Systematic definition and reconstruction of receptor-ligand binding sites

Rob H. Meloen

Pepscan Systems BV, PO Box 2098, 8203 AB Lelystad, The Netherlands;

r.h.meloen@pepscan.nl

Introduction

Recognition of one macromolecule by another is the key event of biological life and the specificity of this interaction is its most important aspect. Indeed, specificity plays a key role at all levels of biological organisation and increases with increasing complexity of the system involved. It is far from absolute when it comes to interacting macromolecules (for instance antibody recognition of antigen), but is higher at the level of interaction between cells, higher still for recognition events that control the integrity of organs and absolute between interacting individuals – it is inconceivable that one member of a (human) couple would not recognise the other one, or would confuse the partner with someone else. Ultimately, each of these recognition events, including the last one, is based on receptor-ligand interactions. The increasing specificity of the interactions is not, however, explained by an increased specificity of single receptor-ligand systems but rather by the concerted action of multiple receptor-ligands, not unlike increasingly complex pincodes.

Biological processes can be modified at all organisational levels simply by altering receptor-ligand interactions, which has made them a key focus of research in the life sciences and pharmaceutical industry. Nevertheless, it is only recently that a real understanding of this interaction has emerged, the result of a concerted application of the latest developments in biology, chemistry, medicine, pharmacology, physics and mathematics. It was as long as twenty years ago, however, when our laboratory (ID Lelystad, The Netherlands) began a systematic study of this interaction, using high-throughput screening of combinatorial peptide libraries specifically aimed at the antigen-antibody interaction. Since then the method has advanced to a point where the systematic resolution of complex discontinuous and conformational binding sites at a resolution of single amino acids is possible. This degree of resolution has provided the solution to the remaining obstacle in the translation of genomics data into new pharmaceuticals, including new

drugs and vaccines. It has also been instrumental in the development of new diagnostic reagents and in food (e.g. flavour purification procedures) and non-food applications (e.g. down-stream processing). The aim of this paper is to review the evolution of the sophisticated methods currently used to define receptor binding sites.

Initially, attempts to define the binding site of receptor-ligand interactions targeted antigen-antibody systems. This approach was driven by the desire to design synthetic peptide vaccines and diagnostic reagents, which would be safer, cheaper, more reproducible, easier to store and more precise in their action than the classical vaccines and reagents based on inactivated or adapted pathogens. At the time, peptide design was based on the three-dimensional (3-D) structure of target proteins, but despite the enormous number of 3-D structures that were known it was still very difficult, if not impossible, to design antigens in this way. More recent systematic methods based on high-throughput screening and combinatorial libraries have enabled the definition of continuous or linear epitopes (Figure 1), subsequent improvements to these methods, including miniaturisation, new chemical techniques and a higher throughput, have allowed new approaches. The result has been the mapping of conformational and discontinuous epitopes (Figure 1). It is anticipated that this approach will work with equal success in all receptor-ligand systems.

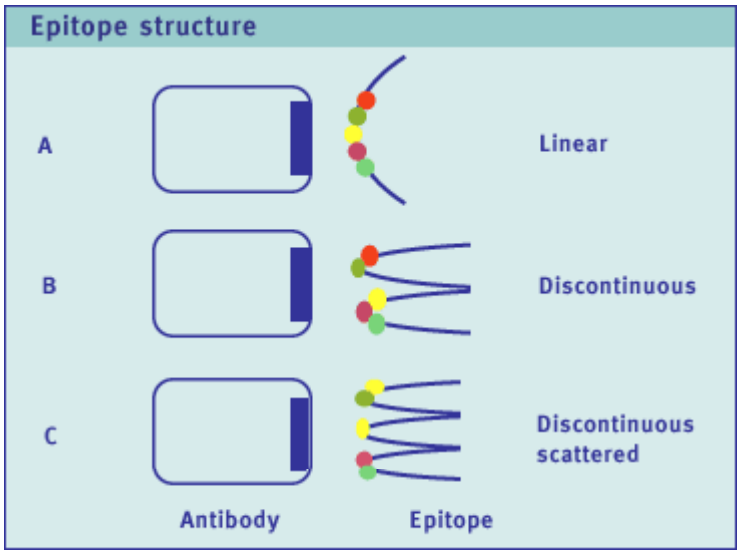

Figure 1. Schematic diagram showing the structure of linear, discontinuous and discontinuous scattered epitopes.

Continuous epitope mapping

Continuous epitope mapping was developed at ID Lelystad twenty years ago [2, 3] and involved the synthesis of peptides on a solid support, initially polyethylene pins. The technique resulted in the compilation of large peptide libraries, which had previously been impossible to obtain. These were used to test the reactivity of both polyclonal and monoclonal antibodies, which marked the beginning of what we now know as combinatorial chemistry. Combinatorial chemistry was first applied to the characterisation of antigen-antibody interactions of foot-and-mouth disease virus (FMDV), and the very first library consisted of all the overlapping hexapeptides (i.e. peptides consisting of six amino acids) of the FMDV capsid protein VP1. When tested against antisera to whole FMDV, a multitude of antibody-binding peptides were obtained, but when the sera were subsequently adsorbed with whole virus, antibodies against only a few peptides were removed [9]. These peptides turned out to form the major immunogenic site of FMDV. The remaining amino acid sequences were apparently located inside the capsid and induced antibodies in animals when the inactivated virus fell apart after administration, which is consistent with the poor stability of the virus. Peptides of the major immunogenic site were used in a vaccine and readily induced virus neutralising antibody production in recipient animals, which however failed to protect the natural hosts against FMDV. This was at variance with the full protection induced by vaccines based on whole-inactivated virus; the explanation for this phenomenon is still subject to debate [12].

The Pepscan methods to be discussed later in this article (Figure 2) were refined still further and the length of the peptides was increased from six to 12 amino acids, increasing the sensitivity of the system. As a result, many more epitopes were mapped, most noticeably the major antigenic site of HIV-1 (the V3-loop) [4, 11] and an antigenic site of Canine Parvovirus (CPV) [15]. Unlike FMDV and HIV, CPV peptides readily induced antiviral activity and protected dogs just as well as the classical vaccine, a preparation based on inactivated virus. This was the first fully protective synthetic vaccine [6, 7, 8, 12] Subsequently the peptide was engineered into Cow Pea Mosaic Virus (CPMV) to create a chimeric plant virus that could be produced in large quantities. This preparation also resulted in full protection of the natural host against CPV [1]. In addition, an antigen of Epstein Barr Virus (EBV) has been identified and synthesised [14], and is now used in commercial

diagnostic assays worldwide. The technique has also been used to prepare site-specific anti-peptide antibodies, which led to the ultimate immunohistochemical test for bovine spongiform encephalopathy (BSE), the demonstration of prion antigen in the brain stem of cattle [17, 18]. Peptide libraries have been used in bioassays [20], enabling the systematic assessment of all binding sites of many receptor-ligand systems.

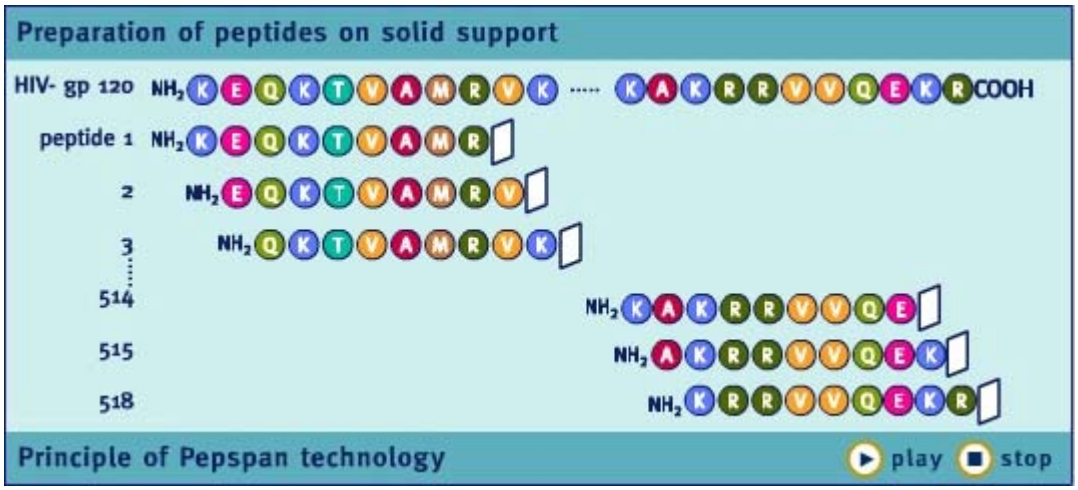

Figure 2. Animation showing the principles of the Pepscan method. **Part 1:** Peptides are prepared on solid (polyethylene) support pins (represented as white squares at the COOH- end of each amino acid sequence). **Part 2:** The pinss with bound peptide are placed into individual wells of a 96-well microtitre plate. Each well contains the test antisera, and after incubation non-bound antibody is washed off the supports. Antibody binding to the respective peptides is visualised using a peroxidase-conjugated secondary antibody in an ELISA system.

During the past few decades, binding sites have been studued in many systems, including viruses, bacteria, parasites and signal molecules [10]. Not unexpectedly, most of them were found to be discontinuous or conformational in nature. In the case of these more complicated binding sites, the overlapping peptide approach normally used for mapping binding sites, or epitopes, could not be applied. This proved to be the major obstacle in the development of a fully synthetic vaccine, for instance against FMDV. It is believed that multiple, discrete antigenic sites are necessary for the induction of a sufficiently wide spectrum of antibodies to prevent FMDV from producing variants that are not recognised by the antibodies. Only one of the three or four antigenic sites of FMDV that induce neutralising activity is linear, and therefore new methods were needed to map and subsequently reconstruct the discontinuous binding sites. During the last

two years these have been developed, based on miniaturisation of the Pepscan methods, new chemical techniques and new combinatorial algorithms.

Development of this most recent technique involved three approaches for the analysis and definition of antigen-antibody interactions – two of which came from recent advances made at ID Lelystad. They were based on:

- the development of mimotopes, useful for investigation of epitopes that are a polysaccharide or nucleic acid
- 'domain scanning', in which long peptides (up to 36 amino acids) are used in overlapping peptide scans
- on a combinatorial approach, e.g. two peptides from different regions of the intact protein molecule combine to form one protein. Using peptide arrays (for a given protein), all overlapping peptides of 12 amino acids can combine.

Together, the three methods have allowed the mapping of all discontinuous epitopes studied so far – a feat that was previously impossible.

Development of mimotopes

This is a generic method that uses only antibody and random peptide libraries to identify peptides that functionally mimic the epitope under investigation. Such peptides are known as mimotopes, the molecules being capable of competing with the native antigen for antibody binding. Mimotope molecules can be used to vaccinate animals and to induce antibodies that have the same specificity as the original, template monoclonal antibody. This approach is especially useful for the production of mimics of polysaccharide epitopes. Although contra-intuitive, several groups have developed peptides that functionally mimic epitopes [see review 13]. The technique is based on the initial screening of random peptide libraries. It normally yields peptides of low binding affinity – which are of no practical use – but these can be upgraded using dedicated small peptide libraries based upon the amino acid sequence of the original low affinity peptides. Typically, affinities can be increased four- to five-fold, thus giving rise to useful peptides. When these peptides mimic an epitope of a protein they often contain amino acid sequences that have nothing in common with the native epitope, thus limiting the use of the method to develop mimotopes. Because the nature of the epitope remains unknown, the approach cannot be used for epitope mapping either, while the following two approaches are suitable.

Epitope mapping through 'domain scanning'

Large sets of much longer peptides (than the former maximum of 12 amino acids) have been obtained with the use of new chemical techniques. Each peptide may be the size of a single domain, hence the term 'domain scanning'. Domain scanning has enabled the mapping of two monoclonal antibodies – from a total of about 30 – specific for follicle stimulating hormone (FSH), none of which could have been mapped using the older method. Peptides that bound the two antibodies represented a loop of the FSH molecule. Deletion of the upper part of the loop left the binding intact, suggesting that the epitope consisted of opposite strands of the loop. Although the binding parts of this epitope were located relatively close to each other in the primary sequence, it is in fact a discontinuous epitope.

However, even domain scanning has been unable to map a large proportion of antibodies, and hence a third approach was developed, based upon the systematic combination of all the subunit parts of the native protein using peptide array systems.

Combinatorial peptide arrays

The introduction of combinatorial peptide arrays not only allowed the mapping of remaining epitopes of the FSH molecule but also of many other discontinuous epitopes.

The technique uses peptides coupled to flat polyethylene surfaces. These are then put in contact with antibody to identify the binding peptides. Typically, dedicated sets of between 40,000 and 100,000 different peptides are tested for each epitope. In its most elementary form two amino acid sequences, both consisting of 12 amino acids, are combined to form linear peptides of 24 amino acids. By combining all overlapping peptides (each consisting of 12 amino acids) with each other, all possible combinations are produced, arrayed and tested for antibody binding. For the FSH protein of about 200 amino acids, some 40,000 peptides are required, each consisting of 24 amino acids. More sophisticated techniques systematically apply branching, cyclisation and the incorporation of spacers, some of which were also used to define the remaining epitopes of FSH. As indicated above, these epitopes were finally found to reside on one tip of the elongated molecule; each had to be classified as discontinuous, because functioning required amino acids from two different loops located very close to one another in the 3-D structure, but widely separated in the primary structure.

Definition of the binding sites of receptor-ligand interactions

Occasionally soluble receptors are available (often as chimeric antibodies), which can be used to define the binding site on a ligand. This was recently done for tumour necrosis factor (TNF) α. Using its soluble receptor, the two binding sites on the TNFα molecule were determined.

When soluble receptors are unavailable, other methods are needed. A generic approach, which can easily be applied in bioassays, makes use of libraries of soluble peptides. This system enabled the direct translation of concepts and algorithms obtained from epitope mapping to receptor-ligand systems. Thus, T-cell epitopes were identified, and the role of the individual amino acids in the binding site was systematically assessed [5, 19, 20].

Subsequently more complicated systems were studied and various bioactive peptides were obtained. In an initial screening the bioactivity of peptides was low; however, just as in the case of the low antibody binding peptides, more active peptides could be obtained by upgrading them step-wise, using small dedicated sub-libraries, similar to the antigen-antibody system. Using this approach a small peptide was produced from an anti-fungal protein, which, after several modifications, had the same bioactivity as the much larger, original protein [16]. Currently, our group is in the process of translating the systematic 3-D mapping of epitopes into one for receptor-ligand systems. Once this has been achieved, a complete set of systematic methods will be available to target most, if not all, receptor-ligand systems.

Conclusion

The development of high-throughput screening and combinatorial peptide libraries started in the early 1980s; systematic use and combination of these techniques has led to methods suitable for the mapping and reconstruction of binding sites of any antigen-antibody system and of many ligand-receptor systems. The binding sites provide building blocks for new drugs, vaccines and diagnostic kits and for many food hygiene-related and other applications. Development of these methods has removed a last obstacle between genomic information and approved pharmaceuticals.

These methods will also help in the production of novel synthetic molecules with pre-designed, biological properties. They may open the way for the development of synthetic antibodies to be used in place of biological ones, which will bring together diagnosis and therapy, and

will eventually allow real-time, tailor-made disease treatments. It is conceivable that designer molecules will be superior to anything taken from nature, because they will be inexpensive to produce, reliable, less variable than natural molecules and thereby more precise in their action.

References:

1. Dalsgaard, K., Uttenthal, Å., Jones, T.D., Xu, F., Merryweather, A., Hamilton, W.D.O., Langeveld, J.P.M., Boshuizen, R.S., Kamstrup, S., Lomonossoff, G.P., Porta, C., Vela, C., Casal, J.I., Meloen, R.H. and Rodgers, P.B. (1997) Plant derived vaccine protects target animals against a virus disease. Nat. Biotechnol. 15, 248-252.

2. Geysen, H.M., Barteling, S.J. and Meloen, R.H. (1985) Small peptides induce antibodies with a sequence and structural requirements for binding antigen comparable to antibodies raised against the native protein. Proc. Natl. Acad. Sci. USA 82, 178-182.

3. Geysen, H.M., Meloen, R.H. and Barteling, S.J. (1984) Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single aminoacid. Proc. Natl. Acad. Sci.USA 81, 3998-4002.

4. Goudsmit, J., Debouck, C., Meloen, R.H., Smit, L., Bakker, M., Asher, D.M., Wolff, A.V., Gibbs, C.J. Jr., Clarence, J. and Gajdusek, D.C. (1988) HIV type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. Proc. Natl. Acad. Sci. USA 85, 4478-4482.

5. Kast, W.M., Roux, L., Curren, J., Blom, H.J.J., Voordouw, A.C., Meloen, R.H., Kolakofsky, D. and Melief, C.J.M. (1991) Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with an unbound synthetic peptide. Proc. Natl. Acad. Sci. USA 88, 2283-2287.

6. Langeveld, J.P.M., Casal, J.I., Cortés, E., van de Wetering, G., Boshuizen, R.S., Schaaper, W.M.M., Dalsgaard. K. and Meloen, R.H. (1994) Effective induction of neutralizing antibodies with the amino terminus of VP2 of canine parvovirus as a synthetic peptide. Vaccine 12,15, 1473-1480.

7. Langeveld, J.P.M., Casal, J.I., Osterhaus, A.D.M.E., Cortés, E., de Swart, R., Vela, C., Dalsgaard, K., Puijk, W.C., Schaaper, W.M.M. and Meloen, R.H. (1994) First peptide vaccine providing protection against viral infection in the target animal: Studies of canine parvovirus in dogs. J. Virology 68, 4506-4513.

8. Langeveld, J.P.M., Martinez-Torrecuadrada, J., Boshuizen, R.S., Meloen, R.H. and Casal, J.I. (2001) Characterization of a protective linear B cell epitope against feline parvoviruses. Vaccine 19, 2352-2360.

9. Meloen, R.H. and Barteling, S.J. (1986) Epitope mapping of the outer structural protein VP1 of three different serotypes of FMDV. Virology 149, 55-63.

10. Meloen, R.H., Casal, J.I., Dalsgaard, K. and Langeveld, J.P.M. (1995) Synthetic peptide vaccines: Success at last. Vaccine 13, 885-886.

11. Meloen, R.H., Liskamp, R. and Goudsmit, J. (1989) Specificity and function of the individual amino acids of a major determinant of HIV-1 that induces neutralization activity. J. Gen. Virol. 70, 1505-1512.

12. Meloen, R.H., Puijk, W.C., Langeveld, J.P.M., Langedijk, J.P.M., Van Amerongen, A. and Schaaper, W.M.M. (1995) PEPSCAN to determine T and B cell epitopes. In: Immunological Recognition of Peptides in Medicine and Biology. Eds. N. Zegers, W. Boersma and E. Claassen. CRC Press, Inc., Florida, pp15-31.

13. Meloen, R.H., Puijk, W.C. and Slootstra, J.W. (2000) Mimotopes: realization of an unlikely concept. J. Mol. Recognit. 13, 1-8.

14. Middeldorp, J.M. and Meloen, R.H. (1988) Epitope-mapping on the Epstein-Barr virus major capsid protein using systematic synthesis of overlapping oligopeptides. J. Virol. Methods 21, 147-159.

15. Rimmelzwaan, G.F., Poelen, M.C.M., Meloen, R.H., Carlson, J., Uytde Haag, F.G.C.M. and Osterhaus, A.D.M.E. (1990) Delineation of canine parvovirus T cell epitopes with peripheral blood mononuclear cells and T cell clones from immunized dogs. J. Gen. Virol. 71, 2321-2329.

16. Schaaper, W.M.M., Posthuma, G.A., Plasman, H.H., Sijtsma, L., Fant, F., Borremans, F.A.M., Thevissen, K., Broekaert, W.F., Meloen, R.H. and Van Amerongen, A. (2001) Synthetic peptides derived from the ß2-ß3 loop of *Raphanus sativus* antifungal protein 2 that mimic the active site. J. Pept. Res. 57, 409-418.

17. Schreuder, B.E.C., van Keulen, L.J.M., Vromans, M.E.W., Langeveld, J.P.M. and Smits, M.A. (1996) Preclinical test for prion diseases. Nature 381, 563.

18. Schreuder, B.E.C., Keulen, L.J.M., van Smits, M.A., Langeveld, J.P.M., Stegeman, J.A. (1997) Control of scrapie eventually possible? Vet. Quarterly 19, 105-113.

19. Wauben, M.H.M., van de Zee, R., Joosten, I., Boog, C.J.P., van Dijk, A.M.C., Holewijn, M.C., Meloen, R.H. and van Eden, W. (1993) A peptide variant of an arthritis-related T cell epitope induces T cells that recognize this epitope as a synthetic peptide but not in its naturally processed form. J. Immunol. 150, 5722-5730.

20. van de Zee, R., van Eden, W., Meloen, R.H., Noordzij, A. and van Embden, J.D.A. (1989) Efficient mapping and characterization of a T-cell epitope by the simultaneous synthesis of multiple peptides. Eur. J. Immunol. 19, 43-47.