Peptides as antigens

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The advent of the solid phase peptide synthesis (SPPS) method as pioneered by Bruce Merrifield (1) made peptide synthesis relatively easier; especially when automated SPPS became available. A significant corollary development is the growing convenience of making antibodies towards whole proteins, yet using short synthetic peptides as antigens. Thousands of antibodies have been raised using this technique and more are on the way.

This article presents a brief introduction to researchers who may be considering custom antibody production from synthetic peptides. It is not meant to be exhaustive in its scope. For this, an excellent review can be found in Ed Harlow and David Lane's "Antibodies, A Laboratory Manual" (2).

ADVANTAGES

Raising antibodies from synthetic peptides is in general quicker and simpler than using a (recombinant) protein. Once the sequence is available, the antibody can be raised immediately according to the predicted amino acid sequence. It also gives the flexibility to target a certain region of a protein, resulting in specific epitope recognition (2). One disadvantage is the possibility that it may not recognize the native protein (2).

HOW TO CHOOSE A SEQUENCE

In order to raise good antibodies from peptides, one has to choose a sequence that shows good antigenicity (for obvious reason), hydrophilicity (for ease of peptide synthesis, specifically peptide purification) and surface exposure (for antibody recognition and accessibility). Different computer programs, such as MacVector (Cary, NC), are available to assist one in choosing an antigenic peptide sequence based on these criteria. The MacVector program can also provide secondary structure predictions. Generally, one epitope consists of 5-7 amino acids; and a 10-15 residue long peptide is adequate to raise good antibodies.

Here we recommend the following criteria for choosing peptide antigens:

- Some sequences to avoid include sequences with a stretch of consecutive hydrophobic amino acids such as Leu (L), Ala (A), Gly (G), Ile (I), Val (V), Phe (F), Trp (W) and Met (M). The number of hydrophobic amino acids should preferably not exceed 50% of the sequence, with 25% or less being ideal.
- Acidic peptides containing Cys will pose difficulty in purification, since the use of a basic solvent in purification will cause cysteines to dimerize.
- Multiple cysteines-containing sequences have to be avoided as well, especially when the conjugation strategy is to use a single thiol group of the Cys for conjugation to carriers. One solution is to use Acm (Acetamidomethyl)-protected Cys for the internal cysteines that are not going to be used for conjugation.
- N-Terminal glutamine (Gln) tends to cyclize to pyroglutamic acid (Pyr), substituting Gln (Q) with Pyr, removing Gln from the N-terminus, adding another amino acid at the N-terminus or acetylating the N-terminus are some of the viable alternatives.
- Because Met is prone to oxidize to methionine sulfoxide [Met(O)],

norleucine (Nle) may be used instead. After a sequence is selected, it is always a good idea to check if the sequence is unique for the protein of interest, i.e. it is not found in another protein. A website such as http://www.ncbi.nlm.nih.gov/blast/ is useful for this purpose.

CONJUGATION TO CARRIERS PROTEINS

With the right conjugation strategies, a sequence with good antigenic index will induce a good immune response. Many peptides contain B-cell epitope, but not T-cell epitope, and may therefore not be immunogenic. Consequently, a peptide conjugated to carriers such as keyhole limpet hemocyanin (KLH) is commonly used in order to elicit an immune response. BSA can also be used as a carrier. However BSA is also a good immunogen (2) and since it has high nonspecific binding, it can be put to better use as a capture antigen when determining antibody titer in ELISA. For example, when using a KLH-conjugated peptide as an immunizing peptide, one can screen the antibodies raised using the same peptide conjugated to BSA. This way, the antibody titer obtained is from the immunizing peptide and not from KLH. Other carriers used include Ovalbumin and RSA (rabbit serum albumin).

The most common linker used for conjugating carrier proteins to peptides is sulfo-SMCC (Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate). Sulfo-SMCC links the carrier protein to the peptide by forming a bond between the maleimide group and the peptide Cys group. Cys is usually introduced on either the N- or the C-terminus. If a sequence is derived from the N-terminus, then Cys goes on the C-terminus, and vice-versa. If the peptide sequence is derived from an internal sequence, Cys can go on either end. For sequences with multiple cysteines, all except one Cys (towards the N or C terminus preferably) must be protected (usually with Acm). Other conjugation methods include the use of EDC (for conjugation to free carboxyl groups) and glutaraldehyde (for conjugation to free amino groups).

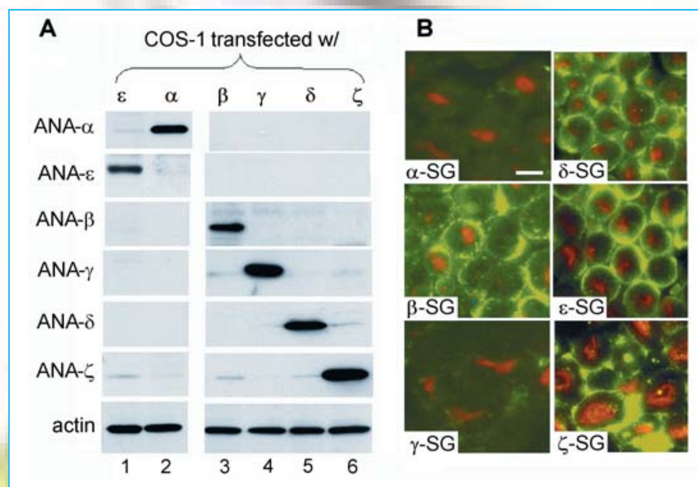
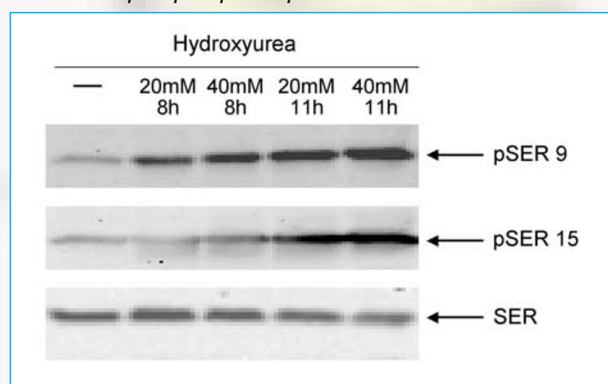


Figure 1 – Specificity of anti-sarcoglycan antibodies (Panel A). COS-1 cells were transfected with ε-SG (lane 1), α-SG (lane 2), β-SG (lane 3), γ-SG (lane 4), δ-SG (lane 5), and ζ-SG (lane 6). Total cell lysate (20 µg) was analyzed by Western blots using antibodies (from AnaSpec) against α-SG (ANA-α), ε-SG (ANA-ε), β-SG (ANA-β), γ-SG (ANA-γ), δ-SG (ANA-δ), and ζ-SG (ANA-ζ). Note that the ANA-ζ and ANA-ε antibodies recognized the corresponding recombinant sarcoglycans and did not cross-react with other sarcoglycans. Actin was used as a loading control. Localization of sarcoglycans in peripheral nerve (Panel B). Cryosections of adult rat sciatic nerves were stained with sarcoglycan antibodies (green) [δ-SG, ε-SG, ζ-SG polyclonal antibodies from AnaSpec, α-SG, β-SG, γ-SG monoclonal antibodies from Novocastra]. Sections were also co-stained with anti-neurofilament antibody (NF-H) to reveal neurofilaments in the axons (red). Bar 12 mm.
Courtesy of Dr. Yiu-mo Michael Chan, Geisinger Clinic, Danville, PA

Figure 2 – Western blots of hydroxyurea-treated COS-7 cells showing an increasing amount of phosphorylated p53 at phosphoryated SER 9 and SER 15, while no signal increase is seen in non-phosphospecific p53



POLYCLONAL AND MONOCLONAL ANTIBODIES

Peptides can be used for raising both polyclonal and monoclonal antibodies. Since these antigens can possess more than one epitope, a variety of B-lymphocyte clones can be induced from the immunized animal, resulting in an antiserum consisting of antibodies that are heterogeneous. These antibodies react with different determinants of the antigen and are called polyclonal antibodies. Monoclonal antibodies, on the other hand, are derived from a single clone of B-lymphocyte and are thus specific for a single epitope. With the fusion of an activated, antibody-producing B cell with a myeloma cell, a hybrid cell called a hybridoma is generated which possesses immortal-growth properties, antibody secretion capabilities and identical antigen recognition specificities.

For polyclonal antibodies, the inclusion of an affinity purification step generally eliminates a lot of background signal resulting in antibodies that are of better sensitivity and specificity.

Applications

Antibodies are used in different applications such as western blots, immunoprecipitations, immunohistochemistry and more recently in flow cytometry. Below are some examples of how different antibodies were used in research studies.

Western Blots and IHCs of Sarcoglycan Subunits Antibodies

In a paper (3) reported by Dr. Yiumo Michael Chan, a staff scientist at the Geisinger Clinic, antibodies each specific to the different subunits of the sarcoglycan (SG) protein were raised. "Some of the SG subunits share significant sequence homology." AnaSpec, the custom antibody provider "did a good job in selecting the right sequences for generating peptides." To confirm specificities of the antibodies, he performed an experiment where COS-1 cells were transfected with different SG subunits (α, ε, β, γ, δ and ζ). Total cell lysate (20 µg) was then analyzed by Western blots using antibodies raised

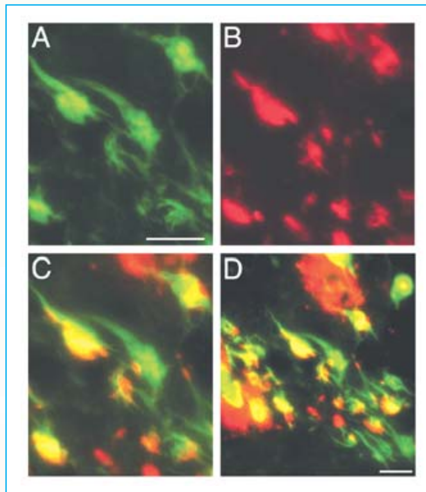


Figure 3 – Coexpression of A^{β} and tau in neurofibrillary tangles.
A–C: Tau [(A0024 labeled with Hilyte Fluor™ 488-labeled goat anti-rabbit IgG (H+L)] exhibiting a green fluorescence (A) and A^{β} 4G8 labeled with Hilyte Fluor™ 555-labeled goat anti-mouse IgG (H+L)] showing red fluorescence (B) are coexpressed in tangle bearing neurons of the entorhinal cortex in a case of AD as shown by the yellow fluorescence in the merged image (C).
D: A lower-power merged image showing a mixture of tangled neurons coexpressing A^{β} and tau (yellow color) and tau only (green color) as well as extracellular A^{β} deposits (red color).
Scale bars: 50 µm in A and 100 µm in D.
Courtesy of Dr. Patrick L. McGeer, Kinsmen Laboratory of Neurological Research, University of British Columbia, Vancouver, Canada

against each of the different subunits (Figure 1, panel A). Immunohistochemistry (IHC) results are shown in panel B of Figure 1 (western blot and IHC data courtesy of Dr. Yiu-mo Michael Chan).

Western Blots of p53 Phosphospecific Antibodies

In normal, undamaged cells, p53, an important mammalian cell cycle checkpoint protein, is rapidly degraded; but when cells are treated with DNA damage-inducing agents, there is a transient accumulation of this tumor suppressor protein and it is activated as a transcription factor. In several types of human cancers, p53 is mutated (4,5). Phosphorylation at serines 6, 9, 15, 20, 33, 37 occurs after cells are exposed either to ionizing radiation or to UV light (6,7). Serines 6 and 15 were demonstrated to be among the strongest and earliest phosphorylated sites in response to DNA damage-induced posttranslational modifications (8,9). As shown in western blots (Figure 2), Cos-7 cells treated with hydroxyurea, a known inhibitor of DNA synthesis, express an increasing amount of phosphorylated serine p53's (pSER 9 and 15), while no signal increase is seen in non-phosphospecific p53.

Since reversible protein phosphorylation/dephosphorylation has been shown to have a principal role in the regulation of essentially all cellular functions and most aspects of cell life, more and more researchers are looking into raising antibodies specific for the phosphorylated version of the proteins and not just the non-phosphorylated protein. AnaSpec, a leader in the production of kinase and phosphatase peptide substrates also offers custom production of phosphospecific antibodies. Our experience has shown that the phosphopeptide immunogen should at least be 90% pure in order to raise good antibodies.

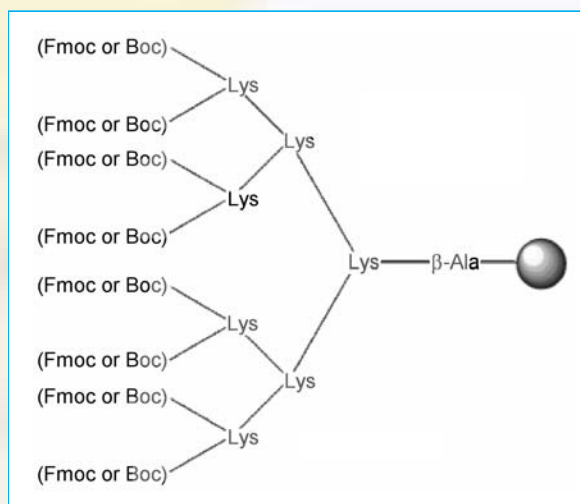
Immunohistochemistry, IHC

An example of antibodies used in indirect IHC is shown in Figure 3 in which secondary antibodies labeled with HiLyte Fluor™ 488 and HiLyte Fluor™ 555 were used in visualizing the co-localization of beta-amyloid and tau (10). For direct IHC, different antibodies labeling kits, such as the AnaTag™ HiLyte Fluor™ 488 Protein Labeling Kit, can be used for directly labeling primary antibodies and using them not only for IHC, but also for flow cytometry.

Multiple Antigenic Peptide System, MAPS

Multiple antigenic peptide system (MAPS) has been used successfully in producing high-titer anti-peptide antibodies (11,12), as well as synthetic peptide vaccines (13). This kind of peptide antigen consists of several copies of the same peptide sequence attached to an inert lysine core (Figure 4). Tetrameric or octameric constructs synthesized from 4-branched or 8-branched MAPS are the most commonly used. The molecular weights of MAPS peptides are difficult to be detected by mass spectrometry as is routinely done for linear peptides. It can only be characterized by amino acid analysis. HPLC profile of MAPS peptides exhibit broad peaks. Since MAPS constructs are big molecules, conjugations to carrier proteins are not necessary. A recent paper by Fukuda *et al.* (14) describes the use of an 8-branched MAPS peptide consisting of 4 amino acids (GWRQ) which was used in raising polyclonal antibodies.

Figure 4 – Structure of an 8-branched MAPS



CONCLUSION

Raising successful antibodies starts with choosing an appropriate peptide sequence, having the peptide synthesized correctly, choosing the right host, getting a reasonable antibody titer and lastly, getting the antibodies to work in the desired applications.

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