Protein Mimicry: A New Dimension for Peptides as Lead Compounds?**

Ulrich Reineke and Jens Schneider-Mergener*

Four publications from 1996 and 1997 in *Science* and the *Proceedings of the National Academy of Science USA* concern the identification of peptides which emulate the biological functions of proteins.[1–4] Many biological processes are regulated by specific protein interactions with receptors located at the cell membrane. A large group of these receptors belong to the family of haemopoietic cytokine receptors. Their members have an N-terminal extracellular part comprising several domains responsible for ligand binding which is connected to the C-terminal signal-transduction domain through a transmembrane helix. Signal transduction takes place by homodimerization or heterooligomerization of the receptors with additional membrane-associated proteins after binding of the cytokine ligand.[5] As shown by X-ray crystal structure analyses of hormone–receptor complexes, such as that of the human growth hormone (hGH) with its receptor (hGHR; Figure 1, right),[6] ligand binding is mediated over a large contact surface area (500–1300 Å^2); around 30 amino acids from each partner are involved in the interaction. Owing to these observations it was long thought almost impossible to imitate such specific, high-affinity interactions with peptides or even smaller organic ligands. Pioneering work in this direction was first published in 1995 by J. Wells and co-workers at Genentec (San Francisco, California). They shortened the atrial natriuretic peptide (ANP) from 28 to 15 amino acids while retaining its activity.[7] The same group also suceeded in shortening the Z domain of protein A, which binds to the Fc region of immunoglobulins, from 59 to 33 amino acids while retaining its binding activity.[8] A breakthrough was achieved in 1996 by the group of W. Dower at Affymax Research Institute (Palo Alto, California) through the identification of a 20-mer peptide which emulates the function of the considerably larger protein erythropoietin (EPO, 165 aa). This protein mimic shows no sequence similarity to the protein, yet it can not only bind the erythropoietin receptor (EPOR), but also activate it. These properties can be explained by analyzing the X-ray crystal structure of a complex comprising two EPO receptors and a dimer of this peptide; the data were obtained by I. Wilson and co-workers at Scripps Research Institute (La Jolla, California).[2]

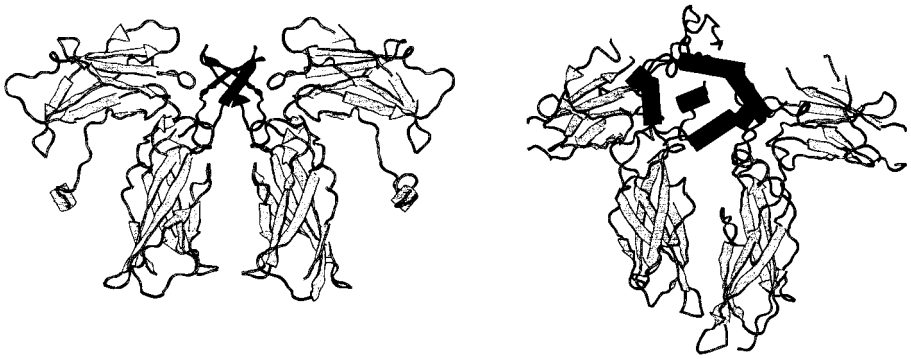

Figure 1. Schematic representation of the X-ray crystal structure of two erythropoietin receptors (gray) complexed with the erythropoietin mimic (black) identified by phage display.[2] The peptide binds the ligand as a homodimer. β-Folded sheets are shown as arrows, and α helixes as cylinders. Right: Schematic representation of the structure of the human growth hormone (hGH, black) complexed with two identical receptor molecules (hGHR, gray).[6] In the case of both receptors, only the soluble extracellular part was crystallized.

The identification of this peptide and the work on the atrial natriuretic peptide and Z domain of protein A were possible with the phage-display technique established in 1985 by G. Smith.[9] Since the beginning of the 1990s, this method has developed into a versatile means of biologically generating peptide libraries on the surface of phage.[10] The principal behind phage display is described in Figure 2. The basis for presenting different peptides on the filamentous phage surface is the cloning of random oligonucleotides in gene III or VIII of the phage genome (Figure 2). The serendipitous sequences in mutated genomes code for all the possible peptides corresponding to the number of randomized triplet

[*] Priv.-Doz. Dr. J. Schneider-Mergener,
Dipl.-Biochem. U. Reineke[+]
Institut für Medizinische Immunologie, Universitätsklinikum Charité
Humboldt-Universität
Schumannstrasse 20/21, D-10098 Berlin (Germany)
Fax: (+49) 30-2802-6460
E-mail: jens@immunologie.charite.hu-berlin.de

[+] Current address: Jerini BioTools GmbH, Berlin

[**] We wish to thank P. Fischer, A. Kramer, and U. Hoffmüller for a critical reading of the manuscript, and R. Stigler for help in creating the figures.

  1433-7851/98/03706-0769 $ 17.50+.50/0

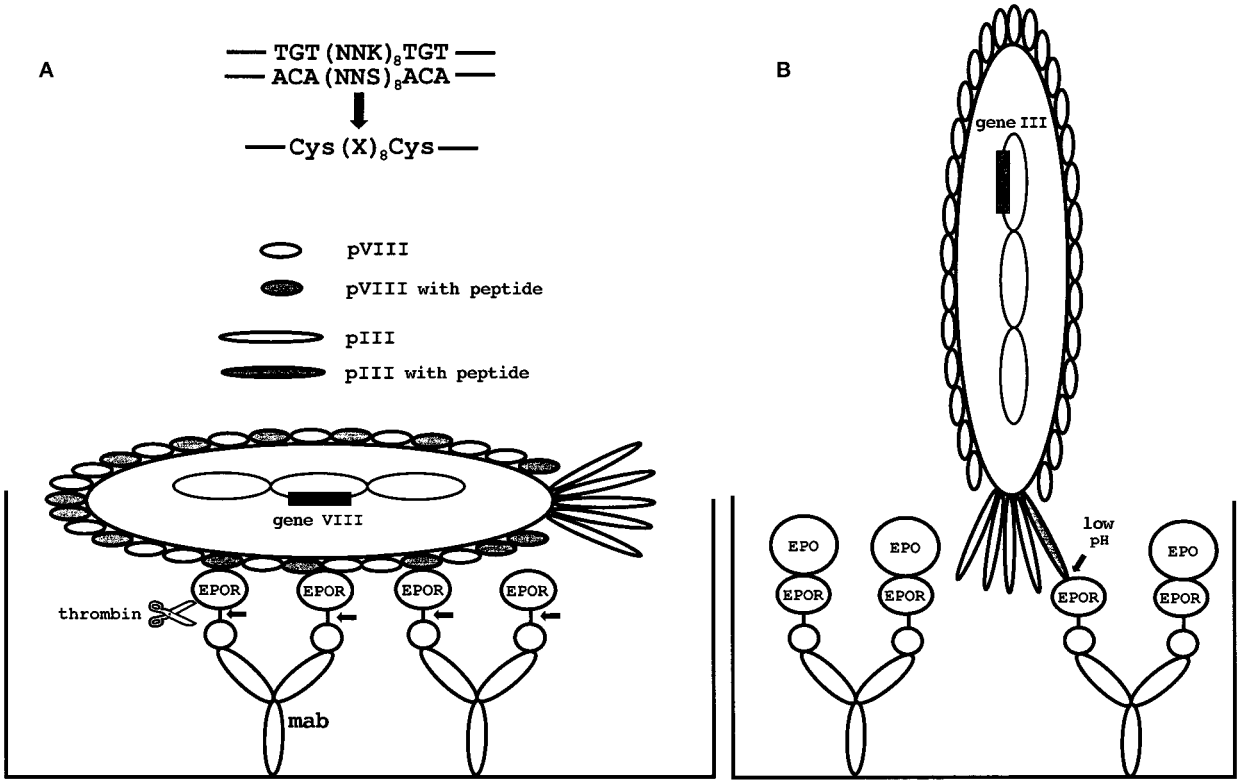

Figure. 2. A) Multivalent phage display on the phage coat protein pVIII. The randomized DNA sequence encoding the library of assorted peptides is cloned into gene VIII.[1, 10] Those triplets coding for all possible 20 amino acids (X) have any of the four nucleotides A, C, G, or T (N) in the first two positions, whereas the third position is occupied by only G or T (K) or, alternatively, A or C (S) in the complementary strand. This selection codes for all 20 amino acids; however, the number of possible stop codons is reduced from three to one. Both flanking triplets code for the cysteine residues that are important for the cyclic conformation. Only about 100 – 200 copies of the protein VIII (pVIII, small gray oval) contain the displayed peptide sequence. The information for the non-mutated coat protein (white oval), which is essential for phage replication, comes either from a second copy of the wild-type gene or through coinfection with a helper phage.[10] To screen the phage library, erythropoietin receptor fusion protein is fixed to microtiter plates by means of an antibody (mab). The bound phages can be eluted by cleavage at a thrombin cleavage site located between EPOR and the fusion region. B) Low-valency display on protein III (pIII) results in the presentation of 1 – 5 peptides. Screening in the presence of competing erythropoietin (EPO) selects for higher affinity binding phages compared to pVIII display. In these experiments, bound phage are eluted from EPOR under acid conditions.

codons. The phages destined to display a peptide sequence fused to the coat protein pIII or pVIII are propagated in bacteria. To do this, the mutated phage DNA must be introduced into the bacteria, a step known as transfection. Libraries of around 10^7 to 10^9 different phages are routine these days. By a step known as "panning", these repertoires are subjected to selection processes based on their affinity to an immobilized ligand. Phages which remain bound after particular selection pressures (e.g. elution conditions, competitive ligands) are subsequently eluted, propagated, and subjected to a further round of selection. After at least three rounds of enrichment, the peptide sequence of several ligand-binding phages can be determined by sequencing the relevant DNA region of gene III or VIII.

How was it possible with this technique to identify a peptide which can bind with high affinity to the erythropoietin receptor and also activate it?[1] The first step was conducted with a phage library in which the cyclic peptide sequence CXXXXXXXXC (C = cysteine, X = any of the 20 genetically encoded L-amino acids) was displayed on pVIII. With this multivalent display, around 100 to 200 copies of the peptide are present on the phage surface. Consequently, even phages displaying lower affinity peptides can bind to the ligand and be amplified (Figure 2 A). The extracellular domain of EPOR as a fusion protein was immobilized through an antibody against the fusion moiety. The EPOR-binding phages were eluted with the protease thrombin, which cleaves an internal amino acid sequence introduced between EPOR and the fusion region. This method identified the peptide CRIGPITWVC, which inhibits the EPO/EPOR interaction with a half-maximal constant (IC_{50}) of 10 μM. To find peptide sequences with higher affinity, a subsequent pIII (Figure 2 B) library of the type GGXXXCRIGPITWVCXXXGG was tested in the presence of EPO as competitor for phage/EPOR interactions. The library was constructed not only to increase the peptide length, but to permit substitutions within the loops. The best peptide obtained (GGT**YSC**HF**GPL**T**W**V**CKP**QGG) inhibited the EPO/EPOR interaction with an IC_{50} of 200 nM; the dissocoiation constant for the natural complex is 200 pM. Amino acids common to all the peptides in the last round of selection plus the cysteine residues essential for activity are highlighted. In addition, a variety of biological assays proved that the peptide (called EMP1 for "EPO-mimicking peptide") is able to activate EPOR, and, hence, is a promising lead compound for EPO mimics. Medical and commercial implications of such a

  1433-7851/98/03706-0770 $ 17.50+.50/0

liason are immense. For example, recombinant human EPO is used in the therapy of renal anaemia following kidney insufficiency. The worldwide turnover volume of EPO in 1995 amounted to 2.6 billion dollars.

The same issue of *Science* in which the identification of EMP1 was published contained an article describing the crystallization and structural resolution of the $EPOR_2/EMP1_2$ complex and shedding light on how the astonishing protein mimicry by a peptide is possible (Figure 1, left).[2] All the amino acids in the EPO mimic EMP1 marked in bold in the previous paragraph are essential either for interactions with the receptor or the peptide conformation. EMP1 forms a double-stranded, antiparallel β sheet and dimerizes with a second EMP1 molecule at an angle of about 90° to form a four-stranded β sheet. This dimerization creates two identical EPOR binding sites. The receptor, as expected for a class-1 cytokine receptor, consists of two seven-stranded β-sheet domains. The interaction of EMP1 dimers with EPOR is achieved through four loops between the β-sheet strands of both receptor domains. Despite some differences in details, a fascinating similarity to the $hGH/hGHR_2$ complex is observed (Figure 1, right). The EPO/EPOR complex itself could not be crystallized to date. The interaction of EMP1 or hGH with their receptors is mediated through homologous amino acids in these receptors. In the hGH/hGHR complex, in addition to the four EPOR loops, two further loops make contact with the cytokine, which mirrors the size of hGH in comparison to EMP1 dimers.

How was it possible purely through selection for affinity to EPOR to obtain at the same time a functional dimeric peptide which dimerizes and activates the receptor? One explaination suggested by the authors is that EPOR is immobilized by a divalent antibody during the panning steps. This process could imitate the three-dimensional situation on the cell surface during receptor dimerization. It is also unclear whether the dimerization of EMP1 already takes place on the phage surface, or if it first occurs between free peptides. In any case, the relatively high affinity for EPOR is impressive (200 nM, as opposed to 200 pM for EPO), as is the specificity (related receptors are not bound) of this 20-mer protein mimic. For the comparable system hGH/hGHR, detailed studies into the contribution to the free binding energy made by particular amino acids were carried out. They revealed that only a few of the contact residues account for most of the free binding energy.[11] Since it probably applies to many systems, this "hot spot of binding" principal should make it possible to mimic larger protein-interaction surfaces by concentrating on the "energetic epitope". Indeed, at almost the same time the group at the Affymax Research Institute, again with the phage-display technique, identified an antagonistic 15-mer peptide which binds the type-1 interleukin-1 receptor with a dissociation constant of 2 nM.[4] This affinity resembles that of the IL-1 receptor antagonist (1.6 nM), a naturally occuring 20-kDa glycoprotein. However, with regard to its biological activity, the peptide is noticably poorer.

A third example of protein mimicry was published one year later, again by W. Dower and co-workers. With a phage display and later *E. coli* display, a 14-mer peptide was found which similarly binds the thrombopoietin receptor (TPOR) with very high affinity ($K_D \approx 2$ nM), although only weakly activating it.[3] However, synthetic dimerization of this peptide through a linker resulted in a half-maximal efficiency constant of 100 pM(!), which is identical to that of the natural ligand thrombopoietin (332 aa) and makes the peptide an attractive pharmacological lead compound. Thrombopoietin is the most important factor involved in the differentiation of bone-marrow stem cells to blood platelets and, in recombinant form, is currently being tested in clinical studies for thrombocytopenia therapy following chemotherapy or bone-marrow transplants. Analogous to the dimerization of the thrombopoietin mimic, W. Dower and co-workers recently reported a pseudosymetrical covalent dimerization of the erythropoietin mimic EMP1 through a lysine residue.[12] Each monomer is simultaeously synthesized either on its ε-amino group or on its α-amino functional end with a β-alanine linker. The two disulfide bridges are selectively joined by an orthogonal protection strategy. Compared to the noncovalently dimerized EMP1, this peptide, $EMP1_{K\text{-dimer}}$, has 100-fold higher affinity for EPOR and more than 10-fold better antagonistic activity in vivo.

The protein mimics described here form a new class of promising lead compounds for the synthesis of readily available active drugs that can be taken orally. Until now, there has existed no general strategy to block larger protein-contact surfaces based exclusively on chemical combinatorial methods; this is currently a bottleneck in drug discovery. The renaissance of peptides as lead compounds is dawning.

German version: *Angew. Chem.* **1998**, *110*, 801 – 804

Keywords: peptides · phage-display technology · protein mimetics · proteins · receptors

[1] N. C. Wrighton, F. X. Farrell, R. Chang, A. K. Kashyap, F. P. Barbone, L. S. Mulcahy, D. L. Johnson, R. W. Barrett, L. K. Jolliffe, W. J. Dower, *Science* **1996**, *273*, 458.
[2] O. Livnah, E. A. Stura, D. L. Johnson, S. A. Middleton, L. S. Mulcahy, N. C. Wrighton, W. J. Dower, L. K. Jolliffe, I. A. Wilson, *Science* **1996**, *273*, 464.
[3] S. E. Cwirla, P. Balasubramanian, D. J. Duffin, C. R. Wagstrom, C. M. Gates, S. C. Singer, A. M. Davis, R. L. Tansik, L. C. Mattheakis, C. M. Boytos, P. J. Schatz, D. P. Baccanari, N. C. Wrighton, R. W. Barrett, W. J. Dower, *Science* **1997**, *276*, 1696.
[4] S. D. Yanofsky, D. N. Baldwin, J. H. Butler, F. R. Holden, J. W. Jacobs, P. Balasubramanian, J. P. Chinn, S. E. Cwirla, E. Peters-Bhatt, E. A. Whitehorn, E. H. Tate, A. Akeson, T. L. Bowlin, W. J. Dower, R. W. Barrett, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 7381.
[5] J. A. Wells, *Curr. Opin. Cell. Biol.* **1994**, *6*, 163.
[6] A. M. de Vos, M. Ultsch, A. A. Kossiakoff, *Science* **1992**, *255*, 306.
[7] B. Li, J. Y. K. Tom, D. Oare, R. Yen, W. J. Fairbrother, J. A. Wells, B. C. Cunningham, *Science* **1995**, *270*, 1657.
[8] A. C. Braisted, J. A. Wells, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 5688.
[9] G. P. Smith, *Science* **1985**, *228*, 1315.
[10] G. P. Smith, J. K. Scott, *Methods Enzymol.* **1993**, *217*, 228.
[11] T. Clackson, J. A. Wells, *Science* **1995**, *267*, 383.
[12] N. C. Wrighton, P. Balasubramanian, F. P. Barbone, A. K. Kashyap, F. X. Farrell, L. K. Jolliffe, R. W. Barrett, W. J. Dower, *Nature Biotechnology.* **1997**, *15*, 1261.

  1433-7851/98/03706-0771 $ 17.50+.50/0