Molecular Profiling of Heart Endothelial Cells

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Background—Endothelial cells that line the vascular lumen can express cell-surface proteins that are specific to the endothelium of a particular tissue. In this study, we probed the heart vasculature for heart-specific endothelial markers by phage display.

Methods and Results—We used a novel combination of in vivo phage selection and a bacterial 2-hybridization scheme against a heart cDNA library, which allows simultaneous identification of peptides that specifically bind to the target endothelium, as well as the endothelial molecules (receptors) recognized by the peptides. We found 5 heart-targeting peptides and their receptors. We confirmed and quantified the selective expression of 4 of the proteins in heart endothelial cells by independent methods. The heart specificity of phages was as high as 300-fold greater than that of nonrecombinant control phages. The proteins selectively expressed by the heart endothelium were in most cases also expressed by cardiomyocytes and, at lower levels, in some other tissues.

Conclusions—These findings provide new markers for the endothelium of heart vessels and reveal a commonality between parenchymal and endothelial gene expression in the heart. The heart-homing peptides provide a means of targeting diagnostic and therapeutic agents to the heart, and their receptors are potential drug discovery targets. (Circulation. 2005;112:1601-1611.)

Key Words: arteries ■ peptides ■ imaging ■ capillaries ■ endocardium

The heart vasculature is at the center of a range of cardiac diseases, which are responsible for more deaths in Western countries than any other disease. Endothelial cells that line the blood vessels have been shown to differ among tissues,1–6 but the specific features of the endothelium in the heart vasculature have not been fully elucidated. Mapping of molecular markers specific for heart vessels could be useful in understanding the susceptibility of these vessels to pathological processes, such as atherogenesis, and might allow specific targeting of therapeutic agents into the heart.

We used in vivo and ex vivo/in vivo screening of phage-displayed peptide libraries and cDNA libraries for the discovery of tissue-specific vascular markers.7,8 We have identified homing peptides for the vessels in a large number of normal organs and tissues, including the brain, kidneys, lungs, breast, prostate, and several other tissues.4,9,10 Others have isolated phage peptides that selectively home to muscle11 or fat12 tissue. We have also identified homing peptides that distinguish tumor blood vessels or tumor lymphatics from normal vessels, and it has even been possible to use homing peptides to show that the blood vessels of premalignant lesions differ from both normal vessels and those of full-blown malignancies in the same tissue.13–17 Atherosclerosis is another pathological condition that has been successfully targeted: The endothelium overlying the lesions expresses markers detectable with phage display.18,19 The success in finding specific markers for so many tissues has led to the suggestion that all normal tissues and perhaps diseases as well put a signature on their vasculature.4

In the present study, we extensively profiled the heart vasculature by phage display. In addition to performing phage screening, we built receptor discovery into the process. We accomplished this by matching pools of heart-homing peptides against a heart cDNA library in a bacterial 2-hybrid system (Figure 1A). We report here on a set of peptides that recognizes 5 proteins, each of which is expressed at higher levels on the endothelial cells of the coronary vessels and chambers of the heart and is largely absent in the vessels of other major organs. Our results show that it is possible to target molecular probes into the heart with high selectivity.

Methods

Animals

BALB/c male and female mice 2 to 3 months old were used for the screening of phage libraries and to test the specificity of phage and synthetic peptides. Where indicated, testing was extended to include FVB and C57BL/6 mice. The animal experiments were approved by the Burnham Institute Animal Research Committee.

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Figure 1. Identification of homing peptide/receptor pairs by ex vivo/in vivo phage display and bacterial 2-hybrid analysis. The principle of parallel homing-peptide isolation and receptor identification. A phage-displayed peptide library is injected into a mouse and allowed to circulate; tissues are harvested and then disrupted into single cells with bound phage. Nonbound phages are washed away; bound phages are recovered and grown in bacteria; and the process is either repeated or the peptide-encoding inserts are amplified by PCR and shuttled into a bait vector of the bacterial 2-hybridization system. Once inside the bacteria, the pool enriched for endothelium-binding peptides can interact with possible receptors and bestow carbenicillin resistance to bacteria. Resistant clones are plated on X-gal–containing agar for a secondary screen; target and bait inserts are then amplified and sequenced from carbenicillin-resistant, LacZ-expressing cells (A). By the third round, a selected pool bound ex vivo heart cells ~230-fold more than nonrecombinant T7 phage (B). When the ex vivo selected pool was further selected for homing to the heart in vivo, strong enrichment was seen in successive rounds of selection, and the final pool accumulated in the heart vasculature nearly 200 times more than nonrecombinant control phage (C). Peptide-encoding inserts from this final pool were subcloned into the bait vector, and some bacteria transformed with both the peptide-bait vectors and heart cDNA library (in target vector) were able to grow on 500 μg/mL carbenicillin plates (D). Some clones were also positive for the secondary marker LacZ, as evidenced by the production of the blue color on plates containing X-gal (E).
Cell Culture
Human coronary artery endothelial cells (HCAECs; Cambrex, Walkersville, Md) and human umbilical vein endothelial cell (HUVECs; Cambrex) were grown in the manufacturer’s growth medium (EGM-2-MV Bulletkit for HCAECs, EGM Bulletkit for HUVECs; Cambrex). HEK 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum.

Ex Vivo and In Vivo Phage Selections
An NIK-encoded CX7C library display on T7Select 415-1 phage (Novagen) was generated as described. Phage selections and validations have been described. We performed 3 rounds of ex vivo selection on heart cells isolated from 2-month-old female BALB/c mice, followed by 3 rounds of selection in vivo. For each ex vivo selection, cell suspensions were prepared from the heart with collagenase IA (1 mg/mL, Sigma) to disperse the tissue. Cells (5 x 10^7) were incubated overnight at 4°C with 5 x 10^10 plaque-forming units (PFU) of a CX7C library. The cells were washed to remove unbound phage. M450 magnetic beads (Dynal) and antimouse CD31 (PharMingen) were used to isolate the vascular endothelial cells. Phage bound to the CD31-positive cell population was rescued, titered, and amplified in Escherichia coli. The ex vivo–selected phage pool (5 x 10^10 PFU) was intravenously injected into a mouse through the tail vein and allowed to circulate for 10 minutes, and the mouse was carefully perfused through the left ventricle with DMEM to remove unbound intravascular phage. The heart and control tissues (brain, kidney, skin, and muscle) were excised. The phage recovered from the heart was reinfected into mice, and the cycle was repeated for a total of 3 rounds. In each experiment, nonrecombinant phage was injected into a separate mouse as a control, and relative phage titer was determined as the ratio of specific phage and nonrecombinant phage recovered from a given tissue.

Bacterial 2-Hybridization Scheme
We constructed the library of bait plasmids by amplifying the peptide-encoding DNA inserts from the heart-homing phage by polymerase chain reaction (PCR). The primers were 5’ TCAGGT-GTATGTCCTGGG (forward) and 5’ GAGTAA CTAGTTAACCC (reverse). The PCR products were digested with EcoRI and XhoI and subcloned into the bait pBT plasmid (Stratagene). We refer to this recombinant library of baits as pBT-hp. The pBT-hp plasmids were transformed into XL1-Blue MRF’ Kan–competent cells, and 2 or 5 μL of the bacterial suspension was plated on 100-mm LB-chloramphenicol plates to determine the total number of primary transformants. The remaining transformants were pooled and plated on 150-mm LB-chloramphenicol plates. Twenty clones were sequenced and found to have proper peptide-encoding inserts. The plates were then scraped and the bacteria harvested. We plated 2 and 5 μL of the harvested library to determine the size of the amplified library and then purified the pool of pBT-hp plasmids from the pooled bacteria by using a commercially available Maxiprep DNA column. This DNA was used to transform the BacterioMatch 2-hybrid system reporter strain–competent cells.

We plate-purified the heart cDNA library plasmids by diluting 2 μL of mouse heart plasmid cDNA library (Stratagene, No. 982303) into 25 μL Super Optimization Catabilome medium and then spread this onto twenty-five 25 x 25-cm LB-tetracycline plates. The plates were incubated at 30°C for 24 hours. We harvested ~5 x 10^6 individual clones and purified plasmid DNA from these.

The bacterial 2-hybridization experiments were performed with the BacterioMatch 2-hybrid system (Stratagene) according to the manufacturer’s instructions. In brief, we cotransformed pBTH and heart cDNA library plasmids with 50 ng of each of the Aliquots (100 μL) of the cotransformants were plated on selective LB plates (500 μg/mL carbenicillin); the plates were then incubated at 37°C for 24 hours and scored for growth. Interactions between a pair of hybrid proteins, as was the case with the positive controls of Gal4–Gal11P hybrid protein, were indicated by growth on selective plates. We streaked individual positive colonies from the carbenicillin plates onto X-gal plates for a secondary screen and incubated the plates for 12 to 14 hours. We cultured cells that showed a dark blue color with X-gal plates and that were able to grow in the presence of LB-chloramphenicol or LB-tetracycline (markers of the target and bait plasmids) overnight at 30°C. We purified the plasmid DNA and retransformed putative bait and target plasmids into the reporter strain clones that were still able to reproducibly grow on selective plates were verified as positive. We then amplified the bait or target inserts by PCR and determined their sequences. Then, sequences from the target vectors were used as queries in a BLASTN search of the nonredundant mouse genome.

Reconstructing Individual Phage Clones
Oligonucleotides that encoded peptides from the selected bait plasmids were synthesized (Integrated DNA Technologies), phosphorylated with T4 PNK (NEB) at 37°C for 1 hour, and annealed at a concentration of 0.08 pmol/μL. The annealed inserts were diluted to 0.04 pmol/μL and ligated into T7Select 415-1 arms (Novagen) with T4 ligase (NEB) overnight at 16°C. The next day, intact recombinant phages were made by mixing the ligation reaction with packaging extracts, as described by the manufacturer. Inserts were confirmed by sequencing, and individual clones were amplified for ex vivo and in vivo testing.

Antibodies and Immunohistochemistry
Custom immunization to produce a rabbit antiserum against mouse cysteine-rich protein-2 (CRIP2) was performed by ProteinTech Inc. Rabbis were immunized with a fusion protein of Glutathione S-transferase (GST) full-length mouse CRIP2. The antibody was affinity-purified on the fusion proteins and absorbed with GST. The resulting antibody preparation (1.6 mg/mL) gave a titer of 1:10 000 against the CRIP2 fusion protein on ELISA.

For immunochenical staining, frozen sections were preincubated with blocking buffer (5% normal goat serum and 0.5% bovine serum albumin in 1 x phosphate-buffered saline [PBS]) for 1 hour, washed 3 times with PBS, and incubated with the primary antibody of interest overnight at 4°C. The antibodies used were rat monoclonal anti-mouse CD31 (1:100, BD Pharmingen), rabbit polyclonal anti-T7 phage (1:500), mouse monoclonal anti-α-smooth muscle actin (1:800, Sigma), and chicken anti-mouse CRIP2 IgY (1:100, GenWay).

The corresponding secondary antibodies were added and incubated for 1 hour at room temperature: AlexaFluor-488 goat anti-rabbit IgG (1:1000, Molecular Probes), AlexaFluor-594 goat anti-rat or rabbit IgG (1:1000, Molecular Probes), and GAYFC-AlexaFluor-594 goat anti-IgY Fc (1:250, GenWay), respectively. The slides were washed 3 times with PBS and mounted in Vectashield mounting medium with DAPI (Vector Laboratories). Blood vessels were also visualized by intravenously injecting Lycopersicon esculentum (tomato) lectin conjugated to fluorescein (100 μg of lectin in 200 μL of PBS; Vector Laboratories).

Peptide Synthesis
Peptides were synthesized in our peptide facility by Fmoc chemistry in a solid-phase synthesizer. The peptides were purified by high-performance liquid chromatography and confirmed by mass spectrometry. Fluorescein-conjugated peptides were synthesized as described.

In Situ Hybridizations
Nonradioactive in situ hybridization has been described. In brief, digoxigenin-labeled antisense RNA probes were generated by PCR amplification and incorporation of an SP6 promoter into the antisense primer and a T7 promoter into the sense primer. In vitro transcription was performed with digoxigenin RNA labeling reagents and T7 RNA polymerase (Roche). Frozen tissue sections were fixed with 4% paraformaldehyde, permeabilized with pepsin, and incubated with the RNA probes (200 ng/mL) overnight at 35°C. A horseradish peroxidase rabbit anti-digoxigenin antibody (Dako) was used to catalyze the deposition of biotin-tyramide (GenPoint kit) for

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signal amplification. Further amplification was achieved by adding horseradish peroxidase rabbit anti-biotin (Dako), biotin-tyramide, and alkaline phosphatase rabbit anti-biotin (Dako). Signal was detected with the alkaline phosphatase substrate fast red TR/naphthol AS-MX (Sigma), and sections were counterstained with hematoxylin.

Quantitative PCR
Quantitative PCR (Q-PCR) was performed as previously described. Primers were designed with the Primer 3 program. Primers were as follows: CRIP2, 5′ ggcacaatgtgtcag3′ (forward) and 5′ acttagttactctcag3′ (reverse); Sigirr, 5′ gaagttcctctgctc3′ (forward) and 5′ aaggtcttttctcgtc3′ (reverse); MpcII-3, 5′ tcctgagaagagaacag3′ and 5′ gcacaacttagctgct3′; and bc10, 5′ gttcactctcagttc3′ (forward) and 5′ aatcagaaacctgatag3′ (reverse). The primers for glyceraldehyde 3-phosphate dehydrogenase that were used as a housekeeping gene control were 5′ ataagcactaagggcttg3′ (forward) and 5′ ctgctgtagccgtattc3′ (reverse). The primers were obtained from Genbase or Integrated DNA Technologies.

To prepare RNA for PCR analyses, total RNAs were extracted from the organs of interest with the RNeasy kit (Qiagen). cDNA was made from 50 ng of whole RNA with use of the SuperScript II first-strand synthesis kit (Invitrogen). Q-PCRs were performed with LightCycler SYBR green DNA master mix (Roche). The amplification protocol consisted of an initial denaturation/antibody inactivation step at 95°C for 10 seconds; followed by 42 amplification cycles with samples heated to 95°C, 56°C for 7 seconds, and 72°C for 20 seconds. Melting analysis was performed after a final melting step at 95°C, followed by a 15-second annealing at 68°C, and then increasing the temperature to 97°C at 0.1°C/s with continuous fluorescence measurements. For relative expression analysis, the level of glyceraldehyde 3-phosphate dehydrogenase–normalized expression value for each receptor in the heart was defined as 100% and used as the basis for comparison. The specificity of Q-PCR was confirmed by melting curves of each PCR product, agarose gel electrophoresis, and DNA sequence analysis.

Receptor Transfections, Phage-Binding Assays, and Fluorescence-Activated Cell Sorting Assays
293T cells were transfected with plasmids (pHMe, Roche) encoding CRIP2, Sigirr, MpcII-3, and bc10 with the Fugene transfection reagent (Roche). In brief, 10 μg of plasmid was mixed with 700 μL of DMEM without serum and 30 μL of Fugene and incubated for 15 minutes at room temperature before being added to the cells. Forty-eight hours after transfection, the cells were detached from the culture dishes with EDTA and washed once with PBS. Phages displaying CRIPPR, CRKRAVR, CRSTRANP, CPKTRRVP, or non-combinatorial control phage (10⁶ PFU) were separately incubated with the corresponding transfected cells for 2 hours at 4°C. Unbound phages were removed by 5 washes with 1% bovine serum albumin in PBS. Bound phages were rescued by adding bacteria, and the binding efficiencies were determined by a plaque assay. Peptide competition of phage binding was studied by adding peptides to the incubation mixture at 100 μg/mL. For fluorescence-activated cell sorting (FACS) analysis, CRIP2-transfected and control plasmid–transfected 293T cells were detached from culture dishes by gentle washing with PBS containing 1% bovine serum albumin. The cells were then stained with 45 μg/mL rabbit anti-mouse CRIP2 or normal rabbit IgG (Sigma) on ice for 1 hour, washed, and incubated with goat anti-rabbit IgG–fluorescein isothiocyanate (40 μg/mL in PBS–bovine serum albumin, Molecular Probes) on ice for 45 minutes. After a final wash, the cells were resuspended with PBS containing 2 μg/mL propidium iodide and analyzed by FACS analysis at the Burnham Institute Cell Analysis Facility.

Results
Identification of Heart Endothelial Ligand-Receptor Complexes
We performed ex vivo phage selection on cell suspensions from murine hearts. To increase the possibility of isolating peptides homing to vasculature in the heart, we isolated endothelial cells from the cell suspension with anti-CD31 magnetic beads before recovering and amplifying the bound phage. Three rounds of selection gave 230-fold enrichment for ex vivo cardiac cell binding compared with nonrecombinant phage (Figure 1B). When this ex vivo selected pool was subjected to in vivo selection for homing to the heart, the homing increased from 5-fold in the first round to nearly 200-fold in the third round relative to nonrecombinant phage. The enrichment over skeletal muscle, skin, brain, and kidneys was 20–50-fold (Figure 1C).

We next amplified the peptide-encoding DNA inserts of the heart-homing phage pool by PCR, introduced the inserts into the bait vector of a bacterial 2-hybrid system, and cotransformed the bacteria with this vector and a target vector encoding a heart cDNA library. A high-stringency screen revealed colonies indicative of a bait-target interaction at a frequency comparable to that of the positive control, whereas no colony growth was observed when only 1 of the 2 vectors was introduced into the bacteria (Figure 1D). A control for leakiness of the system eliminated 75 of 100 colonies as likely false-positives (Figure 1E).

The in-frame sequences from the 25 remaining cDNAs were queried for sequence matches from the National Center for Biotechnology Information nonredundant database. Nineteen of the 25 were abundant proteins in cardiac muscle, such as myosin, myoglobin, ferritin, and oxoglutarate dehydrogenase, whereas the other 6 were membrane or cell-surface proteins (Table). Thus, the combination of in vivo phage display and bacterial 2-hybridization proved to be a method that rapidly identified heart-homing peptides and their putative receptors.

Receptor-Peptide Pairs and Heart-Homing Efficiency of the Peptides
Receptor clone 5 represents the C-terminal 92 amino acids of heart LIM protein (HLP, also known as CRIP2 and ESP1). CRIP2/HLP is an LIM domain–containing protein discovered on the basis of its homology to Crp2 and as a protein expressed in the vascular endothelium of the heart. Crip2 does not have an apparent transmembrane domain or signal sequence. There were 3 peptides present in the 5 colonies sequenced from the HLP clone: CRPRP was present twice, CGRKSSTVC once, and CGNQVDSRC twice. These peptides were cloned back into the T7Select415-1 display vector and individually tested for their ability to home to the heart vasculature after intravenous injection. Relative to nonrecombinant phages, the CRPRP-displaying phage homed with >300-fold selectivity to the heart, whereas the selectivity was ∼50-fold for CGRKSSTVC and insignificant for CGNQVD-SRC (Table and Figure 2A).

Receptor clone 9 is an unannotated RIKEN expressed sequence tag (EST). We found 3 peptides present of the 5 colonies sequenced; 2 of them homed to the heart and 1 did not (Table and Figure 2B).

Receptor clone 15 is a single immunoglobulin interleukin-1 receptor–related protein (Sigirr, TIR8). This protein contains the transmembrane domain and an ∼190–amino acid N-terminal extracellular portion. Sigirr expression
has been found in epithelia of the kidneys, lungs, gut, and some other tissues, but its expression in the heart or in endothelial cells has not been reported. The 3 peptides in this group showed 20- to 30-fold heart-homing selectivity (Table and Figure 2C).

Receptor clone 27 is a hypothetical protein annotated as a glutamine-rich region–containing protein identified from an olfactory cDNA library. The 5 bait clones sequenced all coded for the same peptide, but it showed no specific homing and was not studied further (Table and Figure 2D).

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<table>
<thead>
<tr>
<th>Receptor</th>
<th>GI (Gene ID)</th>
<th>Region (CDS)*</th>
<th>Peptides</th>
<th>Heart Homing</th>
<th>Fold Homing†</th>
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<tr>
<td>Clone 5: Cystein-rich protein 2 (CRIP2; HLP; ESP-1)</td>
<td>NM_024223 (68337)</td>
<td>380–1140 (40–666)</td>
<td>CRPPR CGRKSKTVC</td>
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<td>Clone 9: EST</td>
<td>20071633 (66194)</td>
<td>710–1261 (16–840)</td>
<td>CPSELLLP CARPAR CKRPR</td>
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<td>3×</td>
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<td>Clone 15: Single immunoglobulin interleukin-1 receptor–related protein (Sigirr; TIR8)</td>
<td>BC010806 (24058)</td>
<td>1–801 (120–1349)</td>
<td>CKRAVR CRNSWKPNPC RGSSS</td>
<td>Yes</td>
<td>26×</td>
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<td>Clone 27: hypothetical glutamine-rich region–containing protein, olfactory</td>
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<td>1038–1877</td>
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<td>Clone 36: unnamed protein product; similar to integral membrane protein Cil-3 (Mpcil-3)</td>
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<td>30–801 (28–537)</td>
<td>CRSTRANPC</td>
<td>Yes</td>
<td>22×</td>
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<td>Clone 46: Mus musculus bladder cancer–associated protein (human) homologue (bc10)</td>
<td>20072483 (53619)</td>
<td>419–1359 (253–516)</td>
<td>CPKTRRVPCC SMARTKC</td>
<td>Yes</td>
<td>62×</td>
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GI is the NCBI GenBank accession number. Gene ID is the accession number for NCBI’s Entrez Gene service.

*This refers to the sequenced insert from the target vector, with the numbers corresponding to the mRNA of each receptor. Each region is within each receptor’s coding sequences (CDS).

†Relative to nonrecombinant T7 phage.

Figure 2. Individual phage-displayed peptides selectively home to the heart. The indicated peptide sequences from a combined screen for heart endothelial homing were reconstituted in T7 phage and intravenously injected into mice. With 1 exception, much higher titers of these phages were recovered from the heart than was the case with similarly injected nonrecombinant phage. The heart homing was specific, because other major organs yielded low phage titers. The highest specificity (>300-fold) was recorded for the CRPPR phage in A.
Receptor clone 36 contains the entire coding sequence of an unnamed protein product from the RIKEN Fantom set. It is annotated as being putatively similar to integral membrane protein CII-3 (MpcII-3), a mitochondrial membrane protein that is part of the succinate dehydrogenase complex. It is not known where this protein is expressed. All 5 colonies sequenced encoded 1 peptide, which had /H11015 20-fold heart-homing selectivity (Table and Figure 2E).

Receptor clone 46 is the mouse homologue of the human bladder cancer–associated protein bc10, a small membrane protein that is downregulated as cancer develops from pre-malignant lesions in the bladder.30 Our clone contained the C-terminal 32 amino acids that are predicted to be extracellular. We found 2 peptides with 60- and 10-fold heart-homing selectivity, respectively (Table and Figure 2F).

Phage-Displayed Peptides That Selectively Home to the Heart Colocalize With a Vascular Marker
We focused further characterization on the 5 most efficient heart-homing peptides among the ones listed in the Table and their putative receptors. We first sought to establish that these peptides were binding to the heart endothelium in vivo, that the binding was to the putative receptors, and that the receptors were expressed in heart endothelial cells.

We intravenously injected each of the phages displaying the peptides into mice. The phage was injected together with fluorescein-conjugated tomato lectin, which stains the blood vessels. Localization of the phage with anti-T7 antibody showed that 5 of the phages were present in heart endothelium (Figure 3A–3C, 3E, and 3F). The CLIDLHVMC phage was shown not to home to the heart by phage counting (Figure 2D) and was also not found in the heart by phage immunostaining (Figure 3D). None of these phages were detectable in other tissues by immunostaining (data not shown).

Homing Peptides Bind to Cells Transfected With the Putative Receptors
We cloned and constructed full-length cDNAs for 4 candidate receptors and expressed the proteins in 293T cells (the EST clone was not studied further). Each of the 4 peptides bound to cells transfected with its putative receptor. The binding was 300- to 500-fold more than that of control phage, and phage binding was inhibited in the presence of 100 µg/mL of the cognate peptide but not by the other homing peptides (Figure 4A–4D). Because antibodies were available for CRIP2, we confirmed the expression and cell-surface localization of the transfected protein by analyzing antibody binding by FACS. Strong binding of the polyclonal anti-CRIP2 antibody to the transfected cells was observed, and propidium iodide exclusion showed that the antibody-binding cells were viable (Figure 4E). These results provide strong support for the identity of these proteins as the endothelial receptors for the homing peptides. They also show that the peptides recognize the receptors in the context of cells other than murine, cardiac endothelial cells that we used for screening.

Receptor mRNAs Are Present in the Heart and Are Expressed by Endothelial Cells
We next studied the expression of the putative receptors in tissues. Q-PCR analysis showed that HLP/CRIP2 was strongly expressed in the heart and at lower levels in the brain.
and lungs (Figure 5A). Several other tissues, including skeletal muscle, were negative for CRIP2 mRNA. Sigirr/TIR8, MpcII-3, and bc10 were also strongest in the heart, with lower levels in some other tissues (Figure 5A). The specificity of Q-PCR was confirmed by gel electrophoresis (Figure 5B), melting curves, and DNA sequence analysis (data not shown).

To confirm the Q-PCR results and to determine whether these receptors are present on endothelial cells in the heart and other tissues, we used in situ hybridization. CRIP2 signal was abundant throughout the heart but exhibited a predominantly vascular expression pattern (Figure 5C, first row). Trace level expression was seen in the brain and lungs, whereas the kidneys were negative. Brain CRIP2 was localized outside the blood vessels, but the vessels themselves were weakly positive in the lungs.

Probing Sigirr/TIR8 by in situ hybridization also confirmed the Q-PCR results. The signal was strong in the heart capillaries as well as larger vessels (Figure 5C, second row), weaker in the lungs and kidneys, and negative in the brain. MpcII-3 mRNA was present throughout the heart but was most prominent in the vessels (Figure 5C, third row). It was also present in some blood vessels of the lungs, whereas those of the brain and kidneys were negative. mRNA for bc10 was detected in heart capillaries and larger vessels (Figure 5C, bottom row); in some lung, kidney, and muscle blood vessels; but not in the brain. Thus, the Q-PCR and in situ hybridization results largely agree: The mRNAs of these 4 proteins are most abundantly expressed in the heart. Moreover, the localization of mRNA for the 4 receptors also agrees with the expectation from the phage experiments that the receptors should be expressed on the endothelium. An exception was that in situ hybridization detected CRIP2 mRNA expression in the lungs, whereas there was little CRIPR phage homing to this tissue (see Discussion).

**Intravenously Injected CRPPR Peptide Colocalizes With HLP/CRIP2 Protein in Heart Vessels**

The availability of antibodies against CRIP2 allowed us to compare the localization of CRPPR peptides and the expres-
expression of CRIP2 protein in the heart. Fluorescence from intravenously injected CRPPR peptide extensively colocalized with staining for the vascular marker CD31, in both heart capillaries and the endocardium (Figure 6A and 6B). A fluorescein-labeled control peptide was not detected in the heart (Figure 6C). Coronary (Figure 6D) and smaller heart (data not shown) arteries were strongly positive for uptake of CRPPR, whereas veins were weakly positive (Figure 6E). Skeletal muscle arteries were negative (Figure 6F). Anti-CRIP2 staining colocalized with both CD31 (Figure 6G) and CRPPR (Figure 6H and 6I) in heart capillary vessels and the endocardium. In agreement with the CRPPR distribution, costaining of anti-CRIP2 and anti–smooth muscle actin showed strong coronary artery (Figure 6J), weaker venous, and a lack of CRIP2 expression in skeletal muscle arteries (Figure 6K and 6L).

To test the specificity of CRPPR-phage binding to heart vessels via CRIP2, we performed competition assays. Both the CRPPR peptide and the anti-CRIP2 antibody, in a dose-dependent manner, blocked the binding of the CRPPR phage to heart-derived cell suspensions (Figure 7A and 7B). Two other heart-homing peptides had no effect on binding. Furthermore, the anti-CRIP2 antibody coinjected with the CRPPR phage inhibited the homing of phage to the heart (Figure 7C). These results show that the CRPPR peptide binds to heart endothelial cells through CRIP2. In addition, we tested CRPPR homing in 3 different mouse strains (BALB/c, FVB, and C57BL/6) and found a similar degree of specific homing in each strain (not shown).

To determine whether CRPPR phage also binds to human heart endothelial cells, we tested the binding of the CRPPR phage to HCAECs. As shown in Figure 7D, the CRPPR phage bound to HCAECs much more strongly than to HUVECs. Binding was specific, because it was inhibited by the soluble cognate peptide but not by a control peptide.

In summary, we have identified and validated 4 heart-homing peptides and their receptors in the heart vasculature. In addition, an EST clone that we did not study may encode the receptor of yet another set of heart-homing peptides identified in this study.

**Discussion**

We have described heart-homing peptides identified through in vivo phage display and identified target molecules (receptors) for these peptides that are preferentially expressed in heart blood vessels and in the endocardium. The selectivity of the receptors to endothelial cells offers new possibilities for targeted drug delivery to the heart. Moreover, the selective expression of the receptors in the heart and the medical importance of heart diseases make these proteins potential drug discovery targets.

In the post genomic era, the challenge is to determine the function, interactions, and spatial and temporal specificities for proteins encoded by the genome. In vivo selection of phage-displayed peptide libraries has proven highly effective in the discovery of ligands for tissue-specific, endothelial cell-surface proteins. Because the identification of endothelial target molecules is a potentially difficult task, simultaneous identification of the heart-homing peptides and their receptors that we have described herein greatly extends the reach of in vivo phage-screening technology.

The receptors for the new homing peptides that we identified represent molecular markers of heart endothelium. Our
Figure 6. CRPPR peptide and CRIP2 protein colocalize with CD31 in endothelial cells of heart vessels. Fluorescein-conjugated CRPPR peptide was intravenously injected into mice, and tissues collected 2 hours later were stained with antibodies against CRIP2, the endothelial marker CD31, and α-smooth muscle actin. The fluorescent peptide (green) colocalized with CD31 (red) in heart capillaries (A) and endocardium (B), whereas the control peptide was not observed in the heart (C). In heart macrovessels, strong peptide fluorescence was seen in coronary arteries (D), whereas veins were only slightly positive (E). Anti-CRIP2 antibody stained heart endothelial cells (red), colocalizing with CD31 (G), the injected CRPPR peptide in heart capillaries (H), and the endocardium (I). The CRIP2 protein expression paralleled that of CRPPR uptake in macrovessels, because CRIP2 was strongly expressed in coronary arteries (J) but was weak in veins (K). Neither the fluorescent CRPPR peptide nor CRIP2 was detectable in skeletal muscle vessels (F and L). Magnification ×200 (A–C, I) and ×400 (D–H, J–L). Scale bar = 100 μm.

Figure 7. CRPPR phage binding to heart cells and homing to the heart are blocked by anti-CRIP2 antibodies. A and B, CRPPR phage was incubated with cell suspensions prepared from the heart, and phage binding was measured in the presence or absence of various concentrations of antibodies or peptides. A chicken anti-CRIP2 antibody blocked phage binding; normal IgY was used as a control (A). Dose-dependent inhibition of phage binding by the free CRPPR peptide confirmed the specificity of the ex vivo phage-binding system (B). In vivo homing of the CRPPR phage to the heart was inhibited by coinjected anti-CRIP2 antibody (C). Binding of CRPPR phage to HCAECs in vitro was inhibited by the soluble cognate peptide (100 μg/mL) but not by the control peptide (D). Shown are the mean and SD from 3 separate experiments.
data show that these markers are not absolutely specific for the heart. However, the expression of peptide receptors outside the heart was observed in only a few tissues and at lower levels than in the heart. The lungs and skeletal muscle were the tissues in which the vasculature most often expressed the heart vascular markers, presumably because of anatomic and developmental similarity to the heart. However, impressive homing selectivity of 20- to >300-fold by the phage displaying the heart-homing peptides was observed, and significant levels of fluorescently labeled CRIP2 peptide were detected only in the heart after intravenous injection. Apparently, the receptor expression level in tissues other than the heart, though detectable, is low enough not to support significant accumulation of the peptide-displaying phage or fluorescently labeled peptide to those tissues.

The heart vascular markers we have identified in this study, though annotated in the databases, have not been extensively studied. In agreement with our data, CRIP2 has been shown to be expressed in heart endothelium during development and in the adult. The other 3 proteins that we have identified as heart endothelial markers have not been studied in this role before. The binding of the phage to heart endothelium in vivo and to cultured cells in vitro implies that the receptors are expressed at the cell surface. Three of the proteins, Sigirr, MpcII-3, and bc10, contain hydrophobic domains and are likely to be membrane proteins. MpcII-3 has been shown to be a component of a mitochondrial succinate dehydrogenase protein complex that generates nitric oxide and reactive oxygen species. CRIP2 possesses no discernible membrane-spanning domain. Moreover, it has been reported to be enriched in the submembranous cell cortex and to be a binding partner of submembranous protein tyrosine phosphatase PTP-BL. An intracellular localization would seem to be at odds with our data indicating that phage (and in the case of CRIP2, antibodies) can recognize these proteins at the cell surface. However, there are several other examples of proteins that lack a signal sequence and are primarily intracellular but are also expressed on the surface of cells, including endothelial cells. The functional role of our homing peptide receptors in the heart is unknown, but that function is likely to be in some manner selectively required in the heart vessels. In this regard, these proteins represent potential drug discovery targets. Finally, we and others have shown that homing peptides can be used to concentrate therapeutic agents in that chosen target tissue. Work is under way to explore potential uses of the new heart-homing peptides in imaging and selective targeting of proangiogenic and antiangiomatic agents into the heart.

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References

1. Garlanda C, Dejana E. Heterogeneity of endothelial cells: specific markers. 
3. Thorin E, Shreeve SM. Heterogeneity of vascular endothelial cells in normal and disease states. 
4. Ruoslahti E, Rajotte D. An address system in the vasculature of normal tissues and tumors. 
9. Rajotte D, Ruoslahti E. Membrane dipeptidase is the receptor for a lung-targeting peptide identified by in vivo phage display. 
11. Samoylova TI, Smith BF. Elucidation of muscle-binding peptides by phage display screening. 
13. Ruoslahti E. Specialization of tumour vasculature. 
21. Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. 
24. Galang CK, Muller WJ, Foos G, Oshima RG, Hauser CA. Changes in the
We describe several peptides that selectively bind to the vasculature of the heart. The receptors we have identified for each of these peptides are proteins that are expressed at high levels in the heart endothelium and, in some cases, in cardiomyocytes, but are not detectable or are expressed only at lower levels in other tissues. The peptides are potentially useful in directing therapeutic agents, such as drugs and gene therapy vectors, into the heart. The high cardiac expression of the receptors suggests that they may be functionally important in the heart and that further studies should be directed at exploring their role in cardiac physiology and disease.
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