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Mapping the Epitopes of Antibodies

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Introduction

Epitope mapping is an important step in characterizing antibodies that bind a potential therapeutic target. By selection from a phage Ab library, one might obtain a few hundred Abs that bind the antigen (Ag) (Hoet, *et al.*, 2005). Epitope mapping might group the Abs into classes that do not compete so that one or two members of each class can be tested for bioactivity. Alternatively or subsequently, one uses an antibody showing a desired biological activity to pick additional candidates by picking Fabs that compete with the active Fab for binding to the Ag, that is, those that have overlapping epitopes. Such sorting could reduce a selected pool of several hundred binders to a few tens warranting further testing. There are many other aspects of epitope mapping that relate to the natural function of the immune system in wellness and disease and to the use of Abs as reagents and structural probes that I describe.

The idea of an epitope (Bonavida and Sercarz, 1971, Kabat, 1970) predates both the invention of mAbs (Kohler and Milstein, 1975) and the determination of the first crystal structure of an Ag-Ab complex (Segal, *et al.*, 1974). The technological combination of mAbs (Kohler and Milstein, 1975), recombinant DNA (Morrow, *et al.*, 1974), peptide synthesis (Merrifield, 1965), transgenic mice (Taylor, L. D., *et al.*, 1992), and display of peptides (Cwirla, *et al.*, 1990, Smith, 1985), and proteins (Roberts, *et al.*, 1992), and Abs on phage (McCafferty, *et al.*, 1990) or yeast (Kieke, *et al.*, 1997) has increased both the need for epitope mapping and the possibility of map-

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Abbreviations: Ab, antibody; FACS, fluorescence assisted cell sorting; mAb, monoclonal antibody; pAb, polyclonal Ab; Fab, fragment, antigen-binding; Ag, antigen; ADE, antibody-dependent enhancement (of disease); MHC, major histocompatibility complex; ECD, extracellular domain; FRET, fluorescence resonance energy transfer; SPR, surface plasmon resonance; PAGE, polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; TJ, tight junction; SAM, selected alanine mutant; CDC, complement-dependent cytotoxicity; KLH, keyhole limpet hemocyanin; ORF, open reading frame; RSOV, right-side-out vesicles; ISOV, inside-out vesicles; RBC, red-blood cells.

ping the epitopes of interesting antibodies to an appropriate level. Epitope mapping is easier today than it was ten years ago.

"Epitope" is also used to describe the short peptides that appear in MHC complexes, *i.e.* T-cell epitopes. This review is concentrated on so-called B-cell epitopes which relate to the binding of antibodies.

Epitopes can be divided into linear epitopes (also known as continuous epitopes) and non-linear epitopes (also known as conformational or discontinuous epitopes). Linear epitopes persist after the protein is denatured or is in small peptide fragments. Conformational epitopes persist only in properly folded proteins or large folded fragments. Some of the mapping methods can determine only linear epitopes and others can detect either type. The boundary is not, however, sharp. "Epitope" can be modified or qualified in several ways. For example, there are "functional epitopes" (Sanchez-Madrid, *et al.*, 1983), "structural epitopes" (Abraham, *et al.*, 1985), "contact epitopes" (Jin, *et al.*, 1992), "binding epitopes" (Bock, *et al.*, 1985), "protective epitopes" (Seyer, *et al.*, 1986), "neutralizing epitopes" (Wimmer, *et al.*, 1984), "extracellular epitopes" (Khan, 2001), and "cytoplasmic epitopes" (Froehner, *et al.*, 1983).

After a brief description of the methods that share the label "epitope mapping" and a discussion of the purposes for mapping epitopes, I give a number of examples from the literature, sometimes the earliest, sometimes the most recent, and sometimes the most interesting. The literature is so immense that all choices are somewhat arbitrary.

What is meant by "epitope mapping"

The word "epitope" came into common usage in the 1960's (Onoue, *et al.*, 1965) and has always meant roughly "the place where an antibody binds its Ag", or by extension to the peptide presented in an MHC molecule to which a T-cell receptor binds. "Where" can mean anything from "the S protein of virus Z" to "residues 323, 324, 337, 345, 362, 363, and 394 of protein Q". Etymological purists might wish to have a sharper definition of "epitope mapping", but it is not to be and should not be attempted. Because of the vagueness of "epitope mapping", some have substituted other terms, such as "immunological analysis", "fine-structure analysis", or "epitope determination". This makes searching the literature much more difficult. It seems preferable that the umbrella "epitope mapping" be invoked, with proper qualification, when one reports the determination of where an antibody binds. The phrase "epitope mapping" in the literature can mean measuring any of:

1. WHICH PROTEIN IN A MULTI-PROTEIN STRUCTURE BINDS AN AB OR GROUPS OF ABS

Here the Ag may be an entire virus and the epitope must first be assigned to one of the viral proteins. Alternatively, the Ag may be a tissue or allergen in an autoimmune or allergic disease, and the epitopes must be assigned to one or more proteins. I will call this activity "assignment mapping". Surprisingly, some Abs that are "neutralizing" in cell culture give rise to "antibody dependent enhancement" (ADE) of infection *in vivo*. It has been determined that Abs that bind particular viral proteins may exhibit ADE of viral disease while those that bind other viral proteins are protective

in vivo (Hohdatsu, *et al.*, 2003). Assignment mapping does not distinguish between linear and conformational epitopes. See examples [1], [2], [3], [4], [5], and [6] considered below.

2. ON WHICH SIDE OF A MEMBRANE IS THE EPITOPE LOCATED

Many Ags are membrane proteins and discovering whether the epitope is extracellular or intracellular may be a first step in characterizing the epitope. When selecting an Ab from a library *in vitro*, one can select so that an Ab binding to an extracellular or intracellular epitope is obtained. When obtaining Abs from animals, this is more difficult, though possible in some cases. One method is to combine small peptide mapping (*q.v.*) with a model of the protein that assigns residues to extracellular, transmembrane, and intracellular compartments. Alternatively, one can determine whether the Ab binds to cells with or without permeabilization or inversion. This is "topological mapping". See examples [5], [6], and [7].

3. COMPETITIVE BINDING TO THE AG

This method can show that two Abs bind to overlapping or to non-overlapping epitopes, but not that they bind to the **same** epitope. One can also use competition between known ligands for the Ag and the Abs of interest. If you know where the ligand binds and an Ab competes with the ligand, then you know approximately where the Ab binds. At least, one can divide a set of Abs into those that compete with the ligand (or a particular Ab) and those that do not. I shall refer to this method as "competitive Ab binding". Often, one Ab or ligand is immobilized, the Ag is bound, and a second, labeled Ab (e.g. biotinylated) is tested in an ELISA assay for ability to bind the captured Ag. This can also be done in a surface plasmon resonance (SPR) device by immobilizing one Ab and measuring the binding of the complex of Ag with a second Ab (or ligand) or measuring the sequential binding of Ag and a second Ab (or ligand) (Wassaf, *et al.*, 2006). Patent claims often use the idea of competitive Ab mapping where one Ab is known and the binding of all future Abs are to be measured against that of the known and claimed Ab. Competitive Ab binding is applicable to linear and conformational epitopes, does not distinguish between them, and is illustrated in examples [9], [10], [11], [14], [39], and [41].

4. PROTECTION OF THE AB-BOUND AG FROM A DEGRADATIVE OR MODIFYING AGENT

There are a number of proteases that do not degrade one or more forms of antibodies. For example, papain cleaves IgGs into Fab and Fc fragments, but leaves most Fabs intact. Pepsin cleaves IgGs into $F(ab')_2$ and Fc and leaves most $F(ab')_2$s intact. Usually, the Ab is immobilized, the Ag is bound, the complex is treated with an agent, such as a protease, and the effect on the Ag is compared to the effect of the agent on naked Ag. I will refer to this method as "protection mapping". When the modifying agent is a protease, one can wash away the fragments of the Ag that do not bind the Ab before eluting the smallest fragment that remains bound to the Ab. Using MALDI/mass-spectrometry, one can determine the molecular mass of the fragment. If the

protease is specific and well characterized and the sequence of the Ag is known, it is often possible to assign the Ab binding site to a small segment of the Ag sequence. Protection mapping can yield linear or conformational epitopes, but does not distinguish the two classes. See example [17], [18], and [19].

5. ENERGY TRANSFER BETWEEN A LABELED AB BINDING TO AN AG AND A SECOND FLUOROPHORE

Fluorescent resonance energy transfer (FRET) can be applicable to pairs of fluorophores (such as labeled Abs) that can bind simultaneously and can disclose the distance between sites separated by 1 to 10 nm. Alternatively, if the Ag contains a fluorophore, then binding of a fluorescently labeled antibody can modify the Ag fluorescence (usually quenching it). I will refer to this method as "energy-transfer mapping". Energy-transfer mapping is applicable to linear and conformational epitopes and is usually carried out in solution. At least one Ab must be labeled with a suitable fluorophore, such as Cascade Blue (CCB) (Nance and Burns, 1990) or bodipy (Dansen, *et al.*, 2001). A second fluorophore is supplied by the Ag or a second labeled Ab. Once energy transfer can be measured for one Ab, one can test other Abs to see if they interfere with the binding of one of the labeled Abs. Energy-transfer mapping is illustrated in examples [7], [8], and [9].

6. BINDING BY LARGE AG FRAGMENTS

This is a very commonly used method and involves producing large fragments of the Ag, either enzymatically or recombinantly. When the Ag comprises several domains, large fragment mapping can show which domain contains the epitope, which can be linear or conformational. I will refer to this method as "large fragment mapping". Recombinant production can be very tedious but allows precise control of fragment boundaries. Proteolytic production is less tedious and risky. If several proteases are used, a suitable collection of fragments can be obtained. If many Abs are to be epitope mapped, one can immobilize several IgGs or Fabs on an SPR chip, such as the FlexChip™, and measure the binding of the various Ag fragments flowed over the Ab array. If the large fragments are made by proteolysis of the Ag, one can determine which fragment binds the Ab by feeding affinity purified fragments into MALDI/mass spec. Large fragment mapping can yield linear or conformational epitopes. See examples [4], [7], [11], and [41].

7. BINDING BY MUTATED AG OR MUTATED LARGE AG FRAGMENTS

Large fragment mapping has limited resolution because protein domains may comprise 50 to 500 amino-acid residues. The resolution can be increased by measuring the effect of mutations in the target domain. One way to direct these mutations is to observe where related proteins (orthologues from other species or homologues from the same or different species) differ. One swaps domains or parts of domains of the related protein. The mutations found in related proteins are unlikely to disrupt the folding of the protein (Jin, *et al.*, 1992). Thus, a loss of binding is likely to be directly

related to a change in the epitope, not to a loss of folding. Alternatively, one can alanine scan the domain found to carry the epitope by large fragment mapping. Alanine scanning can introduce mutations that prevent proper folding of the Ag. For example, changing a Leu or Trp to Ala could disrupt a protein to a greater or lesser extent and alter the binding of Abs to the mutant protein. Thus, loss of binding by a single Ab to a mutant Ag does not always mean that the mutated AA is in contact with the Ab in the Ab-w.t. Ag complex. Often, one has several Abs that bind conformational epitopes which can be used to determine whether a particular mutant is correctly folded. If all the Abs lose binding, the protein is not folded. If only some lose binding, then the mutation is in the epitopes of those Abs. Loss of folding on alanine substitution is more likely at hydrophobic residues (Phe, Trp, Leu, Met, Ile, and Val). Charged residues are almost always on the surface of the protein. Thus replacing Arg, Lys, Glu, or Asp with Ala is unlikely to disrupt the protein fold and a loss of binding in such a mutant indicates that the altered residue is part of the epitope with high reliability. Jin and co-workers (Jin, *et al.*, 1992) have distinguished between a functional epitope (the residues which cannot be changed without greatly reducing binding) and a contact epitope (those residues of the Ag that touch the Ab). In a survey of 21 murine mAbs against human growth hormone, Jin and colleagues (Jin, *et al.*, 1992) found that AAs in the functional epitope were most often Arg, Pro, Glu, Asp, Phe, and Ile. In other Ab-Ag interfaces, lysine residues are key (Li, Y., *et al.*, 2003). I will refer to this method as "large mutated fragment mapping" or "mutated Ag mapping". Linear or conformational epitopes can be determined by "mutated large fragment mapping" and the resolution can be very high, although substantial effort is required to produce a large number of mutants. Quantitative measurement of the binding of mutated Ag fragments to Abs can show which residues contribute most to the binding energy. See examples [20], [21], [22], [35], [36], [37] and [39].

8. BINDING BY SMALL PEPTIDE FRAGMENTS OF THE AG

Linear epitopes are often found by synthesizing a series of overlapping peptides, each having part of the amino-acid sequence of the Ag and measuring which bind the Ab. Typically, the peptides are 15 to 20 AAs in length. There are reports in which every 15-mer in the Ag has been tested. Others move the window by 5 or more AAs so that 1-15, 6-20, 11-25, … are tested. "Small peptide mapping" usually cannot discover conformational epitopes. See examples [4], [23] and [39].

9. THE SEQUENCE OF PEPTIDES SELECTED FROM A LARGE NAÏVE LIBRARY OF PEPTIDES

There are numerous reports in which an Ab is used as the target for selection of linear or cyclic peptides using phage display; parts of the Ag sequence are identified with the selected peptide sequences. I call this method "peptide-selection mapping". Making a library of peptides that can all be found within the sequence of the Ag is more difficult than making an unrestricted library. Recently, Mumey and coworkers (Mumey, *et al.*, 2003) have reported determination of conformational epitopes using peptides selected from peptide libraries (displayed on phage) combined with computation. They first determine a consensus AA sequence from 25 to 100 selected phage

and then assign the AAs of the consensus to particular AAs in the Ag. They report that they can determine linear and conformational epitopes. Such predicted conformational epitopes have been confirmed by mutations to the Ag. From conformational epitopes, they can construct 3D models of proteins in a manner similar to the determination of small proteins structure through NMR *via* NOEs. See examples [7], [8], [9], [11], [12], [22] and [24].

10. DIFFERENCE IN BINDING WHEN GLYCOSYLATION IS ALTERED.

There are studies in which alteration of glycosylation of an Ag alters the binding of one or more mAbs, possibly indicating where the Abs bind. Sometimes removal of glycosylation prevents proteins from folding properly and destroys conformational epitopes even though the site of glycosylation is far from the epitope. The alteration of glycosylation could be wrought chemically, enzymatically, or genetically. Genetic alteration could be in the gene encoding the Ag or in the glycosylation enzymes of the organism that produces the Ag. I call this method "glycosylation mapping". See examples [25], [27], [28], [29], and [38].

11. DIFFERENCE IN BINDING WHEN THE PROTEIN IS REDUCED OR OTHERWISE ALTERED IN A SITE-SPECIFIC WAY

Ags that contain disulfides in some domains but not in others can be reduced, disrupting the disulfide-containing domains. If the Ab ceases to bind, the epitope is a) conformational, and b) in a disulfide-containing domain. This is called "reductive mapping". See example [30].

12. COMPETITION BETWEEN PEPTIDES AND AG FOR BINDING THE AB

An alternative to small peptide mapping is measuring the effect of small peptides in solution on the ability of the Ab to bind the Ag. This is called "peptide competition mapping". Unlike immobilized peptides, the concentration of the peptides can be controlled so that the strength of the interaction can be measured. Binding between Abs and Ags is often determined in an ELISA format in which one component is passively bound to a surface. Abs and other proteins are often denatured when bound to polystyrene (Butler, *et al.*, 1992). If the Ag is immobilized and the Ab and peptides are in solution, then the peptide is presented in as natural a state as possible. So long as a reproducible fraction of the Ag retains the ability to bind Ab the assay can detect which peptide can compete with the Ag. Thus, competition of soluble peptide with Ag for binding to Ab removes one area of uncertainty. See example [10].

13. DIFFERENCES IN SEQUENCE BETWEEN ORTHOLOGUES WHERE ONE BINDS AND ANOTHER DOES NOT

Comparison of AA sequences of orthologues, where one or more of the orthologues binds the Ab and others do not can reveal where the Ab binds. This is called

"orthologue mapping" and is closely related to mutated large fragment mapping. The difference being that one need not prepare the mutated large fragments if the orthologues are available. See examples [14] and [31].

14. THE STRUCTURE OF AN AG-AB COMPLEX BY NMR

Using NMR, it is possible to determine which AAs of the Ag are in contact with an Ab fragment in solution. In one mode, this is applicable to small Ags which can be synthesized with ^{15}N, ^{13}C, or ^{2}H and gives high definition of the AAs involved. An alternative use of NMR is saturation-transfer-difference (STD) NMR in which the transfer of NMR energy between Ab and Ag can show which atoms of the Ag are in close proximity to the Ab. In STD NMR, separating the proton resonances of the Ag from those of the Ab is important. Since sugars contain different chemical environments than found in proteins and peptides, this separation is easier for glyco groups than for peptidyl groups. This is a fortunate situation, since it is especially difficult to prepare precisely modified sugar groups. This is a large and complex field: http://bionmr-c1.unl.edu/991/Lectures/NMR-Protein-Ligand.ppt contains an interesting survey of the use of NMR to study protein-ligand interactions. These methods are called "NMR mapping". See examples [32] and [33].

15. THE INTERACTION OF AG AND AB IN A CRYSTAL STRUCTURE

Perhaps the ultimate definition of an epitope is the determination of the 3D structure of an Ag/Ab complex. Since Fabs are not glycosylated and contain all of the binding site, one usually uses Ag/Fab complexes. This method depends on obtaining high-quality crystals of the complex, an unpredictable circumstance. This is called "crystallographic mapping". See examples [15] and [16].

16. LOCALIZATION OF BINDING BY ELECTRON MICROSCOPY

Electron microscopy has been used to visualize where Abs can bind molecules such as collagen fragments or spore walls. Typically a secondary mAb is labeled with colloidal gold and is used to localize the primary mAb. This allows localization of the epitope with respect to larger structures and is called "electron microscopic mapping". See example [34].

Each approach considered above requires a certain amount of effort, involves a certain risk of failure, and gives a certain level of exactness in saying where an Ab binds an Ag. No single method can give a complete description of the binding of an Ab to an Ag. Although an X-ray structure of an Ab-Ag complex might show where all the contacts are and could be regarded as the ultimate description of the epitope and the paratope, it does not show which residues in the interface contribute most of the binding energy or specificity. Many studies of Ab binding use two or more of mapping methods, usually working from low resolution to higher. Below, I will discuss several papers so that each method is exemplified at least once. Each of these approaches can give information about where a particular Ab binds the Ag.

Uses of epitope mapping

Surprisingly, the uses to which epitope mapping has been put are almost as numerous as are the methods used. There are reports in which epitope mapping has been used to: **(**1) Determine the mechanism of a biological process, see examples [10], [9], [20], and [21]; **(**2) Identify an epitope of practical value, such as the binding site of a therapeutic Ab, see examples [1], [2], and [3]; (3) Connect a SNP or other polymorphism to protein structure or Ab binding, see example [35]; (4) Describe Ab binding in patents, see example [41]; (5) Evaluate Abs raised with a vaccine or design a vaccine, see examples [1], [2], [3], and [17]; (6) Understand autoimmune diseases, see examples [18] and [28]; (7) Qualify an Ab for IHC, diagnostic use, Western blot analysis, trans-species assays, distinguishing isoforms of Ag, allergen characterization, or therapeutic use, see example [36]; **(**8) Discover peptide mimic of Ag, see example [40]; (9) Determine structure of Ag, see example [9]; and (10) Discover an Ag that can differentiate between antibodies that arise from immunization and those that arise from infection, see example [36].

Examples with methods and uses

I have selected recent and very early papers that illustrate each of the methods and purposes of epitope mapping. The literature is enormous with 50,831 papers in PubMed having "epitop*"[1] in title or abstract; 1,677 have "epitope mapping" in title or abstract.

ANTIBODY-DEPENDENT ENHANCEMENT AND VACCINE DESIGN

Example [1]: Chou and colleagues (Chou, *et al.*, 2005) report an evaluation of antibodies raised by five mice in response to a chemically inactivated SARS virus preparation. Of the 12 mAbs which bind the denatured virus in ELISA, seven could be assignment mapped to recombinant S protein and five could not be mapped to any recombinant viral protein. The binding to S was refined by large-fragment mapping to show that five of the Abs bind to S1 (AA 12-798) and two bind to S2 (AA 797-1192). The non-virologist must note that an antibody that is neutralizing *in vitro* may not be protective *in vivo*. Indeed, some Abs that neutralize a virus *in vitro* cause Antibody-Dependent Enhancement (ADE) of viral disease *in vivo* or enhanced viral reproduction *in vitro*. Chou and colleagues (Chou, *et al.*, 2005) refer to Sui and coworkers (Sui, *et al.*, 2004) as having shown that the S protein is immunodominant and that the Abs that bind S protein are neutralizing. Sui and coworkers (Sui, *et al.*, 2004), however, reported only Abs which were selected from phage libraries *in vitro* and which neutralized interactions between cells *in vitro*. ADE is reviewed by Tirado and Yoon (Tirado and Yoon, 2003). They report that ADE is seen almost exclusively for enveloped viruses and exclusively via Abs that bind the outer proteins of viruses. ADE can lead to infection of macrophages and monocytes via the Fc receptors and other cell types through the complement receptor.

[1] "epitop*" represents "epitope", "epitopes", "epitopic", etc.; search done on 2006.08.15.

Example [2]: Hohdatsu and colleagues (Hohdatsu, *et al.*, 2003) studied a corona virus (Type II feline infectious peritonitis virus (FIPV)) related to SARS and found that vaccination with the N protein was protective, though not completely, while some antibodies to S caused ADE. The antibodies made in response to vaccination with N protein were not neutralizing and the protection is due to a cellular response. The N protein is sequestered within the membrane and is not available to bind Abs. Thus, Abs that bind N cannot mediate ADE infection. Abs to S are neutralizing in feline kidney cell-based *in vitro* assays. Given the similarity between SARS and FIPV, the predominance of Abs to the S protein in Chou's paper (Chou, *et al.*, 2005) is not encouraging (Marshall and Enserink, 2004).

Example [3]: Similar concerns arise with regard to influenza. De Filette and colleagues (De Filette, *et al.*, 2005) report that mice vaccinated with M2e (an epitope of 23 AAs which is highly conserved among human flu isolates) linked to hepatitis B virus core (HBc) particles respond strongly and are fully protected against challenge by influenza A. Since M2e hardly varies among human isolates, it is tempting to think that a vaccine that is protective against many strains may be possible. Zharikova and coworkers (Zharikova, *et al.*, 2005) showed that virus infecting SCID mice treated with a mAb against M2e produced a very limited range of escape mutants in response to the Ab pressure. Heinen and colleagues (Heinen, *et al.*, 2002) report, however, that pigs vaccinated with a fusion of M2e to HBc suffer severe, fatal ADE when challenged with swine flu. Thus, M2e-based vaccines may be worse than nothing.

COMPLEMENT-DEPENDENT-ADE AND FCR-DEPENDENT-ADE IN HIV-1

Example [4]: Fust (Fust, 1997) has reviewed ADE in HIV-1 and explores the mechanism by which an Ab can enhance the infectivity of HIV-1 *in vitro*. ADE in AIDS has been divided into complement-dependent ADE (C-ADE) and FcR-dependent ADE (FcR-ADE). C-ADE is mediated by Abs that bind an immunodominant epitope in gp41 while FcR-ADE is mediated by Abs that bind epitopes in the V3 loop of gp120. The C-ADE mAbs had been mapped to gp41 by finding large recombinant fragments of gp41 that bind the mAb (Robinson, *et al.*, 1990a). Synthetic peptides were also made to block the C-ADE activity of two mAbs (Robinson, *et al.*, 1990b). The peptide RILAVERYLKDQQLLGIWGCSGKLICTTAVPWNAS blocked the C-ADE effect while, WGCSGKLICTTAVPWNAS was ineffective (Robinson, *et al.*, 1990b). Trishmann and coworkers (Trischmann, *et al.*, 1995) report on *in vitro* enhancement of infection of macrophages via Fc gamma RIII and independently of CD4.

EPITOPES ON TORPEDO ACETYLCHOLINE RECEPTOR

Example [5]: Froehner and co-researchers (Froehner, *et al.*, 1983) made 14 murine mAbs to the acetylcholine receptor (AChR) of *Torpedo californica* in Balb/c mice by immunization with affinity purified AChR (both in native form and denatured) and fusion of spleen cells. They assignment mapped the epitopes of each mAb with respect to the four subunits (α, ß, γ, and δ) and topologically mapped each mAb with

respect to the membrane. Two bound the α subunit, 3 bound ß, 1 bound γ, and 5 bound δ. Two mAbs bound both γ and δ. They tested mAbs for binding to intact muscle cells and determined that 3 of the 14 could bind intact cells, indicating extracellular epitopes. Three mAbs could bind membrane preparation in which both surfaces were exposed and a further three could bind to cells permeabilized with lithium diiodosalicilate, these are assigned to binding cytoplasmic epitopes. (Many other permeabilizing agents are available now.) A further five mAbs could bind only when the proteins were solubilized from the membrane.

TOPOLOGY OF THE C-TRMINUS OF THE SODIUM HYDROGEN EXCHANGER.

Example [6]: Kahn (Khan, 2001) prepared polyclonal Abs (pAbs) to the C-terminal sequence of rat Na+/H+ exchanger isoform 1 (a membrane protein found in red-blood cells(RBC)) using a glutathione S-transferase fusion as Ag. RBC vesicles were resealed and the right-side-out vesicles (RSOV) were separated from the inside-out vesicles (ISOV). The pAbs to the C-terminal sequence bound much more to the RSOVs and to intact RBCs, suggesting that the C-terminus of the exchanger is extracellular.

STRUCTURE OF NEUTROPHIL CYTOCHROME *B*

Example [7]: Neutrophil cytochrome *b* (Cyt *b*) is an intrinsic membrane protein comprising two polypeptide chains, gp91^{phox} and p21^{phox}, which are not amenable to X-ray crystallography or NMR structure determination. The antibody 44.1 was first mapped to the intracellular portion of p21^{phox} by its inability to bind cells unless the cells had been permeabilized (Burritt, *et al.*, 1995). Ab 44.1 was labeled with CCB and FRET was measured between the hemes of Cyt *b* and 44.1-CCB. Burritt and colleagues (Burritt, *et al.*, 1995) selected peptides from phage display libraries for binding to 44.1. The consensus sequence is nearly identical to ^{181}GGPQVNPI^{188} of p21^{phox}.

Example [8]: Burritt *et al.* (Burritt, *et al.*, 1998) extended these results by showing in a colony-lift screen that colonies could be assigned to two groups: dark staining and light staining. Peptides in the light-staining groups matched the sequence of residues 181-188 of p21^{phox} at two to four positions. Peptides in the dark-staining group matched the sequence of residues 183-188 of p21^{phox} at several positions plus other residues that were seen to match p21^{phox} in the region 29-33. Burritt and colleagues (Burritt, *et al.*, 1998) also studied the interaction of selected peptides with Ab 44.1 by NMR and established a peptide conformation that gives a low-resolution model of the epitope. These analyses show that Ab 44.1 recognizes a complex epitope created by the juxtaposition of 29-36 with 183-188 and establishing some structural features of p21^{phox}.

Example [9]: Taylor and fellow researchers (Taylor, R. M., *et al.*, 2004) studied a panel of six antibodies that bind p22^{phox}, a component of flavocytochrome *b* (Cyt *b*) which is an integral membrane protein. Efforts to establish even the topology of the components of Cyt *b* have been unsuccessful to date. The Abs NS1, NS2, NS5, CS6,

CS8, and CS9 were raised in mice. None of the Abs bind intact cells but bind $p22^{phox}$ in Western analysis, suggesting that the epitopes are all intracellular. Each Ab was used to select peptides from a phage library. Five of the Abs produced a single consensus sequence. Four of these could be related to an AA sequence in $p22^{phox}$. Ab NS5 gave two families of sequences that were related to residues 78-83 and 51-57, a conformational epitope. CS6 and CS8 selected peptides highly similar to those selected by 44.1. Competition of these Abs and Ab 44.1-CCB was used for competition mapping. Two of the Abs (CS6 and CS8) blocked the binding of 44.1 indicating that the epitopes overlap, as indicated by the selected peptides. CS9 and NS1 could bind simultaneously with 44.1-CCB and FRET showed that their epitopes are near to the 44.1 epitope while NS5 binds further away. Cell-free assays show that CS9 can markedly inhibit the oxidase activity of Cyt *b*.

The assignments of epitopes for 44.1 and NS5 allow Taylor and colleagues (Taylor, R. M., *et al.*, 2004) to exclude a region from being a transmembrane segment and establishing a new model of the topology of $p22^{phox}$. Since the protein firmly resists other structural approaches this 3D imprinting with Abs may be the best that can be done.

MECHANISM OF $\alpha_v ß_6$ ACTIVITY

Example [10]: Weinreb *et al.* (Weinreb, *et al.*, 2004) have obtained a panel of 14 murine mAbs that bind human and mouse $\alpha_v ß_6$, a receptor for transforming growth factor-ß1 latency-associated peptide (TGFß1-LAP). Some of the Abs have RGD tri-peptide sequence in HC CDR3 and block the binding of $\alpha_v ß_6$ to TGFß1-LAP. Many ligands of $\alpha_v ß_6$ and other integrins contain RGD; it is common that antibodies share short sequences with the natural ligands of the Ag. Other Abs have RYD tri-peptide sequences in HC CDR3 and block the binding to TGFß1-LAP. The third class of Abs contained neither RGD nor RYD and do not block ligand binding. The 3D structure of an RGD-peptide binding a different integrin shows that the RGD-binding site is situated between the α and ß subunits; a similar arrangement is expected in $\alpha_v ß_6$. The RGD-containing Abs were internalized by cell that display $\alpha_v ß_6$ which led the authors to investigate a novel mechanism for the activation of TGFß1-LAP, *viz.* internalization and proteolysis in a vacuole. The mapping methods used included competition among Abs, between Abs and ligands, and between Abs and peptides. The major use cited is understanding the mechanism of $\alpha_v ß_6$ activity.

EPITOPES ON JUNCTIONAL ADHESION MOLECULE-1 RELATED TO FUNCTION

Example [11]: Junctional adhesion molecule-1 (JAM-1) is an IgG-superfamily transmembrane glycoprotein found on epithelial cells and is involved in the formation of tight junctions via intercellular calcium-dependent homodimerization. Liu and colleagues (Liu, *et al.*, 2000) raised murine IgG1 mAbs J3F.1, J4F.1 and J10.4 against a fusion of the extracellular domains of JAM-1 fused to alkaline phosphatase. Mandell *et al.* (Mandell, *et al.*, 2004) added antibody 1H2A9 and mapped the epitopes of J3F.1 and J10.4 which inhibit recovery of barrier function in epithelial monolayers after calcium depletion. The Fab of J10.4 was essentially as effective in blocking TJ

regeneration as was the IgG. By competitive Ab mapping, they show that J3F.1 and J10.4 cannot bind to JAM-1 simultaneously, suggesting they bind overlapping or identical epitopes while 1H2A9 binds a non-overlapping epitope.

Example [12]: Mandell *et al.* (Mandell, *et al.*, 2004) carried out peptide-selection mapping using AEX_9GP linear (LL9) and $AECX_{10}CGP$ cyclic (CL10) phage-display libraries. The sequences of selected peptides were similar but distinct and were used to identify the subsequence of JAM-1 (^{111}VSEEGGNSYGEVK^{123}) as contributing to the epitopes of J3F.1 and J10.4. For J10.4, they obtained from LL9 a peptide containing the subsequence GGNS which matches residues 115-118 of JAM-1. From CL10, they obtained several peptides containing GSN and related sequences. For J3F.1, they obtained binding peptides only from CL10 and these contained either GGN or GSN subsequences. To confirm this assignment, they synthesized three selected peptides (two constrained and one linear), one subsequence of JAM-1, and two negative control scrambled peptides. One of the constrained peptides blocked binding of J3F.1 to JAM-1 with an IC_{50} of 50 µM. The best conformation comes from mutations in JAM-1. If N117 is changed to lysine, J3F.1 and J10.4 cease to bind while 1H2A9 continues to bind, indicating that the mutant protein folds correctly. They show a previously published 3D structure of a dimer of JAM-1 and indeed N117 is located within the interface. The model would match well for two JAM-1 molecules attached to the same cell, but do not appear to match for two molecules coming from different cells unless there is considerable flexibility between D1 and D2. The peptide JAM-1 110-121 is not very effective in blocking the Abs, EC_{50} > 3 mM. J10.4 has an EC_{50} of 3 µg/mL or approximately 20 nM so it probably has reasonably high affinity for JAM-1. JAM-1-N117K does not concentrate at the cell-cell junctions as does the w.t. JAM-1.

INTERACTIONS BETWEEN MAB HYHEL-5 AND HEN EGG-WHITE LYSOZYME

The epitopes of hen egg-white lysozyme (HEL) have been studied extensively because the protein is easily accessible, the 3D structure is known, it is antigenic in mice, many similar sequences are available from related and distant species, and the enzymatic properties have been worked out.

Example [13]: Atassi and Lee (Atassi and Lee, 1978) synthesized short peptides that were intended to mimic the antigenic features of HEL. Their daring hypothesis was that HEL might have as few as three antigenic sites and that these could be simulated by small peptides. Had this worked, immunology would be much simpler. The peptides could block the binding of polyclonal Abs to HEL, but subsequent work showed that Ab-Ag interactions are much more complicated and that essentially every exposed part of a protein can be the epitope for some Ab. The lesson of this project is that designing mimics of conformational epitopes from 3D protein structure is very difficult, not that short peptides cannot represent discontinuous epitopes. Nevertheless, people have been slow to accept methods in which peptides are selected from very large libraries and do simulate discontinuous epitopes with short linear or cyclic peptides, see examples [8] and [9].

Example [14]: Smith-Gill and coworkers (Smith-Gill, *et al.*, 1982) mapped the epitope of mAb HyHEL-5 on hen egg-white lysozyme (HEL) in a three-fold approach. HyHEL-5 was shown to inhibit the activity of HEL, indicating that the Ab bound near the active site. HyHEL-5 blocks the binding of penta- and hexasaccharides can block HyHEL-5 binding while mono- and disaccharides cannot. Biebrich Scarlet, a dye that is know to bind in the F site blocks HyHEL-5 binding. Finally, comparison of the binding of 7 other lysozymes that differ by 4 to 27 amino acids showed extensive areas where mutations have no effect on HyHEL-5 binding although other mAbs are affected. Together, these methods indicated that the epitope comprises residues R45, T47, D48, and R68; R68 had been identified previously. None of the antigenic structures identified by Atassi and Lee corresponded to the epitope identified here.

Example [15]: Sheriff and colleagues (Sheriff, *et al.*, 1987) described the complex of Fab proteolytically derived from mAb HyHEL-5 with HEL determined with data to 2.5 Å and extensive refinement until the R factor was 0.245. Structures of Fabs with haptens had shown the haptens fitting into grooves and holes in the Fab and leaving a large fraction of the CDR residues unbound. Although R45 and R68 form a ridge on HEL that fills a groove in HyHEL-5 and forms salt bridges to HC residues E35(CDR1) and E50(CDR2), the interface of about 750 $Å^2$ between HEL and HyHEL-5 is predominantly flat. The Ab surface is highly complementary to the surface of lysozyme. The flatness of the interface is now known to be common in Ab-protein complexes. No water molecules were identified in the Ag-Ab interface of this model.

The HEL epitope for HyHEL-5 comprises three segments of AA sequence: a) Q41-Y53, b) N65-S71, and c) L84. The paratope of HyHEL-5 comprises residues from every CDR.

Example [16]: Using recombinant Fab, improved X-ray equipment and methods, and low temperature (95°K), Cohen and co-researchers (Cohen, *et al.*, 2005) re-examined the HyHEL-5 Fab-HEL structure with refined data to 1.7 Å (~3 times as many reflections as in Example [15]). The basic description of the interface was confirmed, but many details were improved. In particular, one can see two cavities in the interface that each contain two water molecules plus three isolated water molecules in the interface. In addition, there are seven localized waters that are solvent accessible. This example illustrates the quality of structure determination needed to define an epitope at atomic resolution. The practical value of such a determination remains to be demonstrated.

ANALYSIS OF THE EPITOPE OF 2F5 WHICH BINDS HIV-1 GP41.

Example [17]: Hager-Braun and Tomer (Hager-Braun and Tomer, 2004) studied the fine structure of a linear epitope found in HIV-1 gp41 and bound by mAb 2F5. An Fc-specific Ab was covalently coupled to cyanogen bromide-activated Sepharose beads and 2F5 was captured by the Ab on the beads. A recombinant gp41-containing fusion protein was incubated with the beads to capture the Ag. The complex was digested first with protease LysC and then with carboxypeptidase Y and then aminopeptidase M. The smallest fragment retained by the immobilized 2F5 was ana-

lyzed by MALDI/MS. Since the AA sequence of the Ag was known, one could pick an appropriate set of proteases that a) do not cleave the Abs, and b) cut the Ag into appropriate fragments. The epitope of 2F5 is linear, NEQELLELDKWAS being the smallest fragment observed. Since mAb 2F5 is neutralizing *in vitro*, the authors propose that knowing the linear epitope will aid in vaccine design. This would be true of other, less dynamic pathogens, but it is unclear how well this will work for HIV-1.

EPITOPES BOUND BY AUTOANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS

Example [18]: Satoh and colleagues (Satoh, *et al.*, 2004) studied the epitopes bound by autoantibodies in systemic lupus erythematosus (SLE). Western blotting is most often used with an antibody that is know to bind a linear epitope. A sample that may contain the protein is run out on an SDS gel and the antibody is applied to reveal if the target protein or fragments thereof are present. In SLE research, the process is reversed and a standard set of denatured proteins that are known Ags involved in SLE are run out on an SDS gel and a preparation of Abs from the patient is applied. Satoh et al. used immobilized Y2 mAbs that bind B/B' and D in the Sm core to capture Sm which was then used to capture U1-A and U1-C. In turn, native U1-A and U1-C were used to capture autoimmune Abs for patient sera. Satoh *et al*. have now shown that Western blotting misses a class of Abs that bind only to native Ags U1-A and U1-C. The epitopes of these Abs have been mapped to the extent that we know that they bind outside the interface between U1-A with the Sm core or between U1-C and the Sm core.

EPITOPE IN HIV P26 PROTECTED BY A MAB

Example [19]: Parker and Tomer used two mapping techniques which they call "epitope extraction" and "epitope excision". In epitope extraction, the Ag is first digested and the fragments are allowed to bind the immobilized Ab, a method that corresponds to large fragment mapping. In epitope excision, the Ag is bound to the Ab and the complex is digested and is one form of protection mapping. They mapped the epitope of a murine mAb named M26 on the Ag HIV p26. M26 is coupled to CNBr-activated Sepharose beads, p26 is bound, and the complex is digested overnight with lysyl endopeptidase (Lys-C). Fragments that do not bind the M26 are washed away. The sequence of p26 was known and it was predicted that Lys-C would not cleave p26 into too many small piece and would spare the M26. After the digestion, MALDI/MS of a small aliquot of the beads shows four species released from M26: L4, L4', L5, and L8. L4' is a fragment of L4. Fragments L1, L2, L3, L6, L7, L9-L12 have vanished. The beads were then digested for 2 h with trypsin-TCPK. After this digestion, L5 is lost, L8 is much reduced in intensity, and two fragments of L4', T5 and T5+T6 are seen. Two aliquots of the beads are then digested with either carboxypeptidase Y (CY) or aminopeptidase M (AM). CY gives a fragment of T5+T6 having the last three AAs removed which still allows retention on M26. In the sequence ^{105}HAGPIAPGQMR^{115}, G112 is either protected or its loss causes loss of the peptide from M26. AM digestion produces bound peptides that indicate loss of

^{89}ETINEEAAEWDRV^{101} from ^{89}ETINEEAAEWDRVHPVHAGPIAPGQMR^{115} still allows binding to M26, but the last peak is very weak. Thus, ^{102}HPVHAGPIAPG^{112} contains the minimum epitope. The process consumed 20-50 µg of M26. The authors do not say how much p26 was used, 50 µg would saturate this amount of M26. In a related electrophoretic study of M26 (Qian and Tomer, 1998), ^{102}HPVHAGPIAP^{111} was shown to bind slightly more tightly than does ^{102}HPVHAGPIAPG^{112} supporting the idea that G112 is protected from digestion.

MECHANISM OF FUNCTION OF ß$_1$ INTEGRIN

Example [20]: Luo et al. (Luo, *et al.*, 2004) mapped the epitopes of five mAbs, including SG/19, by making a series of cell lines in which human ß$_1$ integrin carried a series of point mutations. The mutations were at the sites where human and mouse ß$_1$ integrin differ. Each cell line was used in FACS to determine which cell lines supported Ab binding. SG/19 is a function blocking Ab and they determined that only the T82M mutation caused loss of binding. This localization of the binding site of SG/19 combined with structural models of ß$_1$ integrin allow the authors to infer the mechanism of action of SG/19.

BINDING OF COLLAGEN-RELATED PEPTIDE BY GPVI

Example [21]: Smethurst and coworkers. (Smethurst, *et al.*, 2004) studied the collagen-binding surface of glycoprotein (GP) VI. They expressed the Ig-like ectodomains (D1D2) of human and mouse GPVI on insect cells. They made four mutants having point human-to-mouse mutations and six human-to-Ala mutants. Both human and mouse D1D2 bind specifically to collagen-related peptide (CRP), but hD1D2 binds more tightly. The K59E (human-to-mouse) mutant greatly reduced the binding of CRP to hD1D2, indicating that K59 is a key residue in the ligand binding site of GPVI. Using a phage Ab library, they obtained clone 10B12 which blocks the binding of GPVI to CRP. 10B12 binds hD1D2(K59E) much less than it binds hD1D2, consistent with K59 being in the GPVI/CRP interface.

MECHANISM OF AB BLOCKAGE OF PAI-1 ACTION

Example [22]: Wind and colleagues (Wind, *et al.*, 2001) used phage-displayed peptides and selected alanine mutants (SAMs) of human plasminogen activator inhibitor-1 (PAI-1) to identify the epitopes of four previously identified murine mAbs: Mab2, Mab6, AD3780, and AD3783. PAI-1 is a serpin comprising 3 ß sheets and 9 α helices which inhibits tissue-type plasminogen activator (tPA) and urinary-type plasminogen activator (uPA). Mab2 blocks the ability of PAI-1 to inhibit uPA and tPA, especially in the presence of vitronectin; AD3783 weakly blocks the activity; and Mab6 and AD3780 have no effect. Mab2 was used as a target for selection of linear or disulfide-constrained heptapeptides. Four of the five peptides obtained showed similarity to the tripeptide ^{118}LFR^{120} of PAI-1. The phage-display approach using Mab6 gave no hits. The structure of PAI-1, however, shows the side group of F119 as buried and so unlikely to be a key part of the Mab2 epitope. Previous work

(Schousboe, *et al.*, 2000) had shown, however, that Mab2 binds residues between 121 and 156. Here only five peptides were isolated and they matched the Ag sequence at only three AAs and imperfectly at that. Where phage display yields a correct location of the epitope, many isolates give a match over four or more AAs. Identification of epitopes by phage display must be confirmed, for example, by testing mutated Ag.

From SAMs, the epitopes of Mab2, Mab6, AD3780, and AD3783 all involve helix F; AD3780 and AD3783 also interact with ß strands 1 and 2 of sheet A; Mab2 and Mab6 involve strand 3 of sheet A. The epitope of Mab2 indeed contains an F and an R, but not in that order. Rather, SAMs showed that R133, R135, and F136 are part of the Mab2 epitope. Based on the teaching of Jin and colleagues (Jin, *et al.*, 1992), the structure of PAI-1, and the previous mapping, Wind et al. (Wind, *et al.*, 2001) picked 14 residues between 102 and 156 for SAM. The functional epitopes identified for AD3780 and AD3783 are essentially identical, involving residues Q102, **R103**, Q125, D127, **E130, R133**, **F136, I137**, and D140 (bold for EC_{50} increases 100-fold, and underscore for EC_{50} increases 10-fold, plain for EC_{50} increases 3-fold). The functional epitopes of Mab2 and Mab6 are similar but distinct. Mab2 involves **R133, R135, F136,** N139, **D140**, L154, and **K156**, while Mab6 involves R133, R135, F136, N139, **D140**, K143, and **K156**. In the region containing these epitopes, human, rat, and mouse PAI-1 are 84% identical. If human, rat, or mouse PAI-1 are immobilized on plastic, Mab2, AD3780, and AD3783 bind well while Mab6 binds only human PAI-1. If the antibodies are immobilized, only human PAI-1 can be captured. The authors argue that immobilization of PAI-1 on plastic partially denatures the protein and allows greater flexibility of the epitopic region and lowered specificity.

Serpins act by first allowing the target protease to cleave a peptide bond in the reactive center loop and then, while the Ser of the protease is still attached to the serpin, undergoing a molecular rearrangement that prevents the protease from breaking the acyl bond. Mab2, it is argued, binds PAI-1 in such a way that the needed rearragement is significantly slowed so that PAI-1 becomes merely a substrate. For this case, mapping the epitope shows not only where the Ab binds but also how the Ab effects the rigidity of the Ag uncovering added insight into the mechanism of serpin activity.

EPITOPE OF RINDERPEST VIRUS NUCLEOCAPSID

Example [23]: Choi *et al.* (Choi, *et al.*, 2004) studied the linear B-cell epitopes on the carboxy terminus of the rinderpest virus (RPV) nucleocapsid protein (NP). First they determined that GST::$NP_{1\text{-}525}$ (the whole nucleocapsid), GST::$NP_{1\text{-}179}$, and GST::$NP_{414\text{-}496}$ all bind to hyperimmune anti-RPV sera, indicating that immunogenic epitopes are present in both the amino-terminal and carboxy terminal sequences. Although GST::$NP_{414\text{-}496}$ contains less RPV-NP sequence than does GST::$NP_{1\text{-}179}$, GST::$NP_{414\text{-}496}$ binds the hyperimmune sera more strongly, suggesting that the immunodominant sequences are found there. In addition, among sera of weakly responding animals, more showed binding to GST::$NP_{414\text{-}496}$ than to GST::$NP_{1\text{-}179}$ (5/8 vs. 1/8). Thus, they decided to map the epitopes in the 414-525 region. Synthetic peptides of length 10 to 19 and with overlaps of zero to 14 (shown in *Table 1*) were synthesized and immobilized on MaxiSorp ELISA plates. Hyperimmune bovine or

guinea pig sera were applied to the peptides and binding was detected with peroxidase-labeled species-specific anti-IgG and *ortho*-phenylenediamine. Four peptides, R440, R470, R473, and R508, showed significant binding and are shown bold in *Table 1*. Since R453 showed no binding, the antigenic epitope must reside in the sequence ^{440}VPQVRKETWASSR^{452}. Similarly, R470 and R473 show strong binding while R467 and R487 do not. Thus the second antigenic epitope is contained in ^{479}PEADTDPL^{486}. The third antigenic epitope is contained in ^{520}DRDLL^{524} which RPV NP shares with measles virus NP. Guinea pigs immunized with recombinant N protein reacted with the same four peptides, but with slightly different intensity. Another virus belonging to the same genus as RPV is peste-des-petite-ruminant virus (PPRV). Distinguishing RPV infection from PPRV had been difficult. The second site (^{479}PEADTDPL^{486}) reacts with all the RPV hyperimmune sera but not with any of the PPRV sera tested, apparently solving this problem.

Table 1. Synthetic peptides of Choi *et al*. (2004)

Name	Bounds	Sequence	Length	Overlap*
R415	415-428	tqvsflrtdqggei	14	-
R426	426-439	geiqhnaskkdear	14	3
R440	**440-457**	**vpqvrketwassrsdryk**	**18**	**0**
R453	452-462	sdrykedtdne	11	5
R457	457-469	kedtdnesvspsv	13	7
R467	467-477	psvktlidvdt	11	3
R470	**470-486**	**ktlidvdttpeadtdpl**	**17**	**7**
R473	**473-490**	**idvdttpeadtdplgskk**	**18**	**14**
R487	487-494	gskksaeal	10	4
R488	488-502	skksaeallklqtma	15	8
R501	501-519	masilegstlgndslrtyn	19	2
R508	**508-524**	**stlgndslrtyndrdll**	**17**	**13**

* Overlap with previous peptide, shown underscored.

CHARACTERIZATION OF ABS THAT BIND TRANSCOBALAMIN

Example [24]: Orning and coworkers (Orning, *et al.*, 2006) sought an antibody that distinguishes holo-transcobalamin (hTC) from apo-transcobalamin (aTC). hTC is the active form of vitamin B-12; shortage of hTC causes illness. Nevertheless, the currently used diagnostic test does not distinguish hTC from aTC. They used SPR to study the binding of nine murine mAbs with either the mAb immobilized and hTC or aTC flowed or hTC immobilized and the mAbs flowed. One mAb, 3C4, had 75 nM affinity for hTC and at least 100-fold less affinity for aTC. Since cobalamin does not compete with hTC for binding to 3C4, the epitope of 3C4 probably consists of parts of TC that are in the proper conformation only in hTC. They report that a second mAb, 3-11, binds hTC and aTC with sub nM affinity at a non-overlapping epitope. Thus, sandwich ELISA assays using 3C4 and 3-11 were demonstrated. Orning *et al.* also used peptide-selection mapping to establish where 3C4 binds hTC. From linear libraries, they obtained no hits, but a constrained (csX_9cs) library gave seven distinct

but similar sequences, three were seen ten times each. Phagemids displaying the 3C4-selected peptides were specific to 3C4 and did not bind other TC-specific mAbs, as measured by SPR. Synthetic peptides did not bind 3C4. None of the sequences could be aligned to a single stretch of the TC sequence, consistent with the epitope of 3C4 being conformation dependent. Fedosov et al. (Fedosov, *et al.*, 2005) constructed a 3D model of TC. From this model, they identified two structures that correspond to the sequences of the selected peptide sequences.

EPITOPES ON POLYSACCHARIDES

Example [25]: McFadden and Casadevall (McFadden and Casadevall, 2004) studied the epitopes on glucuronoxylomannan (GXM) which is the major component of the carbohydrate capsule of *Cryptococcus neoformans*. Since the tools of deletion, substitution, fragmentation, and crystallography routinely used on polypeptide Ags are not available, they genetically manipulated the enzymes that catalyze the synthesis of GXM. In mice, protective Abs to GXM almost always contain V_H7183, J_H2, V_κ5.1, and J_κ1 and bind only to GXM that has a high degree of O-acetylation. These Abs are termed "class II" Abs. One such Ab is named 2H1.

C. neoformans cells were UV irradiated and three strains were selected for loss of binding by 2H1 without complete loss of capsule. Each of the mutant strains produced a novel form of GXM that had very low levels of O-acetylation (as demonstrated by NMR and the Hestrin assay). *Cas1* encodes an enzyme that catalyzes O-acetylation of GXM. When the mutants were transformed with *CAS1*-containing DNA, the capsule regained O-acetyl groups and 2H1 binding. A different class II IgM mAb, 12A1, binds to w.t. and each of the mutant GXMs quite well yet differs from 2H1 by only a few amino acids. A class II IgG1, 18B7 arose by mutations to 12A1 yet binds the mutant GXMs less well than it binds w.t. GXM. Most of the class II mAbs have very little affinity for the mutant GXMs. Although all class II Abs come from the same germ line, their binding to mutant GXMs are affected differently showing that they bind different epitopes.

Example [26]: Vives and colleagues (Vives, *et al.*, 2004) studied the residues on the CC chemokine regulated on activation, normal T-cell expressed, and secreted (RANTES) which binds to heparin sulfate (HS). HS was adhered to beads, the carboxylic acid groups on HS were activated with EDC/NHS, and RANTES was allowed to bind. The activated acid groups react with amino groups in the protein. The protein is then partially degraded with a protease and the fragments analyzed. Although RANTES is not an Ab, the method could be used on Abs and any Ag that contains carboxylic acid groups or other activatable groups. The method is applicable to Ags that cannot be genetically manipulated, easily synthesized, or crystallized with Abs that have lysines in the combining site.

EPITOPES ON CD52 ON HUMAN SPERM

Example [27]: Hasegawa et al. (Hasegawa, *et al.*, 2003) studied the epitopes seen by mAbs H6-3C4, 1G12, and Campath-1 on CD52 on human sperm. Each of these Abs

are sperm immobilizing (SI-Abs) in a complement-dependent manner. Similar SI-Abs are found in the serum of some infertile women. Understanding the efficient epitopes for this binding could lead either to long-term contraception or to relief of unwanted immune infertility. Hasegawa et al. used two-dimensional PAGE and Western blot analysis to show that Campath-1 and 1G12 bound the core peptide of CD52 (GQNDT**SQTSSPS** (N carries N-linked glycosylation and the **S** and **T** carry O-linked glycosylation) when all the sugars are removed. CD52 extracted from sperm is heterogenously glycosylated at S6, T8, S9, S10, and S12; thus the partially deglycosylated CD52 gives rise to several Campath-1- and 1G12-binding species. H6-3C4 loses all binding when the N-linked sugar is removed with N glycosidase F. Polyclonal rabbit Abs raised to the unglycosylated peptide conjugated to diphtheria toxoid were able to bind the glycosylated CD52 and to immobilize sperm.

GLYCOSYLATION MAPPING OF EPITOPES ON P68

Example [28]: The glycoprotein autoantigen p68 is a target of autoantigens and autoreactive T cells in rheumatoid arthritis (RA). Blass *et al.* (Blass, *et al.*, 1998) characterized the reactivity of autoimmune Abs and rabbit polyclonal Abs raised in response to native glycosylated p68. The glycosylation mapping comprised: a) removal of CHO with NaOH (alkaline ß elimination), b) enzymatic deglycosylation, c) oxidation with periodate, d) blocking with lectins, and e) competition with N acetyl glucose amine (GlcNAc). In all cases, the RA patient-derived anti-p68 antibodies completely lost binding to p68 while the bind of rabbit polyclonals was unaffected. Thus, the autoepitope is glycosyl in nature and contains terminal GlcNAc groups, not previously reported in humans. The p68 used in the study comes from HeLa cells, not from RA patients. It is not clear why HeLa-derived p68 should have this unusual glycoform.

Example [29]: The need for care in glycosylation mapping is illustrated by an article by Toryer and colleagues (Troyer, *et al.*, 1995). They sought the epitope of murine mAb 7E11-C5.3 on prostate specific membrane antigen (PMSA), a membrane glycoprotein highly restricted to prostate tissues. Initial experiments with periodate destroyed the binding of 7E11-C5.3 to PSMA, suggesting that the epitope at least involved the carbohydrate. When subsequent experiments with $NaBH_4$, tunacamycin, and enzymatic deglycosylation failed to confirm this assignment, they mapped the epitope with peptide scanning and found the epitope resides in the N-terminal 6 residues. Tests with periodate oxidized these AAs in such a way the 7E11-C5.3 no longer binds. mAb 7E11-C5.3 linked to ^{111}In is the active ingredient of ProstaScint®, an approved imaging agent for prostate cancer.

EPITOPES OF *MACACA FASICICULARIS* AND *HOMO SAPIENS* CD38

Example [30]: Ferrero and coworkers (Ferrero, *et al.*, 2004) cloned the gene for CD38 from *Macaca fascicularis* and expressed the protein on NIH/3T3 cells (NIH/mac38 cells). Murine mAbs were made by immunizing mice with NIH/mac38 cells. The mAbs were tested for binding to NIH/mac38 cells by flow cytometry. Other

antibodies had been made to human CD38. Some of the antibodies were species specific and others cross reacted; human and *M. fascicularis* CD-38 are 92% identical and 94% similar. CD38 contains 12 cysteines in the extra-cellular domains and these form disulfides in the pattern 1-2, 3-7, 4-8, 5-6, 9-10, and 11-12. Surprisingly, all the species-specific mAbs were insensitive to reduction of the disulfides while all the cross-reactive mAbs lost all binding when the disulfides were reduced with DTT.

EPITOPES OF HUMAN PAPILLOMAVIRUS L1 CAPSID PROTEIN

Example [31]: Oclese and coworkers (Olcese, *et al.*, 2004) studied the epitopes of domains of the human papillomavirus (HPV) type 16 (the most common type) L1 capsid protein, a component of the recently approved vaccine against HPV infection. There are many strains of HPV and infection with one strain does not confer protection against other strains. Each strain of HPV has a distinct potential to cause genital warts or cervical cancer. Serological studies have shown that Abs that bind type-common epitopes are usually not neutralizing and are unlikely to be protective. Strain-specific epitopes have been shown to be more immunogenic that are strain-common epitopes.

Canine oral papilloma virus (COPV) is a member of the papilloma virus family. mAb 16A binds folded HPV16 L1 but not to folded COPV, HPV18, HPV11, or HPV6 L1 capsid nor to denatured HPV16 L1. mAb 16A is not neutralizing. To determine which parts of HPV16 L1 are the epitope of 16A, Oclese *et al.* made chimeras HPV16 and COPV: BB22 having COPV L1(1-169)::HPV L1(170-505) and BB23 having HPV L1(1-399)::COPV(400-505). A structural model of L1 suggests that the type-specific epitopes reside in five surface exposed loops that vary considerably from strain to strain. BB22 has the first two hypervariable of COPV and the three final hypervariable loops of HPV. BB23 has all the hypervariable loops of HPV. Both constructs form virus-like particles, indicating that they are well folded. mAb 16A binds BB23 but not BB22. In addition, they made a proteins called D12345, D1234, D123, D12, and D234 in which the indicated hypervariable loops of HPV L1 were inserted into COPV L1. D12345, D1234, D123, and D12 bind mAb 16A nearly as well as does folded HPV L1 while D234 does not bind 16A. This supports the hypothesis that the conformational epitope of mAb 16A of HPV L1 is in hypervariable loop 1.

MAPPING EPITOPES IN LIPOPOLYSACCHARIDE FROM LEGIONELLA

Example [32]: Kooistea and colleagues (Kooistra, *et al.*, 2002) studied a virulence-related epitope of *Legionella pneumophila* polysaccharide by surface plasmon resonance and saturation-transfer-difference (STD) NMR. *L. pneumophila* is a facultative intracellular Gram negative pathogen that causes legionellosis. The outer surface contains a polymer of highly modified lipopolysaccharide (LPS). It has been observed that *L. pneumophila* of serogroup 1 undergo a phase change that relates to virulence and that the phase shift involves loss or gain of a 29 kilobase unstable genetic element. mAb 2625 binds the PS of the virulent RC1 strain but not to the PS of the avirulent 811 strain or the strain 5215 (constructed by deletion of Orf 8-12).

Since it has not been possible to depolymerize the OPS of *L. pneumophila*, Kooistra *et al.* (2002) have studied the interaction between RC1 PS and mAb 2625 to determine the molecular nature of the virulence factor. They showed that mAb 2625 binds to the OPS that contains specific N-methyl groups. This Ag is not amenable to crystallographic study and demonstrating contact between the mAb and the specific N-methyl groups could not be accomplished by other methods.

A MIMETIC OF ONE EPITOPE OF HUMAN CHORIONIC GONADOTROPIN HORMONE

Example [33]: Ferrat and co-researchers (Ferrat, *et al.*, 2002) used the X-ray structure of a ternary complex between human chorionic gonadotropin hormone (hCG) and two F_vs recognizing its α and ß subunits and pepscan synthesis to obtain a peptide mimetic, "PepH14", of the epitope recognized by one of the F_vs (Fv 3299). The peptide sequences are shown in *Table 2*. Pepscan introduced 9 changes, but two of these could be reverted to wild-type. Using an ^{15}N-containing version, they studied the structure of PepH14 in solution and found, surprisingly, that it has a well-defined structure very similar to that seen in the F_v-hCG-F_v ternary complex. A V_{HH} antibody from llama that binds the α chain of hCG, "V_{HH}-H14", was obtained. The x-ray (Spinelli, *et al.*, 1996) and NMR solution (Renisio, *et al.*, 2002) structures of free V_{HH}-H14 have been reported. Because V_{HH}-H14 is only 117 amino acids, the interactions between V_{HH}-H14 and PepH14 could be studied using heteronuclear (^1H - ^{15}N) single-quantum correlation (HSQC) spectrometry. From the NMR data, they built a model of the V_{HH}-H14-PepH14 complex. There are significant interactions between PepH14 and the CDRs, as expected. The interaction surface between framework and PepH14 was larger than surface between CDRs and PepH14. V_{HH}-H14 has only modest affinity (300 nM) for PepH14. The HSQC spectra were obtained with [V_{HH}-H14] = 300 µM and [PepH14] as high as 1.5 mM. This led to some non-specific binding of the peptide to V_{HH}-H14. Nevertheless, the model is reasonable and could be extended by other studies, such as the effects of mutations in the V_{HH} or the peptide.

Table 2. Peptide sequences from Ferrat *et al.* (2002)

Name	Sequence	Δ1	Δ2	Δ3
1. A-hCG	T12-LQENPFFSQPGAPILQC29	-	9	7
2. pepscan	DWINPFTMQPYAIVLQV	9	-	3
3. PepH14	DQINPFTMQPGAIVLQK	7	3	-

MABS TO *ENTEROCYTOZOON BIENEUSI* SPORES

Example [34]: Zhang and colleagues (Zhang, *et al.*, 2005) produced and characterized murine mAbs against *Enterocytozoon bieneusi* spores purified from rhesus macaques. Although *E. bieneusi* is clinically the most significant microsporidian parasite associated with persistent diarrhea and wasting in individuals with AIDS, there are no mAbs that are commercially available, partly because *E. bieneusi* cannot

be cultured. *E. bieneusi* spores were purified from macaque feces by density sedimentation and used to immunize mice. Twenty mAbs that were positive on human- and macaque-derived *E. bieneusi* were obtained; five were IgGs. mAb 8E2 is IgG1, mAbs 7G2 and 7H2 are IgG2a, and mAb 12G8 is IgG2b. Competition ELISA on disrupted spores showed that 8E2 recognized an epitope distinct from the other mAbs. 7G2, 7H2, and 12G8 all bind the same 40 KDa protein but only partially inhibit each others binding. Immune electron microscopy showed that all four mAbs bind the spore walls.

SINGLE NUCLEOTIDE POLYMORPHISMS IN ß3 INTEGRIN

Example [35]: Watkins and colleagues (Watkins, *et al.*, 2002) studied single nucleotide polymorphisms (SNPs) in ß3 integrin. It was known that most humans have Leu^{33} in ß3 but that Pro^{33} occurs through a SNP. With Ab CAMTRAN-007 which is specific for Leu^{33}, they phenotyped 6000 donors and found one that reacted only weakly with CAMTRAN-007. The donor was genotyped and was found to be heterozygous at codon 33 and also carried a novel SNP that introduces the mutation R93Q. Antibodies specific to αIIb/ß3 bound normally. This indicates that residues 33 and 93 are near in space and contribute to the epitope recognized by CAMTRAN-007 but not to the epitopes recognized by αIIb/ß3-binding mAbs.

AG TO DISTINGUISH ILLNESS FROM IMMUNIZATION

Example [36]: Seco-Mediavilla and coworkers (Seco-Mediavilla, *et al.*, 2003) studied the epitopes of *Brucella melitensis* periplasmic protein BP26. *B. melitensis* causes brucellosis. When animals are vaccinated with attenuated strains of *B. melitensis*, they produce antibodies that confuse immunodiagnostics that involve the proteins encoded by the vaccine strain. It was found that a vaccine strain lacking *bp26* gave protection in mice. Full-length BP26 could be made in E. coli, but the serum of *Brucella*-free sheep gave false positive reactions with full-length BP26. Seco-Mediavilla *et al.* (Seco-Mediavilla, *et al.*, 2003)sequenced *bp26* in four *Brucella* species and found them to be essentially identical (contrary to some literature). A panel of 18 murine mAbs to BP26 was obtained. The immunodominant epitopes were determined by measuring the binding of the mAbs, Brucella-free sera, and Brucella-infected sera to 12 amino-terminal truncation mutants, one carboxy-terminal truncation mutant, and one mutant truncated at both ends. The double truncated protein that comprised BP26(55-152) did bind to five of the mAbs, but BP26(55-152) bound to all the *Brucella*-infected sera and to none of the *Brucella*-free sera.

INTERACTIONS BETWEEN MHC I MOLECULES AND LY-49G

Example [37]: Osman et al. (Osman, *et al.*, 2005) studied the interactions between Ly-49G2 (an inhibitory receptor in murine natural killer (NK) cells) and MHC I by mutated Ag mapping. MAbs CK-1 and Cwy-3 were obtained from hybridomas from BALB/c mice immunized with IL-2 activated C57BL/6 murine spleen cells. CK-1 can block the interaction between Ly-49G2 of BL/6 mice and MHC-I while it does

not bind Ly-49G2 of BALB/c mice. Cwy-3 binds Ly-49G2$^{BL/6}$ but not to Ly-49G2$^{BALB/c}$. Cwy-3 does not inhibit the interaction of Ly-49G2 with MHC I. The extracellular domains of Ly-49G2 BALB/c and BL/6 differ at only 4 positions. Ly-49G2$^{BALB/c}$ was mutated at each of these sites and expressed in COS-7 cells; binding was measured by fluorescent activated cell sorting (FACS). The mutation D159N allows Cwy-3 to bind Ly-49G2$^{BALB/c}$ but does not change the binding of CK-1. The mutation G251D allows CK-1 to bind Ly-49G2$^{BALB/c}$ but does not affect Cwy-3 binding. They also showed that CK-1 and Cwy-3 can bind Ly-49G2$^{BL/6}$ at the same time. They fused the ECD of Ly-49G2$^{BL/6}$ to the transmembrane and intracellular domain of Ly-49W (an activator of NK cells). CK-1 could block activation of cells transfected with this fusion, showing that the epitope of CK-1 is a key site of binding to MHC I. From a model of Ly-49A, they deduced that D251 is in the loop between ß strand 4 and ß strand 5.

DISTINCTIVE EPITOPE ON HIV-1 GP120 RECOGNIZED BY 2G12

Example [38]: Trkola and colleagues (Trkola, *et al.*, 1996) studied the mAb 2G12 obtained from a human HIV-1 patient. 2G12 does not block the binding of gp120/IIIB to CD4. mAb 2G12 is unusual in that its epitope is not linear; 2G12 does not bind reduced and denatured gp120. The epitope is not, however, classically conformational in that it survives boiling gp120 in 6 M urea. The epitope is also destroyed by enzymatic deglycosidation of gp120 or genetic alterations that abolish the N-linked glycosylation at AAs 295, 332, 339, 386, 392, 397, or 448.

EPITOPES ON CD20

Example [39]: Teeling and colleagues (Teeling, *et al.*, 2006) mapped the epitopes of a panel of human mAbs, murine mAbs, and rituximab (the active ingredient of Rituxan®) against human CD20 using mutagenesis and overlapping synthetic 15-mer peptides. The human mAbs 2F2 and 7D8 were obtained by immunizing mice having human immunoglobulin genes with mouse NS/0 cells that had been transfected with a human CD20 gene (Teeling, *et al.*, 2004). CD20 is an integral membrane protein that cross the bilayer four times giving two extracellular loops. The epitopes of these mAbs were mapped in several ways: 1) peptides of the form $CX_{15}C$ (X is a sequence taken from CD20) were coupled to dibromoxylene and used in ELISA, 2) peptides of the form XA_7-G-XB_7 (XA and XB are two heptapeptides taken from different parts of CD20, 3) 17-mer peptides having sequences that are part human and part mouse were synthesized and used in ELISA, and 4) mutant CD20 molecules were expressed in HEK293F cells. The murine mAbs and rituximab all required A_{170} and P_{172} which are found in the larger, second extracellular loop of CD20. Surprisingly, the human mAbs bind to a different epitope comprising the first, very small extracellular loop and residues (N163 and N166) in the second, large loop. The human mAbs have much slower off rates than does rituximab and cause complement-dependent cytotoxicity (CDC) to a greater extent. To dissect these effects, they engineered a human mAb, 2C6, that had an off rate comparable to rituximab but that has an epitope similar to 2F2 and 7D8. Suprisingly, 2C6 has CDC superior

to rituximab and similar to 2F2 and 7D8 which have very slow off rates. Thus the epitope is more important than the kinetic properties.

USING THE EPITOPE OF A THERAPEUTIC AB AS A CANCER VACCINE

Example [40]: Li *et al.* (Li, M., *et al.*, 2006) used linear 12-mer peptide phage display to determine an amino-acid sequence that binds to rituximab. Seven distinct sequences were identified and all bound rituximab when displayed on phage. Three of the mimotopes were tested for the ability to block binding of rituximab to Raji cells; one worked. Two of the peptides were synthesized and conjugated to keyhole limpet hemocyanin (KLH). Both peptide conjugates were immunogenic in mice. Sera from vaccinated mice bound to Raji cells ($CD20^+$) but not to Jurkat cells ($CD20^-$). Li *et al.* argue that such a treatment may be far cheaper than repeated dosing with purified mAbs.

CONFORMATIONAL EPITOPES OF HEPATITIS C VIRUS

Example [41]: On 15 August 2006, Foung *et al.* were awarded US patent 7,091,324 entitled "Prevention and treatment of HCV infection employing antibodies directed against conformational epitopes". They identified conformational epitopes in the E2 envelope protein of hepatitis C virus (HCV) using a series of deletion mutants of E2. One set of Abs bind a conformational epitope in the 411-644 region and inhibit the binding of E2 to CD81, the receptor for HCV. A second group binds a conformational epitope in the 470-644 region and inhibit E2-CD81 binding. A third set bind in the 470-644 region but fail to block E2-CD81 binding.

Their first claim reads,

> "1. An isolated antibody that binds to a conformational epitope of a Hepatitis C virus E2 protein, wherein the epitope is found in Hepatitis C virus of more than one genotype, and wherein the antibody is selected from the group consisting of CBH-2, CBH-4G, CBH-5, CBH-7, CBH-8C, and CBH-11, or binds to the same conformational epitope as that bound by an antibody selected from the group consisting of CBH-2, CBH-4G, CBH-5, CBH-7, CBH-8C, and CBH-11."

The aim of this claim is to cover all future Abs that bind to the epitopes defined by the binding of the named mAbs. This is an important consideration in the biotechnology industry. Ab are so versatile that one can find new mAbs to bind an epitope but with no apparent sequence similarity to the epitope-defining mAb. Thus having a claim to a particular epitope gives broader protection than claiming only the amino-acid sequence of one or more Abs.

Concluding remarks

Epitope mapping is a very widely practiced art. Without appropriate details, the phrase "epitope mapping" does not accurately describe what has been done. The

range of tools available today makes determination of where an Ab binds accessible to almost anyone working with Abs. The tools are now well understood as to how much effort is needed and what level of detail can be obtained. The majority of recent papers use two or more methods to first find the epitope roughly and then refine the site.

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