Mapping Antibody-Defined Linear Epitopes

Introduction

An epitope, or antigenic determinant, was defined as the site on an antigen at which an antibody binds, by virtue of the antibody's antigen-combining site (called the paratope) [1]. The word epitope derives from the Greek epi, meaning "upon", and topos, or "place", and thus it is the place on the antigen upon which the antibody binds.

As antigens can be recognised by two distinct groups of receptor molecules of the immune system, namely antibodies (Ab's) or T-cell receptors (TCR's), we need to distinguish whether we are talking about epitopes defined by antibodies or by TCR's. This article will deal only with antibody-defined epitopes. See Mimotopes Application Note PT3-012 "T Cell Epitope Mapping with PepSets Peptides", for a guide to mapping of peptide epitopes defined by TCR's.

Topics in this article:

Classification of Antibody-defined Epitopes

Antibody-defined epitopes of protein antigens can be broadly classified as linear (=sequential, continuous) or assembled (=discontinuous) [2]. This classification is based on whether (or not) the amino acids of the protein antigen, which interact with the antibody, are close together in the PRIMARY sequence of the protein antigen. The surface of a protein is made up largely of the side chains of the amino acids comprising the protein. The surface of the antigen which interacts with the antibody surface could thus either consist of amino acids which are close together in the primary sequence of the protein, or of amino acids which are well separated in the primary sequence, but are brought together as a result of the natural folding of the protein to its native, fully functional shape. Epitopes consisting of residues close together in the primary sequence are called linear, continuous, or sequential epitopes, whereas epitopes consisting of residues separated in the primary sequence are by contrast called discontinuous; or "assembled" epitopes, for obvious reasons. Another term used to describe epitopes is "conformational", but as all antigen- antibody binding involves a particular conformation of the antigen, the use of this term adds nothing to the qualitative description of an epitope.

Approaches to Epitope Mapping

The primary structure (amino acid sequence) of many proteins is now known. However, for most of them, the detailed 3- dimensional structure is unknown. The 3-D structure of proteins is only readily solved by crystallographic methods, such as X- ray crystallography, although the rapid development of NMR methods may soon provide an alternative source of such structural information. Mapping epitopes by solving the structure of antigen-antibody complexes using crystallography is impractical in the vast majority of cases, because antigen- antibody crystals are very difficult to make. Therefore, a number of other methods are used to deduce the nature of individual epitopes [2,3,4,5]. One of these methods is the use of synthetic fragments (peptides) of the protein antigen, which can be similar enough to the homologous parts of the whole antigen to permit binding by the antibody. For this method to be practical, the affinity of the antibody for the peptide has to be such that the peptide/antibody complex does not dissociate significantly under the conditions of an immunoassay. This situation occurs with linear epitopes, thus allowing the use of peptides to define those epitopes.

Based on our unpublished epitope mapping studies with monoclonal antibodies, it appears that approximately 5-10% of all antibodies directed to native antigens bind to linear epitopes. For those antibodies, and for polyclonal antisera in general, Multipin Peptide Synthesis Technology provides a rapid, cost-effective and adaptable means of identifying linear epitopes [6].

Prerequisites for Successful Epitope Mapping

In the following sections, strategies for epitope location and characterization on protein antigens will be outlined. Clearly, there are two prime requisites before epitopes can be mapped using synthetic peptides:

1. The amino acid sequence of at least part of the protein antigen must be known. Sequences are available for a vast number of antigens due to the sequencing of cloned genes, and for some antigens via protein sequencing.

2. A defined antibody population with specificity for that antigen must be available. The cleanest examples of such antibody populations are monoclonal antibodies (MAb's). "Polyclonal" antisera can be used, particularly if they are from animals/subjects hyperimmune to the particular antigen in question. Other antibody preparations worthy of study include: affinity-purified fractions from sera, sera depleted of particular (unwanted) antibody specificities, and defined (classified) sets of sera from unimmunized subjects. These defined sets of sera can be identified in a conventional serological test, and can be used to establish a correlation between positives in that serological test and binding to peptide epitope(s). Control antibody preparations should always be studied in parallel wherever possible.

The first and simplest approach to defining linear epitopes of a protein antigen is referred to simply as "scanning" [6]. This scanning strategy has two distinct phases. The first phase allows location of the area of the sequence in which an epitope is to be found. The second phase defines the limits or boundaries of each epitope at a resolution of a single amino acid, i.e. indicates which parts of an antibody-binding peptide are "inside" and "outside" the epitope.

Scanning - Phase 1, "General Net" Synthesis

The General Net or "Gnet" synthesis consists of a set of peptides designed to catch all the antibody-binding regions of a protein (hence the term "net"). All overlapping peptides of a defined length, homologous with the protein, are synthesised. The use of highly overlapping peptides ensures that epitopes are not missed by being "cut" at a critical point, which could easily occur if abutting rather than overlapping sequences were made. As long as the peptides are of sufficient length, no linear epitopes should be missed. One approach is to synthesize overlapping octamers (8mers), offset by one residue. The terms offset and overlap are often confused, overlapping residues are those common to two peptides covering an area of a protein sequence while the offset is the distance in residue number between the N-terminal ends of two overlapping peptides. For example the sequences:

ACDEFGHI

and

CDEFGHIK

overlap by seven and are offset by one.

The sequences:

ACDEFGHI

and

EFGHIKLM

overlap by five and are offset by three.

For long protein sequences, where cost of peptide synthesis is a major consideration, a larger offset e.g. 2 to 4 residues, can be used. This reduces the number of peptides which need to be made. In this case, we would recommend longer peptides e.g. 9- to 11-mers respectively, to eliminate the chance of cutting an epitope at a critical point. With the use of longer peptides/larger offsets, there will be a loss of resolution of individual epitopes.

Example of a General Net

The Sperm Whale Myoglobin sequence consists of 153 residues. A general net of octamers offset by 1 is:

Peptide 1: VLSEGEWQ
Peptide 2: LSEGEWQL
Peptide 3: SEGEWQLV
Peptide 4: EGEWQLVL
..
..
Peptide 145: KYKELGYQ
Peptide 146: YKELGYQG

The peptides are reacted with appropriate polyclonal or monoclonal antibodies to the original protein and binding is detected by ELISA. The strongest antibody binding was to four peptides of the following sequences:

Peptide 119(-126): HPGDFGAD
Peptide 120(-127): PGDFGADA
Peptide 121(-128): GDFGADAQ
Peptide 122(-129): DFGADAQG

with the sequence 122-126 DFGAD common to all four peptides. Peptides which strongly bind the antibody such as these are considered to contain epitopes. In this example, epitopes were identified when the peptides were reacted with a 1/1000 dilution of rat anti-sperm whale myoglobin serum, and the ELISA was developed with an anti-rat conjugate.

Scanning - Phase 2, "Window Net" Synthesis

The next phase of the strategy, called a Window net or "Wnet", is to identify the precise boundaries of the epitope(s) located from a Gnet scan. The Window net terminology comes from the concept of this process as "looking at the linear protein sequence through a series of moving windows of different sizes". This involves the synthesis of all of the shorter overlapping sequences covering antibody-binding peptides identified in the Gnet synthesis, e.g. 4mers, 5mers, 6mers and 7mers could be made in a Wnet if the Gnet scan had been done at 8mer length.

Example: The strongly-binding area of sperm whale myoglobin as described above was used as the basis for synthesizing every overlapping 4-, 5-, 6-, and 7-mer starting on residues 118 to 125 of myoglobin:

Myoglobin sequence ...118RHPGDFGADAQGAM131...

PEPTIDES SYNTHESISED

Peptide 1: RHPG
Peptide 2: RHPGD
Peptide 3: RHPGDF
Peptide 4: RHPGDFG
Peptide 5: HPGD
Peptide 6: HPGDF
Peptide 7: HPGDFG
Peptide 8: HPGDFGA
..
..
Peptide 29: ADAQ
Peptide 30: ADAQG
Peptide 31: ADAQGA
Peptide 32: ADAQGAM

The result of testing these peptides with the same rat antiserum as scanned in the Gnet test show that the sequence of the shortest peptide to bind strongly was the pentapeptide starting on residue 122, DFGAD. All the longer peptides containing this pentapeptide also strongly bound antibodies from this polyclonal serum, showing that DFGAD comprised a dominant linear epitope as defined by this serum.

Confirmatory Tests on Mapped Linear Epitopes

The interpretation of binding to a linear peptide by a monoclonal antibody is relatively straightforward. For a pure MAb, specific binding to a peptide, demonstrated by showing that other randomly selected MAb's or isotype-matched controls do not bind to the peptide, is sufficient to show that the peptide is the linear epitope recognised by that MAb. If the MAb is impure (e.g. is in the form of ascites, contaminated with other mouse antibodies), additional criteria may need to be employed to demonstrate specificity.

Initial location of an epitope in polyclonal antisera, by the methods described above, is not of itself sufficient proof of the relevance of the epitope to the overall antibody response to that antigen. It is possible that the antibodies binding to the peptides are those raised against denatured forms of the antigen [7] or are crossreactive with another, unrelated antigen. We recommend using one or more of a range of confirmatory tests on serum/epitopes found in screening experiments [6,8]. For examples, see Mimotopes Application Note PT3-015 "Antigen Competition to Identify Sequential (Linear) Epitopes".

Phase 3. "Replacement Net" Synthesis

While the primary scanning technique followed by the window net analysis provides basic information about the location and the boundaries of epitopes, no information about the contribution to antibody binding by individual residues within the epitope is obtained. This information can be obtained, for each residue in a peptide, by comparing the antibody binding ability of analogs containing single amino acid substitutions. The replacement net or "Rnet" analysis uses such a synthesis of systematic singly substituted peptide analogs. In addition to choosing substituting amino acids from among the 20 genetically coded amino acids, other residues such as uncommon amino acids, D-optical isomers of the genetically coded amino acids, and synthetic amino acids can be used to increase the variety of substitutions. This can give a better understanding of the requirements for antibody binding in terms of both residue constraints and structural requirements.

The epitope found in the Gnet and Wnet scans (above) was examined in detail using substitution with each of the 19 alternative genetically- coded residues. The results show that a residue may be replaceable by a range of other residues without complete loss of binding (e.g. the A replaced by S or P); it may be replaceable only by residues clearly sharing a property with the replaced residue (e.g., E for the second D); or in the extreme case it may be essentially unreplaceable (e.g. the central G in DFGAD).

In an extensive study of replacement nets, sufficient data was gathered to allow creation of a replaceability matrix based on antibody recognition of linear epitopes [9]. A table

showing the replaceability patterns, with logical relationships such as D (aspartic acid) for E (glutamic acid), and high interchangeability of the small amino acids G, S and A may be obtained from Mimotopes if required.

Applications of the Strategies

At least two experimental methods are available for application of synthetic peptides to antibody-binding scans: synthesis of peptides on the surface on which the binding assay is done (pins) [6]; and synthesis of peptides which are subsequently cleaved into solution before use in a binding assay [10]. Peptides made on the surface of a pin can be tested for binding, then "regenerated" by disrupting the antibody-peptide interaction with detergent/reducing agent/sonication prior to the next binding assay. Cleaved peptides can be coated directly on microtitre trays or strips [11,12]; or biotinylated cleaved peptide can be captured on a surface after precoating the surface with avidin or streptavidin [10]. Having a choice of methods then raises the question as to when each should be used, a question which can only be answered in the context of the nature of the information sought.

The most important difference between the two methods, pin- bound peptide versus cleaved peptide, is detection threshold. In our experience, antibodies give discernible signals at much lower concentrations, or lower antibody affinities, with pin- bound peptides than with peptide-coated plates. The higher sensitivity of pin-bound peptide for antibody binding is probably due to the close spacing of pin-bound peptides, allowing multivalent interactions with antibody. Work with antibodies known, from X-ray crystallographic solution of the Ag-Ab complex, to recognize discontinuous epitopes, demonstrates the ability of pin-bound peptides to detect binding to a part of the complete epitope. The high sensitivity of pin-bound peptides makes feasible the identification of mimotopes, mimics of epitopes determined without knowledge of the primary sequence of the antigen of interest [13].

The relative strengths and weaknesses of pin-bound and cleaved peptides can be summarized as follows: - Compared with cleaved peptides pin-bound peptides detect a more complete set of determinants, detecting both linear and parts of discontinuous epitopes. - The data obtained from the pin-bound peptides may be more difficult to interpret. - Assay resolution is good with all types of peptides but it should be noted that the use of nonbiotinylated peptides usually requires longer peptides to achieve simultaneous adhesion to the plate and the antibody. Without a specific capture mechanism failure to bind the plate [11] and some epitope masking can occur. - For pin-bound peptides the number assays which can be performed is >50. The peptides are regenerated between assays by disrupting the antibody-peptide interaction. Cleaved peptide from a single pin is sufficient to perform several hundred assays. - Assay reproducibilty declines for pin-bound peptides as the number of assays increases, this is due to declining peptide reactivity because of incomplete peptide-antibody disruption between tests. Assays using cleaved peptides are fully reproducible. - The amount of serum required for assays with pin-bound peptides is typically 0.1 microlitre/peptide compared with 1 microlitre/cleaved peptide. - Assays performed with cleaved peptides can be done concurrently while only one assay/day can be performed with pin-bound peptides. - Standard ELISA equipment is required for assays with all types of peptides, however to disrupt the pin-bound peptide- antibody interaction an ultrasonic bath is also required.

In general, we prepare both forms (biotinylated cleaved peptides, and permanently pin-bound) of the peptides, giving ourselves the greatest latitude in subsequent testing, with the added advantage of having the biotinylated solution-phase peptide available for competition experiments with the pin- bound peptides or other uses.

To obtain the maximum information about peptide binding antibodies present in polyclonal sera (e.g. human serum) we would recommend that both sets of peptides (pin-bound and biotinylated) be used.

Problems in Epitope Mapping

We recommend that the above strategies be used routinely for the scanning of epitopes. However, problems in interpretation of the resulting data can arise. It is not uncommon when using human sera for multiple weak reactivities to be reported . The poor signal to background ratios are not improved by increasing the concentration of the test sera. However, one approach to extracting meaningful data from scans on a set of sera is to analyse them for the strongest and the most common reactivities by making a "consensus plot".

Our interpretation of weak binding over a high background (poor signal/noise ratio) is that the serum tested lacks a major population of antibodies directed to a linear epitope of the antigen. Many or all of the small peaks present are believed to be due to segments of discontinuous determinants, a conclusion drawn from the similarity of the signals to those obtained when testing a monoclonal antibody known to recognise a discontinuous epitope.

While, in some cases, the results of a scan on a polyclonal serum may initially be disappointing because linear epitopes appear to be few or weak, in many instances it is only necessary to identify the location of any linear epitope(s), as these then represent a viable target for (e.g.) an antipeptide response. The fact that a linear epitope has not elicited a strong response (i.e. is not a dominant epitope) as a result of either a natural infection, or immunisation with a protein antigen, does not diminish its potential value as a peptide immunogen.

Assay Procedures for ELISA with Peptides

Assay methods are given in the instruction manuals supplied with peptide synthesis kits, and with peptide sets. Software which assists with reading and plotting the results of the assays is also supplied. Practical guides are available in the Mimotopes Application Note series, including the following titles: "Antigen Competition to Identify Sequential (Linear) Epitopes" and "ELISA Method Using Biotinylated Peptides".

Conclusions

Epitope mapping with overlapping synthetic peptides is the most efficient way to identify the linear antigenic determinants defined by a particular serum or monoclonal antibody. A large proportion of the total epitope "universe", the epitopes which are assembled or discontinuous, are not accessible by this method. However, linear epitopes are amenable to detailed investigation, including establishing the contribution of each residue to the overall binding requirements of the epitope. The relevance of peptide epitopes detected with polyclonal antibodies should be checked using a secondary technique such as competition with native antigen; or elution of antibody from the binding peptide, followed by a test of the eluted antibody in a supplementary immunoassay.

Epitope mapping with synthetic peptides is ideally done with pin-bound peptide (for high sensitivity) and with solution- phase peptide. Alternatively, to scan only for the high affinity or high concentration antibodies, use biotinylated solution-phase peptides alone.

References

1. Jerne, N.K. (1960) Immunological speculations. Ann. Rev. Microbiol. 14; 341-358.

2. Benjamin D.C. et al. (1984) The antigenic structure of proteins: A reappraisal. Ann. Rev. Immunol. 2; 67-101.

3. Atassi, M.Z. (1984) Antigenic structures of proteins. Eur. J. Biochem. 145; 1-20.

4. Getzoff, E.D., Tainer, J.A., Lerner, R.A. and Geysen, H.M. (1988) The chemistry and mechanism of antibody binding to protein antigens. Adv. Immunol. 43; 1-98.

5. Jemmerson, R. and Paterson, Y. (1986) Mapping antigenic sites on proteins: implications for the design of synthetic vaccines. BioTechniques 4; 18-31.

6. Geysen, H.M., Rodda, S.J., Mason, T.J., Tribbick, G. and Schoofs, P.G. (1987). Strategies for epitope analysis using peptide synthesis. J. Immunol. Methods 102; 259-274.

7. Jemmerson, R. (1987) Antigenicity and native structure of globular proteins: low frequency of peptide reactive antibodies. Proc. Natl. Acad. Sci. USA 84; 9180-9184.

8. Tribbick, G., Triantafyllou, B., Lauricella, R., Rodda, S.J., Mason, T.J. and Geysen, H.M. (1991) Systematic fractionation of serum antibodies using multiple antigen homologous peptides as affinity ligands. J. Immunol. Methods 139; 155-166.

9. Geysen, H.M., Mason, T.J. and Rodda S.J. (1988) Cognitive features of continuous antigenic determinants. J. Molecular Recognition 1; 32-41.

10. Weiner, A.J., Geysen, H.M., Christopherson, C., Hall, J.E., Mason, T.J., Saracco, G., Bonino, F., Crawford, K., Marion, C.D., Crawford, K.A., Brunetto, M., Barr, P.J., Miyamura, T., McHutchinson, J. and Houghton, M. (1992) Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. Proc. Natl. Acad. Sci. USA 89; 3468-3472.

11. Geerligs, H.J., Weijer, W.J., Bloemhoff, W., Welling, G.W. and Welling-Wester, S. (1988) The influence of pH and ionic strength on the coating of peptides of herpes simplex virus type 1 in an enzyme-linked immunosorbent assay. J. Immunol. Methods 106; 239-244.

12. Tainer, J.A., Getzoff, E.D., Alexander, H., Houghten, R.A., Olson, A.J., Lerner, R.A. and Hendrickson, W.A. (1984) The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein. Nature 312; 127-134.

13. Geysen, H.M., Rodda, S.J. and Mason, T.J. (1986) A priori delineation of a peptide which mimics a discontinuous antigenic determinant. Mol. Immunol. 23; 709-715.

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