involving this motif, leading to a zipper-like model for cadherin-mediated cell adhesion[10]. These antiparallel 'adhesive interactions' are not seen in the Ecad12 crystal structure, although sterically Ecad12 could be accommodated in the zipper-like arrangement. Regardless, we have shown that the building block of any higher-order organization is a parallel cadherin dimer whose structure is promoted by and dependent on the presence of bound calcium ions. The dimerization, rigidification and resulting mechanical stabilization of E-cadherin explains, at least in part, the calcium requirement for the integrity of cell junctions[19]. □

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1. Takeichi, M. *Science* **251,** 1451–1455 (1991).
2. Mareel, M., Bracke, M. & Van Roy, F. *Molec. Biol. Rep.* **19,** 45–67 (1994).
3. Birchmeier, W. *Bioessays* **17,** 97–99 (1995).
4. Takeichi, M. *A. Rev. Biochem.* **59,** 237–252 (1990).
5. Blaschuk, O. W., Munroe, S. B. & Farookhi, R. *Can. J. Oncol.* **4,** 291–301 (1994).
6. Overduin, M. *et al. Science* **267,** 386–389 (1995).
7. Nose, A., Tsuji, K. & Takeichi, M. *Cell* **61,** 147–155 (1990).
8. Kemler, R. *Trends Genet.* **9,** 317–321 (1993).
9. Pokutta, S., Herrenknecht, K., Kemler, R. & Engel, J. *Eur. J. Biochem.* **223,** 1019–1026 (1994).
10. Shapiro, L. *et al. Nature* **374,** 327–337 (1995).
11. Tong, K. I. *et al. FEBS Lett.* **352,** 318–322 (1994).
12. Ozawa, M., Engel, J. & Kemler, R. *Cell* **63,** 1033–1038 (1990).
13. Brown, E. M., Vassilev, P. M. & Hebert, S. C. *Cell* **83,** 679–682 (1995).
14. Ringwald, M. *et al. EMBO J.* **6,** 3547–3653 (1987).
15. Overduin, M., Tong, K. I., Kay, C. M. & Ikura, M. *J. Biomol. NMR.* (in the press).
16. Oda, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **91,** 1858–1862 (1994).
17. Risinger, J. I., Berchuck, A., Kohler, M. F. & Boyd, J. *J. Nature Genet.* **7,** 98–102 (1994).
18. Blaschuk, O. W., Sullivan, R., David, S. & Pouliot, Y. *Devl Biol* **139,** 227–229 (1990).
19. Volberg, T., Geiger, B., Kartenbeck, J. & Franke, W. W. *J. Cell Biol.* **102,** 1832–1842 (1986).
20. Kabsch, W. *J. appl. Crystallogr.* **21,** 916–924 (1988).
21. Otwinowski, Z. in *Proceedings of the CCP4 Study Weekend* (eds Sawyer, L., Isaacs, N. & Bailey, S.) 56–62 (SERC Daresbury Laboratory, Warrington, UK, 1993).
22. Tesmer, J., Stemmler, T., Penner-Hahn, J., Davisson, V. & Smith, J. *Proteins* **18,** 394–403 (1994).
23. Furey, W. & Swaminathan, S. *Meth. Enzym.* (in the press).
24. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, *Acta crystallogr. A* **47,** 110–119 (1991).
25. Brünger, A. *X-PLOR, v3.1 A System for X-ray Crystallography and NMR* (Yale University Press, New Haven, USA, 1992).
26. Evans, S. V. *J. molec. Graph.* **11,** 134–138 (1993).
27. Kraulis, P. J. *appl. Crystallogr.* **24,** 946–950 (1991).
28. Glusker, J. P. *Adv. Prot. Chem.* **42,** 1–76 (1991).

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CORRESPONDENCE and requests for materials to be addressed to J.M.R. (e-mail rini@gene4d.med.utoronto.ca).

Organ targeting *in vivo* using phage display peptide libraries

Renata Pasqualini & Erkki Ruoslahti

La Jolla Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, California 92037, USA

PREFERENTIAL homing of tumour cells[1,2] and leukocytes[3,4] to specific organs indicates that tissues carry unique marker molecules accessible to circulating cells. Organ-selective address molecules on endothelial surfaces have been identified for lymphocyte homing to various lymphoid organs and to tissues undergoing inflammation[5–8], and an endothelial marker responsible for tumour homing to the lungs has also been identified[9]. Here we report a new approach to studying organ-selective targeting based on *in vivo* screening of random peptide sequences. Peptides capable of mediating selective localization of phage to brain and kidney blood vessels were identified, and showed up to 13-fold selectivity for these organs. One of the peptides displayed by the brain-localizing phage was synthesized and shown to specifically inhibit the localization of the homologous phage into the brain. When coated onto glutaraldehyde-fixed red blood cells, the peptide caused selective localization of intravenously injected cells into the brain. These peptide sequences represent the first step towards identifying selective endothelial markers, which may be useful in targeting cells, drugs and genes into selected tissues.

We injected phage libraries[10–13] intravenously into mice and subsequently rescued the phage from individual organs. Within the time frame of the experiments, the bulk of the phage remained in circulation (not shown). Some organs, such as liver and lung, captured too many phage to be used as target organs for selection; we focused on peptide sequences that directed phage binding to the brain and kidney, because these organs bound relatively few phage from the unselected libraries.

To select peptides that home to the brain, phage were injected intravenously, recovered from the brain, amplified repeatedly *in vitro*, and re-injected to obtain sufficient enrichment. Although more of the injected phage were recovered from an equivalent amount of kidney than from brain after the first injection, 6- and 13-fold more phage were recovered from the brain in the second and third rounds of the selection, respectively. This enrichment for the brain was reproducible in several experiments. Results from representative experiments performed with two different mixtures of libraries are shown in Fig. 1.

Sequencing of the inserts from 48 brain-localizing phage from library pool I revealed three dominant amino-acid sequence motifs. Peptides containing an SRL motif represented 54% of the clones, followed by a CENWWGDVC motif (29%). Other motifs that appeared more than once included CKDWGRIC, CVLRGGRC and CTRITESC. Many of these less common motifs shared the sequence RI/RL with the more common ones. Eight sequences were seen only once and probably represent background. From the library pool II phage, 25 sequences revealed only one motif, WRCVLREGPAGGCAWFNRHRL, which comprised 40% of the sequences.

The SRL tripeptide was found in several sequence contexts, indicating that the sequences were derived from a number of independent phage. Moreover, the DNA sequences of phage displaying the same peptide were in some cases not identical. The strong selection for predominant motifs and their internal diversity clearly shows that the peptide displayed by the phage, rather than some incidental mutant property of the phage, is responsible for the selective binding.

When tested as isolated phage, the CLSSRLDAC, CNSRLHLRC, CENWWGDVC and WRCVLREGPAGG-CAWFNRHRL phage each targeted the brain several-fold more effectively than the kidney. The brain/kidney ratios (number of phage recovered from brain divided by number of phage recovered from the same amount of kidney tissue) were about 8 for the CLSSRLDAC and the CNSRLHLRC phage, 4 CENWWGDVC, and 9 for WRCVLREGPAGG-CAWFNRHRL. A phage that had not been selected for brain or kidney binding gave a brain/kidney ratio of approximately 1.

Phage that would home selectively into the kidney were isolated from a mixture of the CX_5C and CX_6C libraries. The enrichment was three- to fivefold (data not shown). Phage carrying two motifs, CLPVASC and CGAREMC, constituted 60% of the 48 phage sequenced; CKGRSSAC appeared three times. The preferential binding to kidney relative to brain was highest, about sevenfold, with CLPVASC phage. Control phage again gave a kidney/brain ratio of about 1.

Immunohistochemical staining of the brain-binding phage displaying CLSSRLDAC revealed staining within the brain capil-

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laries (Fig. 2*a*). No preference for any part of the brain was seen. Injection of the kidney-binding CLPVASC phage did not cause staining of the brain capillaries (Fig. 2*b*). In contrast, the kidney-binding phage was found in the glomeruli and in between the tubules (Fig. 2*c*). Only slight staining was seen in the kidney with the brain-binding phage (Fig. 2*d*).

A soluble cyclic peptide was synthesized according to one of the brain-binding phage sequences, CLSSRLDAC. We chose to synthesize this peptide because it gave a slightly higher (8.2 versus 7.6) brain/kidney ratio than the most prevalent motif, CNSRLHLRC. The CLSSRLDAC peptide inhibited the preferential localization into the brain of the phage carrying the same

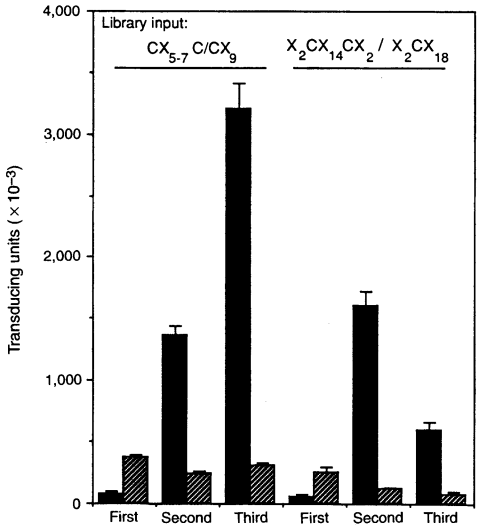

FIG. 1 Selective localization of phage to the brain. Two library pools, $CX_{5-7}C/CX_9$ (pool I)[10–12] and $X_2CX_{14}CX_2$, X_2CX_{18} (pool II) (E. Koivunen and E.R., unpublished data) were injected in the tail vein. After 1–4 min the mice were killed and the phage rescued from tissues. Phage recovered from the brain were amplified and re-injected in two consecutive rounds. The number of phage (transducing units) recovered from brain (black bars) and kidney (hatched bars) tissue in a representative experiment with each library pool is shown. The number of phage recovered from different organs varied to some extent, but the ratios were consistent across experiments. The bars show standard error of the mean (s.e.m.) from plating in triplicate.

METHODS. The libraries were prepared as described[10–13], and display mostly cyclic peptides, which often bind with higher affinity than non-cyclic peptides[11,12]. Balb/c (2-month-old females; Jackson Laboratories, Bar Harbor, ME) were anaesthetized with Avertin (0.015 ml g^{-1}) and injected intravenously (tail vein) with a mixture of phage libraries containing 10^{16} (pool I) and 10^{14} (pool II) transducing units diluted in 200 μl DMEM. At the end of the experiment the mice were snap-frozen in liquid nitrogen, while in a state of deep anaesthesia. To recover the bound phage, the carcasses were partly thawed at room temperature, organs were removed, weighed, and ground in 1 ml DMEM-PI (DMEM containing the protease inhibitors phenyl methyl sulphonyl fluoride (1 mM), aprotinin (20 μg ml^{-1}) and leupeptin (1 μg m^{-1})). The tissue were washed three times with ice-cold DMEM-PI containing 1% BSA and incubated with 1 ml of bacteria for 1 h. NZY medium (10 ml) containing 0.2 μg ml^{-1} tetracycline was added, the mixture was incubated in a 37 °C shaker for 1 h, and 200-μl portions were plated in agar plates in the presence of 40 μg ml^{-1} tetracycline. About 200 individual colonies were grown separately for 16 h in 5 ml NZY medium containing 40 μg ml^{-1} tetracycline. The bacterial cultures were then pooled and the amplified phage were injected into mice as described above.

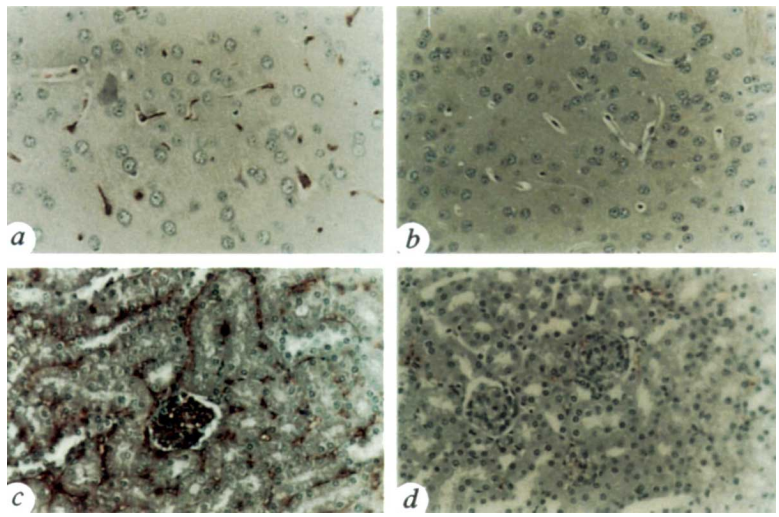

FIG. 2 Immunohistochemical staining of phage in brain and kidney tissue. Phage were amplified individually and injected into mice. Tissue sections were prepared after perfusion of the mice through the heart with DMEM, and the organs were fixed in Bouin solution. An antibody against M13 (Pharmacia Biotech, Piscataway, NJ) was used for the staining, followed by a peroxidase-conjugated secondary antibody (Sigma, St Louis, MO). Brain-selective phage displaying CLSSRLDAC in brain (*a*) and in kidney (*d*), and the kidney-selective phage displaying CLPVASC in kidney (*c*) and in brain (*b*) are shown. Magnification: *a*, ×400; *b*–*d*, ×200.

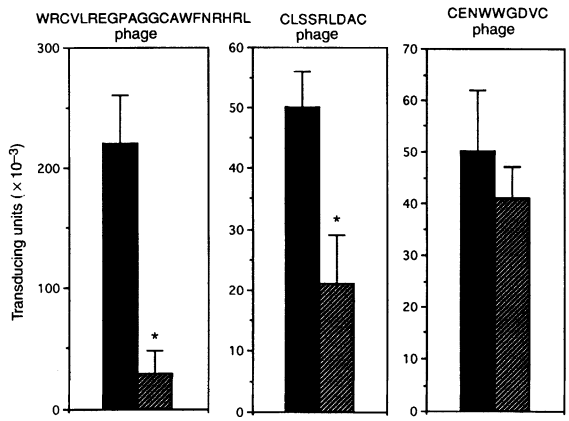

FIG. 3 Effect of the CLSSRLCAC synthetic peptide on the *in vivo* localization of phage. Brain-binding phage displaying CLSSRLDAC, CENWWDGVC and WRCVLREGPAGGCAWFNRHRL motifs were titrated to the same concentration and 10^8 transducing units were injected into mice either on their own (black bars) or together with 500 μg of the CLSSRLDAC synthetic peptide (hatched bars). Shown is the number of phage (transducing units) recovered from the brain. The CLSSRLDAC peptide was synthesized and purified by high-performance liquid chromatography (Immunodynamics, La Jolla, CA). Bars show s.e.m. from triplicates. Asterisks indicate statistically significant differences (unpaired Student *t*-test, $P < 0.05$).

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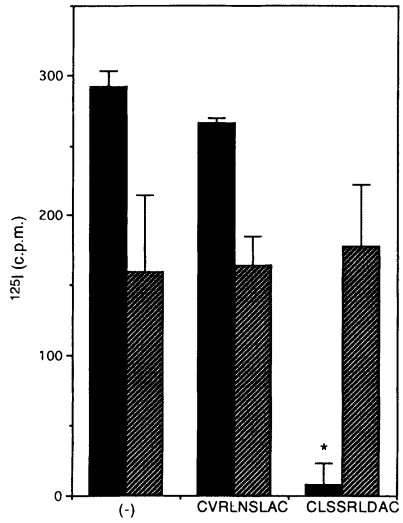

FIG. 4 Tissue localization of red blood cells coated with CLSSRLDAC peptide. Inhibition of brain localization by the corresponding soluble peptide. Iodinated CLSSRLDAC peptide was coupled to red blood cells and injected into the tail vein of mice in the presence or absence of unlabelled peptides. Radioactivity in perfused brain (black bars) and kidney (hatched bars) tissues is shown.

METHODS. The CLSSRLDAC peptide (1 mg) was labelled using the Bolton Hunter reagent (Amersham Life Science, Arlington Heights, IL), and purified by reversed-phase chromatography on Sep-Pak cartridges (Waters, Millipore, Milford, MA). The labelled peptide (100 μg) was coupled to 1 ml glutaraldehyde-stabilized sheep red blood cells (Sigma, St Louis, MO) according to the manufacturer's instructions. The coated cells (50 μl, 200,000 c.p.m.) were injected into the tail vein of mice in the presence or absence of 10 mM of unlabelled CLSSRLDAC peptide. An unrelated peptide, CVRLNSLAC, was used as a control. The mice were killed 2 min later, perfused through the heart with 50 ml DMEM, and their brain and kidneys were removed and assayed for radioactivity. The animals were treated in accordance with the Institute's Animal Facility Guidelines. Bars show s.e.m. from triplicates. Asterisks indicate statistically significant differences (unpaired Student's *t*-test, $P < 0.05$).

sequence and of the WRCVLREGPAGGCAWFNRHRL phage, but had no effect on the brain localization of the CENWWDGVC phage (Fig. 3). Thus the first two peptides, which were obtained from different libraries in two independent experiments, seem to bind to the same target molecule, possibly because of the similarity of the C terminus of the long peptide with the SRL motif. Differences in binding parameters may explain the greater susceptibility of the long motif to inhibition by the CLSSRLDAC peptide. The third peptide is likely to have a different target.

We also showed that CLSSRLDAC could target a particle other than the phage to the brain. Coupling the peptide onto the surface of red blood cells resulted in their accumulation in the brain to a greater extent than in the kidney (Fig. 4). Moreover, the brain localization of the red blood cells was blocked by co-injection of the soluble peptide, whereas the accumulation in the kidney was not affected (Fig. 4).

Future studies will be needed to identify the molecules to which the peptides bind in the brain and kidney. The sequences of the binding motifs are not helpful in this regard, because they do not reveal any significant similarities with known receptor ligands. Our initial attempts to identify the target molecule for the CLSSRLDAC peptide by affinity chromatography of brain extracts have not been successful, possibly because endothelial cell molecules would only be present as minor components in a brain extract. The receptors for the peptides are likely to be endothelial cell molecules, because the phage were allowed to circulate only for a few minutes, making it unlikely that the phage would have left the circulation. Moreover, immunohistochemical staining of phage after injection showed that they remain in the lumen of blood vessels in the targeted organs.

To our knowledge this is the first time an *in vivo* selection procedure has been applied to a random library. So far we have targeted only two organs, the brain and the kidney, and were in each case able to recover organ-selective phage. This initial success suggests that it will be possible to apply this procedure to the identification of selective binding sequences for other organs as well, although organs that capture a large number of phage, such as liver and lung, may prove rather more troublesome. The method should be applicable to phage display libraries expressing larger proteins including the antibody variable binding region and the binding domains of specific ligands, as well as random libraries based on principles other than phage display; the only requirement is the ability to identify the compound in the tissue after the *in vivo* binding.

Organ-selective targeting molecules isolated from random libraries following the procedures described here may have a variety of uses. It may be possible to graft motifs to surface molecules of viruses or cells used in gene therapy. Other possibilities include their use in the preparation of drug conjugates or liposomes with specific targeting properties. Tumour vasculature, which undergoes active angiogenesis and contains specific markers[14,15], would be a particularly attractive future target, as it might allow therapies to be directed into tumours while sparing other tissues. □

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1. Fidler, I. J. & Hart, I. R. *Science* **217,** 998–1003 (1982).
2. Johnson, R. C. *et al. Cancer Res.* **51,** 394–399 (1991).
3. Springer, T. A. *Cell* **76,** 301–314 (1994).
4. Salmi, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **89,** 11436–11440 (1992).
5. Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A. & Seed, B. *Science* **243,** 1160–1165 (1989).
6. Siegelman, M. H., Rijn, M. & Weissman, I. L. *Science* **243,** 1165–1171 (1989).
7. Cepek, K. L *et al. Nature* **372,** 190–193 (1994).
8. Rosen, S. D. & Bertozzi, C. R. *Curr. Opin. Cell Biol.* **6,** 663–673 (1994).
9. Johnson, R. C., Zhu, D., Augustin-Voss, H. G. & Pauli, B. U. *J. Cell Biol.* **121,** 1423–1432 (1993).
10. Smith, G. P. & Scott, J. K. *Meth. Enzym.* **217,** 228–257 (1993).
11. Koivunen, E., Wang, B. & Ruoslahti, E. *J. Cell Biol.* **124,** 373–380 (1994).
12. Koivunen, E., Wang, B., Dickinson, C. D. & Ruoslahti, E. *Meth. Enzym.* **245,** 346–369 (1994).
13. Pasqualini, R., Koivunen, E. & Ruoslahti, E. *J. Cell Biol.* **130,** 1189–1196 (1995).
14. Brooks, P. C. *et al. Cell* **79,** 1157–1164 (1994).
15. Rak, J. W., St. Croix, B. D. & Kerbel, R. S. *Anticancer Drugs* **6,** 3–18 (1995).

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CORRECTION

Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor gene in mice

Lutz Hein, Gregory S. Barsh, Richard E. Pratt, Victor J. Dzau & Brian K. Kobilka

Nature **377,** 744–747 (1995)

REFERENCES 22 and 25 of this paper should be replaced as follows:

22. Nakajima, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **92,** 10663–10667 (1995).
25. Viswanathan, M. & Saavedra, J. M. *Peptides* **13,** 783–786 (1992). □