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Selection of peptide ligands binding to the basolateral cell surface of proximal convoluted tubules

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Background. Recently, we have reported a novel approach of screening phage-display peptide libraries on microdissected intact renal tubular segments and identified an RGD-containing peptide ligand that specifically binds to the basolateral membrane of cortical collecting ducts (CCD). However, screening phage libraries on proximal convoluted tubules (PCT) did not yield a tubule segment-specific ligand. Here, we describe the successful modification of our previously developed phage-display approach and the identification of two distinct ligands that bind specifically to receptors expressed at the basolateral membrane of PCT.

Methods. Ex vivo screening of phage-display peptide libraries for specific ligands was adapted for PCT. The previously developed method was significantly extended by applying it to a distinct tubular segment, varying the number of rounds of biopanning and incubating phage libraries with absorber cells prior to biopanning. Binding specificity and cellular localization of selected peptide-displaying phage or the corresponding synthetic peptide were analyzed using various epithelial cell lines as well as competition assays and confocal immunofluorescence microscopy.

Results. Screening phage-display peptide libraries, depleted of ligands binding to ubiquitously expressed receptors by preincubation with HEK-293 cells, led to the identification of two PCT-specific ligands. Phage expressing peptides with the consensus sequence GV(K/R)GX$_3$(T/S) or RDXR mediated 15-fold and 13-fold higher binding to PCT than control phage, and binding to PCT was 13-fold and 21-fold higher than binding to CCD, respectively. Neither phage mediated significant binding to various epithelial cell lines, and binding of both ligands was abolished by the addition of the corresponding synthetic peptide. Immunofluorescence experiments revealed a submembrane localization of both ligands upon incubation with PCT.

Conclusions. Exploiting the versatility of phage-display and biopanning allowed the identification of two distinct peptide ligands that bind specifically to the basolateral membrane of PCT. Tubule segment-specific ligands, such as the described PCT ligands, may be useful for the analysis of cell-extracellular matrix interactions and may contribute to the development of new therapeutic strategies for renal diseases.

Key words: phage display, peptide ligands, proximal convoluted tubule, cortical collecting duct, microdissection, basolateral membrane.

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Proximal tubules express numerous transporters, channels, and receptors at the luminal and basolateral membrane for the reabsorption of electrolytes, water, amino acids and substances like glucose from the tubular fluid. While many investigators have studied the expression of apically located membrane proteins in proximal tubules [1], little is known about basolateral epithelial markers and their ligands in this segment. Since the expression of cell surface receptors may be significantly altered upon culturing of cells [2, 3], a native system for the identification of kidney tubule-specific ligands is advantageous.

Recently, we described the screening of phage-display peptide libraries ex vivo on microdissected intact renal tubular segments [4]. This approach allowed the identification and characterization of a ligand that binds specifically to cortical collecting duct (CCD), whereas peptides selected from proximal convoluted tubule (PCT) were not tubule segment-specific. Here, we report the successful selection of two ligands that mediate preferential binding to PCT after biopanning phage-display peptide libraries that were pretreated with absorber cells to remove unspecific phage.

METHODS

Sprague-Dawley rat kidneys were perfused in situ via the abdominal aorta, and tubular segments were isolated by microdissection as described recently [4], with minor modifications. The slightly modified perfusion solution contained 120 mmol/L NaCl, 5 mmol/L KCl, 0.25 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 0.2 mmol/L Na_2HPO_4, 0.15 mmol/L NaH_2PO_4, 5 mmol/L glucose, 2 mmol/L lactate, 1 mmol/L pyruvate, 4 mmol/L essential and nonessential amino acids, 20 mmol/L HEPES, pH 7.4, and the osmolarity was adjusted to 400 mOsm/kg by the

addition of mannitol. The left kidney was treated with perfusion solution containing 3.0 mg/mL (0.9 U/mg) of collagenase Clostridium histolyticum (Serva, Heidelberg, Germany). For the removal of endothelial cells and extracellular matrix, small cortical pieces were incubated in perfusion solution containing 0.65 mg/mL collagenase for approximately 45 minutes at 31°C, and PCT and CCD were microdissected in aerated perfusion buffer containing 0.25 mmol/L $CaCl_2$ and 1 mg/mL bovine serum albumin (BSA) at 0°C to 4°C. Intact nephron segments of a total length of 25 or 50 mm, corresponding to approximately 7500 and 15000 cells, respectively, were subjected to incubation with phage or synthetic peptides as described below.

Microdissected intact PCT of a total length of 50 mm (15,000 cells) were preincubated in 200 μL of phage incubation buffer (perfusion solution containing 1% BSA and 100 μmol/L chloroquine) for 15 minutes at 37°C. Tubules were then incubated for 40 minutes at 37°C with 10^{10} plaque forming units (PFU) of phage from the linear random nonapeptide library LL9 or the circular random decapeptide library CL10 in a first set of biopanning experiments or from depleted LL9 libraries (discussed later in this article in a second set of experiments). The ratio of phage per cell was 6.7×10^5. Unbound phage was removed by extensive washing, followed by an acid wash and neutralization with perfusion solution. PCT-associated phages were then recovered and amplified as described previously [4]. In subsequent rounds of biopanning, 10^{10} PFU of purified selected phage were reapplied to freshly isolated PCT (50 mm, 15,000 cells), and after three or four rounds individual phage clones were isolated and the sequences of the expressed peptides determined.

In an attempt to deplete library LL9 [5, 6] of phage binding to various types of renal tubular segments, two different conditions were chosen. In one set of experiments, 10^{10} PFU of phage from library LL9 were incubated with CCD (50 mm, 15,000 cells) for 30 minutes at 37°C, followed by centrifugation of the tubules at $150 \times g$ for three minutes. The ratio of phage per CCD cell was 6.7×10^5. Subsequently, the supernatant containing the unbound phage was incubated with isolated PCT, and PCT-binding phage were isolated as described above. This procedure of depleting the phage pool on CCD and selection of phage binding to PCT was repeated four times, followed by analysis of individual phage clones. In a second set of experiments, the depletion of library LL9 was performed with HEK-293 cells. Upon incubation of 10^{10} PFU of phage with 5×10^6 HEK-293 cells (ratio of 2×10^3 phage/cell), unbound phage were separated from the cells by filtration through 0.45 μmol/L pore size filters (Millipore, Bedford, MA, USA).

Phage-binding to isolated tubules was quantitated according to the selection procedure described above by incubating tubules (25 mm) at 37°C for 40 minutes with 10^{10} PFU of an individual phage clone. In competition experiments, the selected phage and various concentrations of its corresponding synthetic peptide or a random control peptide with the sequence PSRHIPPQL were incubated simultaneously with tubule segments. After acid wash, cell-associated phages were recovered and quantitated by plaque assay.

The binding specificity of selected PCT-binding ligands was assessed by simultaneous incubation of 10^{10} PFU of an individual phage clone with either 10^5 cells of different epithelial origin (MDCK-II, kidney CCD origin; MCF-7, breast; SUT, lung; SW-620, intestine; HELA, cervix; LLCPK-1, kidney PCT origin; rat GMC, kidney glomerular origin) or the corresponding synthetic peptide (Macromolecular Resources, Fort Collins, CO, USA) at various concentrations (Fig. 1). After extensive washing, cell-associated phage was determined as described above.

For intracellular localization of peptides, microdissected PCT (25 mm) were preincubated in phage incubation buffer (perfusion solution containing 1% BSA and 100 μmol/L chloroquine) for 15 minutes at 37°C prior to the addition of 10 μmol/L of synthetic biotinylated peptide and incubation for one hour at 37°C. Reactions were terminated by adding ice-cold phage incubation buffer, followed by the removal of unbound peptides by extensive washing and acidic wash. The pelleted PCT were taken up in 100 μL of microdissection buffer, subjected to cytospin centrifugation at $100 \times g$ for three minutes onto glass cover slides and fixed with 4% paraformaldehyde supplemented with 4% sucrose in 100 mmol/L sodium phosphate buffer, pH 7.4, for 10 minutes at 25°C. Immunostaining and analysis by confocal microscopy were performed as described previously [4], whereby the Na/HCO_3 cotransporter was detected with a rabbit polyclonal antibody (Chemicon, Temecula, CA, USA) and ALEXA-594-conjugated goat anti-rabbit IgG, and synthetic peptides were visualized using fluorescein-conjugated mouse monoclonal anti-biotin antibody (Molecular Probes, Leiden, The Netherlands).

Statistical analysis of phage binding specificity was performed using the Wilcoxon signed rank test. A P value less than 0.05 was considered as statistically significant.

RESULTS

Screening phage display peptide libraries for PCT-binding ligands

In a previous screening of phage-display libraries, four rounds of selection on isolated PCT yielded phage-expressing peptides of the consensus sequence KX_3TNHP, which were not tubule segment-specific. Due to the competitive nature of biopanning, ligands binding to receptors with lower expression levels or ligands with lower affinity may have been lost after four rounds of selection.

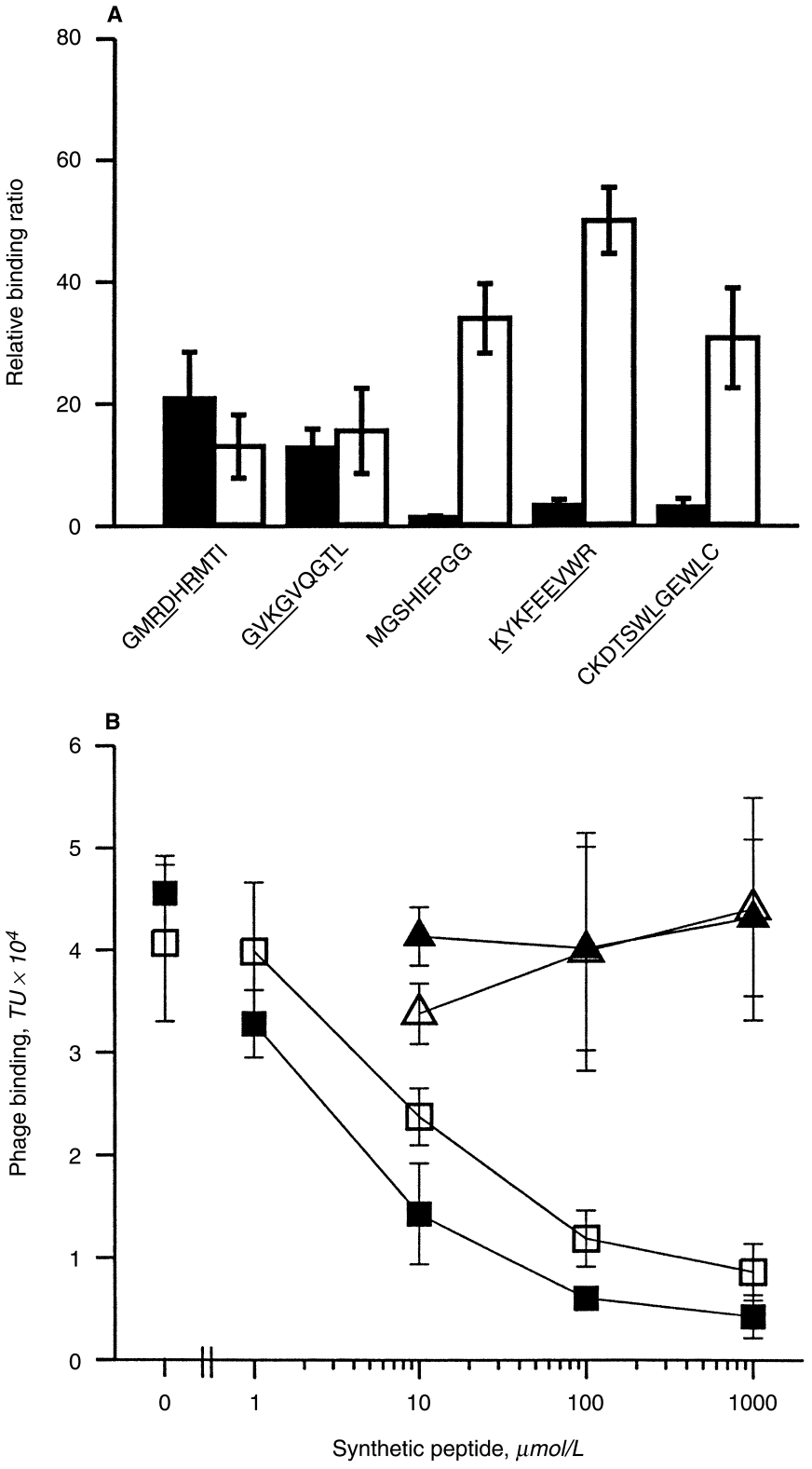

Fig. 1. Analysis of phage-binding specificity. (*A*) Ratios of phage binding to proximal convoluted tubule (PCT) versus cortical collecting duct (CCD) (PCT/CCD, ■) and of phage binding versus control to PCT [PCT(specific phage)/PCT(control phage); □]. (*B*) Inhibition of PCT binding of GV-phage by a random control peptide (△) or by the synthetic peptide GVKGVQGTL (□), and inhibition of PCT binding of RD-phage by a random control peptide (▲) or by the synthetic peptide GMRD HRMTI (■) was determined. Data represent mean ± SE of the number of phage plaques (A) or of phage binding (PFU × 10^4) per cm of tubule (B) from three to five independent experiments.

Therefore, in the present study, individual phage clones were analyzed after three rounds of selection on PCT. Phage expressing the consensus sequence $KX_2FX(E/D)VW$ were recovered from panning the linear library LL9 in addition to the motif KX_3TNHP (Table 1). Three rounds of biopanning the circular library CL10 yielded the circular motif C-X_2(T/S)SWLXEWL-C (Table 1), which mediated 30-fold higher binding to PCT than con-

Table 1. Peptides selected from microdissected proximal convoluted tubules (PCT)

Type of library *N of biopanning rounds*	Selected sequence	*N* of identical sequences
Linear Library LL9	KMGGTNHPE	(2)
(3 rounds, 12 clones were sequenced)	KSAVTNHGI	(2)
	HLNHPMSIM	(1)
Consensus	KXXXTNHP	
	KYKFEEVWR	(4)*
	KMAFQDVWM	(2)
	KSGFNEVWP	(1)
Consensus	KXXFX(E/D)VW	
Constrained Library CL10	CKDTSWLGEWLC	(4)*
(3 rounds, 8 clones were sequenced)	CEKSSWLAEWLC	(2)
	CLLTSFLGEVYC	(1)
	CLISQRHVASSC	(1)
Consensus	C(T/S)SWL(A/G)EWLC	
Linear Library LL9	MGSHIEPGG	(4)*
Depleted on CCD	TGSFGVAGG	(2)
(4 rounds, 12 clones were sequenced)	GGMVSQGSK	(1)
	GGMGEHGSS	(1)
Consensus	G(G/S)XXXG(G/S)	
Linear Library LL9	GVKGVQGTL	(3)*
Depleted on HEK-293	HGVRGNLIS	(2)
(4 rounds, 16 clones were sequenced)	GVRGQLATP	(1)
Consensus	GV(K/R)GXXX(T/S)	
	GMRDHRMTI	(4)*
	ETMQRDVRA	(2)
	YRDFRDIWA	(1)
	SLRDRGFT	(1)
	HLNMWRDGG	(1)
	GGAIKDTQN	(1)
Consensus	RDXR	

Alignment of phage-displayed peptides isolated from screening the linear nonapeptide library LL9 or the circular decapeptide library CL10 on microdissected PCT. To deplete library LL9 of ubiquitously binding phage, a library aliquot was incubated with either CCD or HEK-293 cells in each round of biopanning, and the pool of unbound phage was screened for phage binding to PCT. The number of isolated phage expressing identical peptide sequences is indicated in parenthesis. The phage ligands used for analytic studies are indicated by an asterisk. Residues characteristic for the binding motif are underlined.

trol phage ($P < 0.05$; Fig. 1A). Unfortunately, all three ligands selected from screening crude libraries were not tubule segment-specific with only two- or threefold increased binding to PCT compared to CCD (Fig. 1A).

Affinity purification of PCT-specific peptide ligands from depleted libraries

In an attempt to deplete the linear nonapeptide library LL9 of ligands binding unspecifically to different nephron segments, we incubated a library aliquot with isolated CCD prior to panning unbound phage on microdissected PCT. This procedure resulted in the selection of phage expressing peptides with the conserved sequence G(G/S) X_4G(G/S) (Table 1), which was not observed when screening the undepleted library. Binding studies using purified phage expressing the peptide MGSHIEPGG revealed 34-fold increased binding to PCT compared to control phage but almost equal binding to PCT and CCD (Fig. 1A).

To achieve a more efficient depletion of ubiquitously binding ligands, a library aliquot was incubated with HEK-293 cells at a ratio of 2×10^3 phage per cell. Comparison of the peptide sequences expressed by 16 isolated individual phage clones revealed the two distinct motifs GV(K/R) GX_3(T/S) (GV-phage) and RDXR (RD-phage) (Table 1). Neither motif was observed in previous biopanning experiments.

Characterization of binding properties of GV-phage and RD-phage

Analysis of binding of GV-phage or RD-phage to microdissected tubules revealed 15-fold and 13-fold increased binding to PCT versus control phage, whereby binding ratios of PCT to CCD were 13-fold and 21-fold, respectively ($P < 0.05$; Fig. 1A). The binding specificity of GV-phage and RD-phage was characterized further by comparing binding of specific phage with that of control phage to various epithelial cell lines. Binding of both GV-phage and RD-phage to isolated CCD or to the cell lines MDCK-II (kidney, CCD origin), MCF-7 (breast), SUT (lung), SW-620 (colon), HELA (cervix), LLCPK-1 (kidney, PCT origin), and GMC (kidney, glomerular origin) was determined by using the plaque assay as indicated in Figure 1A, yielding binding that was not significantly higher than control phage binding (data not shown). Highest binding of the RD-phage, at only 2.5-fold higher levels than control phage binding, was observed for LLCPK-1 cells. In competition assays, coincu-

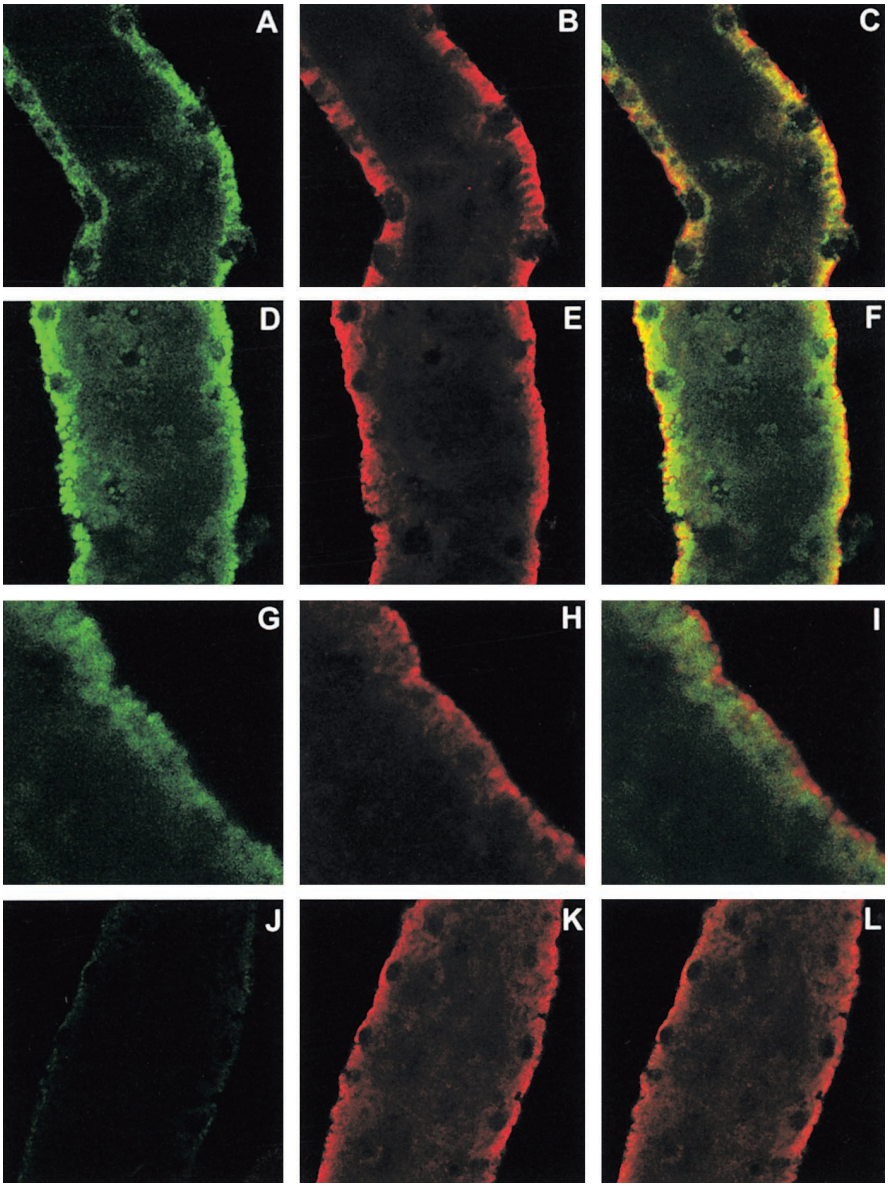

Fig. 2. Localization of PCT-binding peptides by immunofluorescence. Microdissected intact PCT were incubated with 10 μmol/L of the biotinylated synthetic peptide with the sequence GMRDHRMTI (*A* through *C*), or GVKGVQGTL (*D* through *F*, enlarged in *G* through *I*), or with an irrelevant control peptide with the sequence PSRHIPPQL (*J* through *L*). Samples were dual stained with fluorescein-labeled anti-biotin antibody to detect biotinylated synthetic petides (A, D, G, J) and with rabbit polyclonal anti-Na/HCO_3 cotransporter antibody and ALEXA-594 labeled goat anti-rabbit secondary antibody to detect the Na/HCO_3 cotransporter located at the basolateral cell surface (B, E, H, K). An overlay of the fluorescein fluorescence from the synthetic peptide and of the ALEXA-594 fluorescence from the Na/HCO_3 cotransporter is shown on the right panel (C, F, I, L). Magnifications: ×200 in A through F and J through L, and ×600 in G through I.

bation of microdissected PCT with varying concentrations of the corresponding synthetic peptide revealed an IC_{50} between 20 and 40 μmol/L for the GV-phage and between 2 and 4 μmol/L for the RD-phage (Fig. 1B). Binding of both phages was not affected by coincubation with an irrelevant control peptide.

Detection of peptide ligand binding by fluorescence microscopy

Immunofluorescence experiments were performed in order to localize PCT-bound peptide ligands (Fig. 2). Samples were stained with an antibody against the Na/HCO_3 cotransporter and an anti-biotin antibody to visualize biotinylated peptides. Analysis by confocal microscopy revealed specific localization of both RD-peptide and GV-peptide at the basolateral surface of the microdissected, centrifuged PCT similar to the Na/HCO_3 cotransporter after incubation for two minutes at 37°C or upon incubation at 4°C (not shown). However, while the staining of the Na/HCO_3 cotransporter was more uniform and most intense at the surface, the staining of the peptides showed a multifocal submembrane staining pattern upon incubation for 40 minutes at 37°C (Fig. 2 C, F, I). Incubation of PCT with a control peptide did not result in significant binding (Fig. 2J), in contrast to RD- and GV-peptides (Fig. 2 A, D), confirming further the observed PCT-binding specificity of RD-phage and GV-phage compared to the control phage.

DISCUSSION

Our recently developed approach of incubating phage-display libraries with microdissected intact renal tubular

segments successfully yielded a ligand binding specifically to CCD, however, no specific ligand was selected for PCT [4]. The aim of the present work was to extend this method in order to make it suitable for the identification of ligands for various defined renal segments such as PCT. For this purpose, the previous screening procedure for PCT was extended by two modifications. First, analysis of individual phage clones was performed after three instead of four rounds of biopanning on PCT. Since the screening of phage-display libraries is a competitive process that ultimately tends to result in the selection of the "best-fit" sequence, ligands binding to receptors with lower expression levels or ligands with lower affinity may have been lost after four rounds of selection in the previous experiments. The reduction of panning rounds resulted in the isolation of two peptide ligands that were not observed previously (Table 1). Their motifs, $KX_2FX(E/D)VW$ and $C\text{-}X_2(T/S)SWLXEWL\text{-}C$, resemble each other when considering conservative substitutions of amino acids, suggesting that both peptides bind to the same receptor. However, these ligands were again not tubule segment-specific. Second, in order to deplete phage-libraries of ubiquitously binding ligands, phages were preincubated with absorber cells prior to selection on PCT. The number of approximately 1.5×10^4 CCD cells and a ratio of 6.7×10^5 phages per cell might have been insufficient for library depletion, leading to the isolation of a peptide ligand with the conserved sequence $G(G/S)X_4G(G/S)$ (Table 1) that bound to PCT but was not segment specific (Fig. 1A). In contrast, efficient depletion of unspecific phage was achieved by incubation of phage with HEK-293 cells at a ratio of 2×10^3 phages per cell. This modification allowed the isolation of two distinct phage containing the motifs $GV(K/R)GX_3(T/S)$ and RDXR (Table 1) which exhibited significant binding specificity for PCT over CCD (Fig. 1A). A database search with the consensus sequences of the two motifs did not reveal their native epitope. However, possible candidates among several proteins containing the motif $GV(K/R)GX_3(T/S)$ are collagen-α subunits. Human and rat collagen α 1 (III) contain the sequence GVKGERGS whereas human collagen α 1 (XI) and collagen α 1 (XVI) contain the sequence GVRGLLKGS and GVRGLPGT, respectively. It should be noted here that none of the ligands selected from PCT contained an RGD sequence as previously found in panning experiments on isolated CCD [4]. Further analysis of binding specificity of these two phage to various epithelial cell lines revealed binding that was less than 2.5-fold that of control phage (data not shown). Importantly, the two motifs did not mediate significant binding to LLCPK-1 cells, which are often used as a cell model for PCT. Isolation and culturing of rat proximal tubule cells have been shown to result in significantly altered expression and distribution of cell surface receptors [2]. These observations emphasize the need for a native cell system for the identification of renal epithelial cell ligands and their cognate receptors.

Detection of GV-peptide and RD-peptide by immunofluorescence suggested the initial binding of the specific peptides to the basolateral cell surface (not shown) and internalization to a submembranous localization upon prolonged incubation at 37°C (Fig. 2). Evidence for internalization also is given by the fact that phage resisted the acid wash after incubation with PCT for 40 minutes at 37°C. The submembranous localization of specific peptides may be explained by a recycling pathway between the cell membrane and early endosomes as described for some receptor-ligand complexes [7]. Alternatively, further trafficking of the PCT-binding peptides beyond early endosomes may have been hampered by a disruption of the cellular microtubule network due to a lack of oxygen or a temperature-dependent effect. Actin filaments and microtubules are involved in endo- and exocytotic pathways, whereby endocytosis from the apical membrane appears to be more affected by cytoskeleton disrupting agents than from basolateral membranes [8, 9]. In proximal tubule cells, ischemia causes a rapid, duration-dependent dissociation of the actin cytoskeleton and a redistribution of some surface membrane proteins [10]. Cold treatment followed by rewarming of renal epithelial cells results in a partially reversible disruption of the microtubule network, especially in proximal tubules, and a redistribution of some membrane proteins [11]. We cannot exclude the possibility that in our experiments microtubules were affected by the microdissection procedure that was performed on ice and did not completely recover within the 15-minute adaption period at 37°C prior to the addition of peptide ligands.

The utility of defining basolateral ligands for PCT is twofold. Such ligands are relevant for putative investigations of cell-matrix interactions and for therapeutic purposes. Delivery of biologically active molecules to the proximal tubule is of interest in some acquired or congenital proximal tubulopathies such as Dent's disease [12] or Fanconi syndrome [13]. Ligands such as those found in the present study may be used for targeting of viral or non-viral vectors to distinct nephron segments. Transduction of renal epithelial cells via the basolateral membrane, however, necessitates the access of the delivery vector from the blood stream across the endothelium and the extracellular matrix to the epithelium, a pathway sown by other groups to be facilitated by coapplication of substances that increase vascular permeability [14].

In conclusion, we have significantly extended our recently developed strategy of screening phage-display libraries ex vivo on microdissected renal tubular segments and identified two peptide ligands that bind specifically to the basolateral cell surface of PCT. The identification and characterization of ligands with tubule segment-specific properties provide a means to extend existing

knowledge about tubular epithelial cell functions such as cell-extracellular matrix interactions and receptor-mediated endocytosis, and contributes to the development of vectors for drug and/or gene delivery to specific renal tubular segments for future therapeutic treatment.

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