their target cells, suggesting binding to different receptors. Phage binding resisted low pH treatment, and low concentrations of the corresponding synthetic peptides competitively inhibited phage binding to target cells, with IC50 concentrations in the high nanomolar range. In addition, the significant decrease in phage recovery in the absence of chloroquine suggests that the CCD- and the T84-peptide ligands are internalized upon ligand binding and undergo lysosomal degradation.

The selection of ligands with an RGD sequence and binding to putative integrin receptors is not surprising. Both polarized cell types, CCD cells and colon epithelial T84 cells, exhibit interactions of their basolateral cell surface with the extracellular matrix. Integrin receptors play an important role by interacting with extracellular matrix proteins and adhesion molecules of neutrophils or pathogenic microorganisms (21).

The estimated number of approximately 50 phage particles bound per cell is relatively low. This phenomenon has been observed previously (8) and may be explained by the large size of phage particles. Phage binding to a receptor might sterically hinder the binding of additional phage to receptors located in close proximity. In addition, phage binding may cause internalization of other still unoccupied receptors.

The CCD and T84 motif both contain an RGD sequence, known to mediate binding to the integrin family of receptors (21). The presentation and the residues that flank the RGD sequence clearly are different between the two ligands and seem to be essential for rendering cell specificity and high affinity binding. The linear CCD-binding motif ELRGD(R/M)AX(W/L) contains a positively charged arginine or a methionine at position +1, relative to the RGD sequence, whereas the circular T84-binding peptide RGDGLGXL(K/R) has a hydrophobic leucine residue at this position. Whereas both the CCD- and the T84-binding motifs have a hydrophobic residue at position +4 (W/L or L, respectively), a positively charged amino acid (K/R) at position +5 is characteristic for the T84-motif. Furthermore, the CCD motif contains a glutamate and a leucine residue at positions −2 and −1, whereas the region upstream of the RGD sequence of the T84 peptide is less conserved but contains an uncharged or a positively charged residue at position −1. Although the identity of the specific integrin receptor for both RGD ligands is unknown, it is unlikely that they bind to the α1 integrin expressed in tumor vasculature (22) and in the vascular endothelium of the kidney (23), because folding and flanking residues of the CCD, the T84, and the α1 integrin ligand (ACDCRGDCFCFG) clearly are different.

RGD-recognizing integrins have been shown to be present in endosomes of a variety of cells (24), and integrin-mediated gene delivery vectors led to enhanced gene transfer into different cell lines (25-27). Using tracheal epithelial cells, Colin et al. (28) presented evidence for a clathrin-coated pit-dependent endocytic internalization of an RGD-oligolysine/DNA complex. In an approach to achieve gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway, Erbacher et al. (29) coupled the peptide CYGGGRG-DTP, present in fibronectin, vitronectin, and type I collagen, to polyethylenimine/DNA complexes. They observed markedly improved efficiency of gene transfer into HeLa cells of up to 100-fold over control. In contrast, the phage expressing the ELRGDMAAL peptide bound preferentially to CCD and showed only slightly increased binding to HeLa cells compared with a random control phage. Both peptides are not structurally constrained. Their main differences involve charge and length of the side chain of the residues in position −2 and −1, relative to the RGD sequence, with Glu, Leu in the CCD motif and Gly, Gly in the fibronectin-derived peptide. Evidence that changes in the amino acid context can dramatically affect the binding properties of RGD ligands has been provided by other investigators (21,30).

The kidney consists mainly of nonproliferating, terminally differentiated cells. Viral vector delivery to these cells is relatively inefficient, probably as a result of the lack of high affinity viral receptors on the cell surface (31,32). Nephron segment-specific peptide ligands, such as the ELRGD(R/M)AX(L/W) peptide, potentially could be used for the development of kidney-specific delivery vectors, either by direct incorporation into viral vectors such as adenovirus (33,34), by coupling to polyethylenimine (29,35) or oligolysine (26,27), or by indirect coupling to viral vectors or liposomes using polyethylene glycol (36). Alternatively, genetically modified filamentous bacteriophage could be used for gene transfer into mammalian cells (37-39). Alternative ligands may be identified by screening other defined tubular segments or isolated CCD using different libraries and conditions, e.g., in the presence of synthetic peptide.

Selective targeting of renal tubular epithelial cells may be achieved either through retrograde infusion, with possible applications to target papilla and outer medulla, or by arterial perfusion, which seems most promising for the targeting of glomerular cells or cortical segments (34,40-42). Targeting of epithelial cells via arterial delivery may be more challenging. Perfusion of the kidney for 40 min with high titer modified adenovirus led to the transduction of kidney cortical vasculature but not parenchymal cells (34). However, McDonald et al. (34) used an adenovirus whose fiber protein contained a ligand that has been shown previously to mediate homing to endothelial cells (5,23). It is likely that this fiber-modified adenoviral vector was trapped in endothelial cells. Access of vectors from the blood stream to parenchymal cells may be facilitated by interventions that act on tight junctions and enhance transendothelial migration. Arap et al. (43) demonstrated that a phage homing specifically to tumor endothelium had spread from the blood vessels into the tumors after circulation for 24 h. Similarly, arterial perfusion of vectors modified with a ligand specific for basolateral epithelial cells and circulation for a prolonged period of time may allow homing to the basolateral epithelial cell surface.

A notable finding in our study is the relatively low but significant cross-reactivity of the CCD-binding phage with T84 cells (13.9-fold over control) and that of the T84-binding phage with CCD (5.4-fold over control), suggesting at least some
expression of both putative receptors on either cell type. CCD and colon epithelium play a role in the regulation of salt transport, and colonic epithelial T84 cells show enzyme activities comparable to those of primary cultured rabbit CCD cells (44).

Surprising, phage binding to MDCK cells, a cell line derived from canine kidney that is widely used as a cell model for CCD, was very low. Culturing of the cells could have led to dedifferentiation resulting in dramatically diminished expression of the putative integrin receptor, probably involved in adhesion of the basolateral membrane with the extracellular matrix. Another explanation is the species difference, because tubules were microdissected from rat and MDCK cells are derived from canine kidney. It is not surprising that the expression of cell surface receptors in cell lines differs from that in the native system. Cell dissociation disrupts intercellular communications and extracellular attachments. It has been shown that kidney tubular cells lose their epithelial polarity within minutes after dissociation (45,46). Our results emphasize the need for a native system of living cells to retain proper differentiation for the identification and characterization of ligands and their corresponding cell surface receptors. In addition to the disadvantage of altered differentiation of cell lines, the basolateral membrane of cells grown on culture dishes is directed toward the solid surface of the dish and is not directly accessible for analysis.

The presented method was designed to select high affinity binding phage undergoing internalization upon binding to their putative cell surface receptors and allowed the identification of a ligand that binds selectively to the basolateral membrane of CCD. Additional binding sequences may be identified by modification of the conditions chosen for the binding or elution of phage, such as temperature, pH, the presence or absence of the ions Mg²⁺ or Ca²⁺, or elution with specific known ligands. Different types of phage-display libraries with distinct presentation and length of the displayed peptide sequences could be applied to search for alternative motifs. In addition, affinity purification can be performed by inclusion of competing synthetic peptides, e.g., ELRGDMAAL in the case of CCD (8). The screening of phage-display libraries ultimately tends to result in the selection of the “best-fit” sequence, and only one or few distinct peptide sequences are obtained after several rounds of panning (5,8,43). Phage with lower affinity to their putative receptors or phage binding to receptors that are expressed at lower abundance may be found by analyzing clones after fewer rounds of affinity purification. Although these sequences may be less abundant or display a lower affinity to their corresponding receptors, they may be highly specific for the cell type of interest and be useful for the development of cell-type specific vectors.

The versatility of phage-display and biopanning using the present approach is expected to lead to the identification of additional ligands with tubule segment-specific binding properties (analogous to the CCD ligand) but will also result in the selection of high affinity non–cell-type specific ligands (analogous to the PCT ligand). Characterization of these ligands may contribute to the refinement of the current knowledge on interactions among endothelium, extracellular matrix, and epithelium and may help in the development of cell-specific, systemic delivery applications in the kidney.

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