Identification of Technetium-99m Binding Peptides Using Combinatorial Cellulose-Bound Peptide Libraries

Reinhard Malin,[†] Renate Steinbrecher,[‡] Jörg Jannsen,[‡] Wolfhard Semmler,[‡] Bernhard Noll,[§] Bernd Johannsen,[§] Cornelius Frömmel,[†] Wolfgang Höhne,[†] and Jens Schneider-Mergener*,[∥]

Institut für Medizinische Immunologie and Institut für Biochemie, Universitätsklinikum Charité Humboldt-Universität zu Berlin, Schumannstrasse 20-21 10098 Berlin, Germany
Institut für Diagnostikforschung GmbH an der Freien Universität Berlin, Spandauer Damm 130 14050 Berlin, Germany
Institut für Bioanorganische und Radiopharmazeutische Chemie, Forschungszentrum Rossendorf e. V. Postfach 510119, 01314 Dresden, Germany

Received May 2, 1995

Technetium-99m (^{99m}Tc) complexed to organic compounds, proteins, or peptides plays an important role in the radioimmunodetection (RAID) of organs and tumors because of its ideal physical properties, low cost, and availability.[1–6] Here we report the use of cellulose-bound combinatorial hexapeptide libraries prepared by spot synthesis[7,8] for the systematic search for ^{99m}Tc binding peptides. Cellulose-bound combinatorial libraries have proven to be useful for the detection of protein, metal and nucleic acid binding peptides.[8–11] We have synthesized a combinatorial $B_1XB_2XB_3X$ library (B = defined position being one of the 20 L-amino acids, X = randomized position) consisting of 8000 peptide mixtures (spots) which was subsequently screened for ^{99m}Tc binding (Figure 2). Our rationale was to identify a ^{99m}Tc binding hexapeptide which could be genetically fused to an anti-CEA single chain Fv antibody fragment for tumor imaging purposes (CEA, carcinoembryonic antigen).[12–14] For that reason, we selected non-cysteine-containing peptides from the libraries to rule out incorrect folding caused by one or more additional cysteines in the engineered antibody fragment. Single ^{99m}Tc binding peptides were identified by incubation of the library $B_1XB_2XB_3X$ with ^{99m}Tc and subsequent selection of single hexapeptide mixtures as starting points to identify single hexapeptides. Incubation of the libraries with ^{99m}Tc revealed binding of the metal preferably to cysteine-containing peptide mixtures as expected,[5,8] but also histidine, lysine, arginine, and methionine contributed significantly to ^{99m}Tc binding (see Figure 2, amplified parts of the complete library).

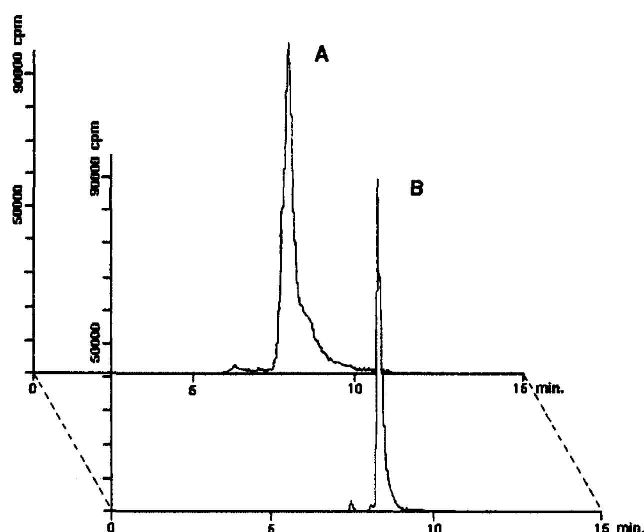

Figure 1. HPLC analysis of the peptides KGHSHV (A) and KAMYHG (B) after incubation with technetium-99m. Following synthesis the two peptides were purified to >98% by preparative HPLC and characterized by LD-TOF mass spectrometry. The analysis of the peptide–technetium complex was performed on a C18 column (Lichrosphen 125*4, Merck, Darmstadt, Germany). A 50 μL portion of the solution described below was analyzed using a gradient starting from 100% A (A = Na_2HPO_4/H_2O, pH 7.5) to 75% A/25% B (B = 50% A/50% CH_3CN) in 6 min and to 99% A/1% B in 4 min followed by an isocratic run of 5 min (flow rate 2.0 mL/min). For detection of the radioactively labeled peptides a Berthold LB506-C-1 detector (EG&G-Berthold, Bad Wildbad, Germany) was used. Before HPLC analysis the peptides were labeled with technetium-99m. Sodium citrate (50 μL; 0.5 mg/mL), 100 μL of TcO_4^- (~1200 μCi), 50 μg of peptide (1 mg/mL) in phosphate buffer (0.1 M, pH 8.5), and 5 μL of $SnCl_2$ dissolved in 0.1 N HCl (20 mM) were added to 250 μL of phosphate buffer (0.1 M, pH 10.5). After 30 min the solution was diluted 10-fold with phosphate buffer.

From the $B_1XB_2XB_3X$ library were chosen several peptide mixtures as starting mixtures to define the three X-positions in two additional syntheses and screening steps. This is demonstrated for the mixtures KXHXHX and KXMXHX. In the second step we selected the peptide mixtures KGHSHX and KAMYHX from the libraries KB_1HB_2HX and KB_1MB_2HX. In the final step the peptides KGHSHV and KAMYHG were selected from two libraries KGHSHB and KAMYHB (Figure 2). The ^{99m}Tc binding capability of these two hexapeptides was investigated by HPLC analysis following technetium labeling of the soluble peptides as described (Figure 1). Both peptides quantitatively incorporated technetium-99m. No ^{99m}Tc starting material or free pertechnetate was observed as judged from the HPLC experiments. The technetium–peptide complexes were stable for 24 h in the incubation buffer as shown by HPLC analysis (data not shown). It is not possible to distinguish whether different amounts of technetium bound to single spots are due to differences in affinity or caused by binding of more than one metal ion to the respective peptide or peptide mixture. The creation of a single species having one metal binding site is desirable with respect to characterization of the product, stability, and potential pharmaceutical application. Therefore,

* To whom correspondence should be addressed.
[†] Institut für Biochemie, Universitätsklinikum Charité, Humboldt-Universität zu Berlin.
[‡] Institut für Diagnostikforschung GmbH an der Freien Universität Berlin.
[§] Institut für Bioanorganische und Radiopharmazeutische Chemie, Forschungszentrum Rossendorf e. V.
[∥] Institut für Medizinische Immunologie, Universitätsklinikum Charité, Humboldt-Universität zu Berlin.

(1) Schwochau, K. *Angew. Chem., Int. Ed. Engl.* **1994**, *33* (22), 2258–2267.
(2) Clarke, M. J.; Podbielsky, J. *Coord. Chem. Rev.* **1987**, *78*, 253–331.
(3) Griffiths, G. L.; Goldenberg, D. M.; Jones, A. L.; Hansen, H. J. *Bioconjugate Chem.* **1992**, *3*, 91–99.
(4) Johannsen, B.; Noll, B.; Leibnitz, P.; Reck, G.; Noll, St.; Spiess, H. *Radiochim. Acta* **1993**, *63*, 133–137.
(5) Morelock, M. M.; Comner, T.; Tolman, G. L. *Inorg. Chem.* **1988**, *27*, 3137–3140.
(6) Fishman, A. J.; Babich, J. W.; Strauss, H. W. *J. Nucl. Med.* **1993**, *34*, 2253–2263.
(7) Frank, R. *Tetrahedron* **1992**, *48*, 9217–9232.
(8) Kramer, A.; Schuster, A.; Reineke, U.; Malin, R.; Volkmer-Engert, R.; Landgraf, C.; Schneider-Mergener, J. *METHODS (Com. Meth. Enzymol.)* **1994**, *6*, 388–395.
(9) Kramer, A.; Volkmer-Engert, R.; Malin, R.; Reineke, U.; Schneider-Mergener, J. *Pept. Res.* **1993**, *6* (6), 314–319.
(10) Schneider-Mergener, J.; Kramer, A; Reineke, U. In *Combinatorial Libraries*; Cortese, R., Ed.; Walter de Gruyter: Berlin, 1995; pp 53–68.
(11) Malin, R.; Reineke, U.; Höhne, W.; Semmler, W.; Steinbrecher, R.; Schneider-Mergener, J. In *Peptides*; Maia, H., Ed.; ESCOM: Leiden, 1995; pp 477–478.
(12) Gold, P.; Freedman, S. O. *J. Exp. Med.* **1965**, *121*, 439–462.
(13) Gold, P.; Freedman, S. O. *J. Exp. Med.* **1965**, *122*, 467–481.
(14) Thomas, P.; Toth, C. A.; Saini, K. S.; Jessup, J. M.; Steele, G., Jr. *Biochim. Biophys. Acta* **1990**, *1032*, 177–189.

0002-7863/95/1517-11821$09.00/0 © 1995 American Chemical Society

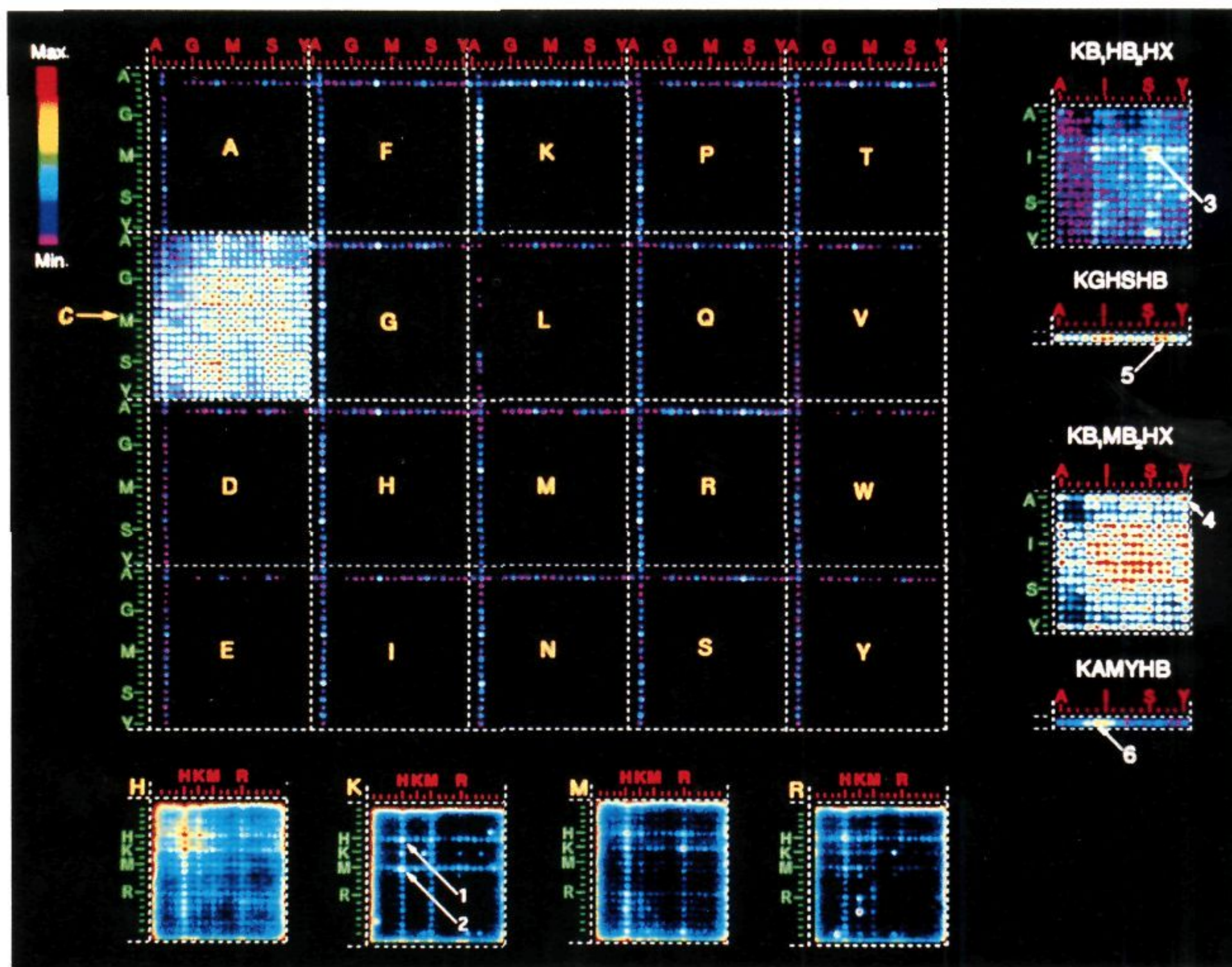

Figure 2. Binding of technetium-99m to the cellulose-bound combinatorial libraries. In each square (white dotted lines) of the $B_1XB_2XB_3X$ library B_1 is one of the 20 L-amino acids (yellow letters). The rows in each square define the B_2 position (green letters) and the columns the B_3 position (red letters). The colors of single spots correspond to the amount of bound technetium-99m from black (no binding) to red (strongest binding) in the order of the spectral colors (see color scale). The four lower libraries represent selected squares (B_3 = H, K, M, and R) of the same library in which the sensitivity was increased by lowering the threshold of detection. These squares contained the strongest binding non-cysteine-containing peptide mixtures (the cysteine-containing peptide mixtures were cut out for ease of interpretation). The two peptide mixtures serving as starting points for the synthesis of two additional combinatorial libraries are indicated by arrows (1 KXHXHX and 2 KXMXHX). The second-generation libraries KB_1HB_2HX (1) and KB_1MB_2HX (2) are displayed on the right side (C, H, K, M, and R are omitted from the B_1 and B_2 positions). Two peptide mixtures KGHSHX (3) and KAMYHX (4) were selected to define the C-terminal amino acid by synthesizing the two final libraries KGHSHB and KAMYHB (C, H, K, M, and R are omitted in the B positions). The peptides KGHSHV (5) and KAMYHG (6) indicated by arrows were selected for the technetium labeling experiments (Figure 1). The libraries were automatically synthesized (Auto Spot ASP 222, Abimed GmbH, Langenfeld, Germany) using an adapted version of the software provided by Abimed. For generation of the sequence files the software DIGEN (Jerini BioTools GmbH, Berlin, Germany) was used. The synthesis of the libraries was carried out as previously described in detail.[7,8] Incubation of the membrane with technetium-99m was performed as described.[8] After reduction of 5 mCi TcO_4^- (+VII) (Behringwerke, Marburg, Germany) using 10 μL of freshly dissolved $SnCl_2$ (25 mM in 0.1 N HCl) and addition of 2 mL of 0.1 M gluconate/H_2O, Tc(V) species were formed. The membrane was incubated for 15 min with the labeling solution in 100 mM phosphate buffer (100 mL, pH 7.5), washed, and analyzed using a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

we excluded the five residues cysteine, histidine, lysine, methionine, and arginine that significantly contribute to binding from the second- and third-generation libraries. Since Tc(V) does not exist as free ion, forming complexes in reversible reactions, measurement of thermodynamic stability constants is impossible.[15] Therefore, challenge experiments[16] with cysteine were carried out with ^{99m}Tc−hexaglycine as representative of ^{99m}Tc-labeled hexapeptides not containing cysteine.[17] A significantly higher stability of the complexes with the two library-derived peptides compared to hexaglycine was observed.

In summary, this novel approach proved useful for the identification of technetium binding peptides and might be generally applicable for the detection of metal binding peptides.[8−10] The two non-cysteine-containing motifs described here are novel and do not display any homology to technetium binding peptides derived from metallothionine sequences such as KCTCCA, which, however, could also be deduced from our library.[5]

(15) Johannsen, B.; Spies, H. *Top. Curr. Chem.* **1995**, *176*, 77−121.

(16) Goedemanns, W. Th.; Paneck, K. J.; Ensing, G. J.; de Jong, M. Th. M. In *Technetium and Rhenium Chemistry and Nuclear Medicine*: Nicolini, M., Bandoli, G., Mazzi, U., Eds.; Raven Press: New York, 1990; pp 595−603.

(17) Vanbilloen, H. P.; Bormanns, G. M.; De Roo, M. J.; Verbruggen, A. M. *Nucl. Biol. Med.* **1995**, 325−338.

Acknowledgment. This work was supported by grants of the BMBF (0310256A and 0310085A) and Fonds der Chemischen Industrie.

JA9514353