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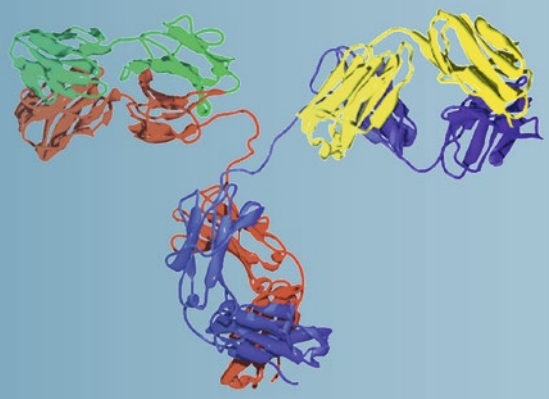

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Peptide Antibodies

Methods and Protocols

Humana Press

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Peptide Antibodies

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Preface

The ability to design and produce peptide antibodies has had a large impact on molecular cell biology and immunology, including all the different techniques involved (immunoassays, immunoprecipitation, immunoblotting, immunohistochemistry, etc).

This large impact is a result of several technical and scientific advances: solid phase peptide synthesis, peptide carrier conjugation and immunization, genomics, transcriptomics, proteomics and elucidation of the molecular basis of antigen presentation and recognition by dendritic cells, macrophages, B cells, and T cells.

Moreover, although peptide antibodies have been available for many years, they continue to be a field of active research and method development. For example, peptide antibodies which are dependent on specific posttranslational modifications are of great interest (phosphorylation, citrullination, etc.) and different forms of recombinant peptide antibodies are gaining interest (nanobodies, single chain antibodies, TCR-like antibodies, etc.).

This volume covers basic and advanced aspects of peptide antibody production, characterization, and uses.

I thank all contributors and editorial staff for their work, especially the series editor, John Walker. Also, I want to thank all my collaborators and students throughout the years.

Copenhagen, Denmark *Gunnar Houen*

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Chapter 1

Peptide Antibodies: Past, Present, and Future

Gunnar Houen

Abstract

Peptide antibodies recognize epitopes with amino acid residues adjacent in sequence ("linear" epitopes). Such antibodies can be made to virtually any sequence and have been immensely important in all areas of molecular biology and diagnostics due to their versatility and to the rapid growth in protein sequence information. Today, peptide antibodies can be routinely and rapidly made to large numbers of peptides, including peptides with posttranslationally modified residues, and are used for immunoblotting, immunocytochemistry, immunohistochemistry, and immunoassays. In the future, peptide antibodies will continue to be immensely important for molecular biology, TCR- and MHC-like peptide antibodies may be produced routinely, peptide antibodies with predetermined conformational specificities may be designed, and peptide-based vaccines may become part of vaccination programs.

Key words Peptides, Antibodies, Epitopes, Three-dimensional, Linear, Continuous, Contact residues, Recombinant, Single chain, TCR, MHC

1 The Development of Peptide Antibodies

Early work on protein structure and epitopes for antibodies (Abs) revealed that most epitopes were three dimensional (*see* **Notes 1** and **2**) and that a small percentage of Abs reacted with linear (continuous epitopes) [1–7]. Peptide Abs (Fig. 1) were described in 1980 and the use of synthetic peptides (coupled to a carrier protein) to induce specific Abs was developed in the following decades together with methods for epitope mapping and a general understanding of immunogenicity and antigenicity (Tables 1 and 2) (*see* **Notes 3–5**). This development was facilitated by the introduction of solid-phase peptide synthesis [41–43], the understanding of immunological T cell help for efficient stimulation of B cells to produce Abs [44–48], and the rapid growth in DNA and protein sequence information (Table 2).

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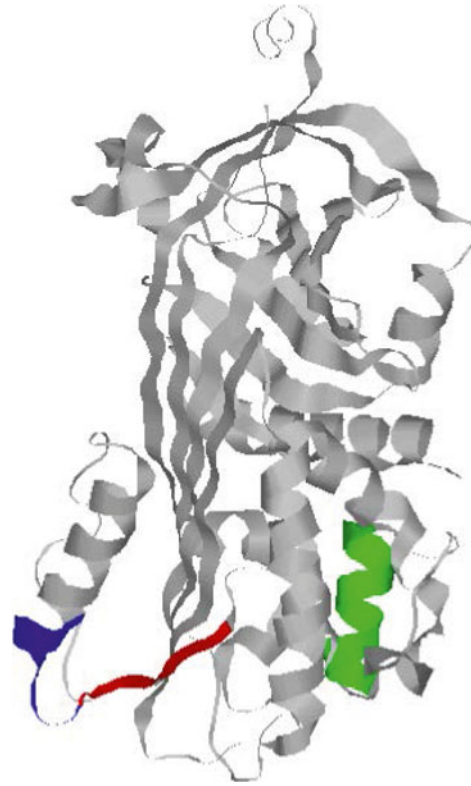

Fig. 1 Examples of peptide epitopes in ovalbumin [52]. Three different linear/continuous epitopes for monoclonal epitopes are marked in *blue*, *red*, and *green* respectively

2 Current Status of Peptide Antibodies

Currently, peptide synthesis, conjugation, and immunization protocols have been optimized and the applications of peptide Abs have expanded to include a variety of immunoassays (e.g., sandwich assays), immunoprecipitation, immunoblotting, immunocytochemistry, and immunohistochemistry (Table 1). Moreover, posttranslational modification-specific Abs (e.g., phosphorylation and citrullination), cleavage site-specific Abs (e.g., amyloid beta 1–40/1–42), tag-specific Abs (e.g., hexa-histidine, FLAG, myc), and conformation-dependent Abs (Tables 1 and 2, [49–51]) are available for the different applications. Methods for epitope prediction have been refined but must always be verified by experimental results and compared with available structural data (*see* **Note 6**).

Table 1
History, status, and future developments of peptide antibodies

A. History of peptide antibodies (selected publications)		
Target[a]	Immunogen (residues (n))	References
MMLV putative protein	C-terminal pentadecapeptide	[8]
SV40 large T	N-terminal heptapeptide, C-terminal undecapeptide	[9]
HBV sAg	Several peptides (5–34)	[10]
FMDV VP1	Several peptides (15–40)	[11]
FMDV VP1	Hexadecapeptide	[12]
TCR	Branched lysine constructs	[13, 14]
RB	C-terminal decapeptide synthesised on carrier protein	[15]
CSFV E2	Dendrimeric peptide construct	[16]
B. Current applications of peptide antibodies (i.e., methods used for detection of proteins and studies of protein modification and processing)		
Application	References	
Immunoblotting	[17, 18]	
Immunoassays (direct, sandwich, etc.)	[19, 20]	
Immunocytochemistry and histochemistry	[21, 22]	
Flow cytometry	[23, 24]	
Immunoprecipitation	[25, 26]	
C. Future developments of peptide antibodies		
Application	References	
TCR-like Abs	[27, 28]	
Therapeutic peptide Abs/vaccines	[29, 30]	
Predesigned, conformation-specific peptide Abs	[31, 32]	
MHC-like Abs	?	

[a]*CSFV* Classical swine fever virus, *FMDV* Foot and mouth disease virus, *HBV* Hepatitis B virus, *MMLV* Moloney murine leukemia virus, *RB* Retinoblastoma protein, *SV* Simian virus, *TCR* T cell receptor

Table 2
Peptide antibody reviews and resources

A. Review and handbooks	
Subject	References
Peptide Abs	[33]
Peptide vaccines	[34]
FMDV vaccines and peptide Abs	[35]
Peptide Ab immunoassays	[36]
Peptide Ab laboratory techniques	[37]
Peptide antigenicity and immunogenicity	[38]
Peptide-based autoimmune serology	[39]
Posttranslational modification-specific peptide Abs	[40]
B. Websites	
Epitope database	www.iedb.org
Epitope prediction	www.cbs.dtu.dk
Human protein atlas	www.proteinatlas.org
NCBI	www.ncbi.nlm.nih.gov
Uniprot/Swissprot	www.expasy.org
Protein database	www.pdb.org

3 Future Developments of Peptide Antibodies

Despite the achievements described above, the potential for peptide Abs has not been exhausted and many new uses have been recently established, are under development, or have been suggested (Table 1) including recombinant peptide Abs, single-chain peptide Abs, TCR-like Abs, predesigned conformation-dependent peptide Abs, and therapeutic peptide Abs. One of the original goals of peptide Abs, the development of clinical useful peptide vaccines, is getting closer to realization but still has to make it into clinical everyday use. MHC-like Abs, i.e., Abs, where the antibody-peptide complex mimics an MHC molecule, would be a desirable, although challenging goal.

4 Notes

1. All epitopes are three dimensional, but this term is here restricted to epitopes containing parts of a polypeptide chain not directly continuous in sequence. Thus, three-dimensional epitopes depend on a folded, native structure of the antigen (Ag). Three-dimensional epitopes may also be denoted "composite" epitopes.
2. Peptide epitopes are usually denoted "linear" or "continuous" epitopes but may also be denoted "simple" epitopes and are smaller than 20 residues continuous in sequence. The difference between peptides and polypeptides is not well defined but lies somewhere between 20 and 30 residues. All proteins are polypeptides, but this term is usually confined to polypeptides larger than 100 residues.
3. Epitope mapping: Mapping of amino acid residues with direct influence on Ab binding (e.g., by peptide scanning, X-ray crystallography, or NMR spectroscopy). Epitope residues may be contact residues or structural (conformational residues) or may contribute through backbone amide bonds.
4. Peptides may be antigenic (i.e., react with Ab (defined by Kd)) but not immunogenic (i.e., incapable of inducing an immune response (i.e., specific Abs and/or T cells)) [38]. The Ab response is quantified by the titers of a serum (defined by midpoint or endpoint titration) and by the average antigenicity of the Abs (Kd).
5. Contact residues: Amino acid residues directly interacting with the epitope or paratope (site on Ab interacting with epitope) as determined by X-ray crystallography and/or NMR spectroscopy of Ag-Ab complexes. The contact may take place between side chains or through backbone amide bonds.
6. See relevant chapters in this volume or see [53–55] for recent reviews.

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Chapter 2

The Structure of Natural and Recombinant Antibodies

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Abstract

Immunoglobulins (Ig) isotypes A, D, E, G, and M are glycoproteins which are mainly composed of a "Y"-shaped Ig monomer (~150 kDa), consisting of two light and two heavy chains. Both light and heavy chains contain variable (N-terminal) and constant regions (C-terminal). Each light chain consists of one variable domain and one constant domain, whereas each heavy chain has one variable domain and three constant domains. However, heavy-chain antibodies consisting of only heavy chains and lacking the light chains are found in camelids and cartilaginous fishes. Unlike other immunoglobulins, the heavy chain of avian antibody IgY (~180 kDa) consists of four constant domains. The single-chain variable fragment (scFv; ~25 kDa) of an antibody contains variable regions of antibody heavy and light chains. The fragment antigen-binding (Fab; ~50 kDa) region has the full antibody light chain but the heavy chain is composed of a variable region and one constant domain.

Key words IgG, IgY, ScFv, Fab

1 Structure of Immunoglobulins

Antibodies, also known as immunoglobulins (Igs), enable antigen recognition in the serum. They are produced by B-cell-derived plasma cells. Antibodies are mainly located in blood, spleen, bone marrow, egg yolk for birds, as well as interstitial fluids and exocrine secretions. Antibodies can be effectively used by the immune system to identify, kill, or neutralize invading bacteria, parasites, toxins, and viruses and to destroy other foreign compounds [1].

Mammalian immunoglobulins are classified into five isotypes, namely IgM, IgD, IgG, IgE, and IgA. The synonymous "Y" shape associated with a basic immunoglobulin unit (Ig) monomer (or subunit) (~150 kDa) consists of two light and two heavy chains, which are connected by disulfide bonds (*see* Fig. 1a) [2]. Each light chain has two regions composed of one variable region (V_L) and one constant region (C_L), whereas each heavy chain contains one variable domain (V_H) and three constant domains (C_H1–3). All of the antibodies perform specific binding to defined antigens through

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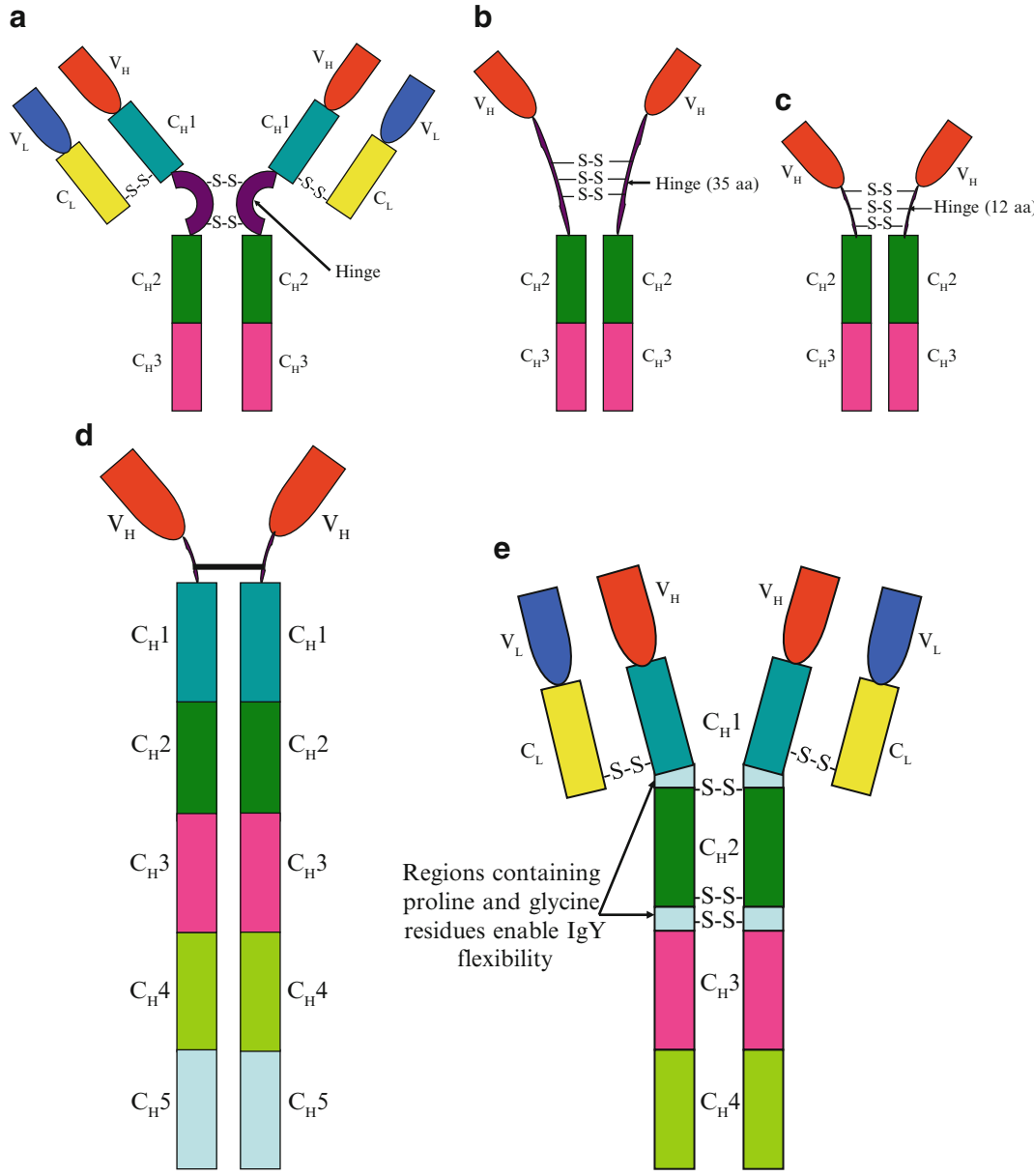

Fig. 1 Structure of basic immunoglobulins. (**a**) Shows structure of a basic immunoglobulin monomer. (**b–d**) Show structure of Camelidae IgG2 (**b**), Camelidae IgG3 (**c**), and avian IgY antibody (**d**). NH_2 = amino group; COOH = carboxylic acid group; V_H = variable region of antibody heavy chain; V_L = variable region of antibody light chain; C_L = constant region of antibody light chain; $C_H1,2,3,4,5$ = constant domain one, two, three, four, and five of antibody heavy chain; S–S = disulfide bond; and aa = amino acid

the variable regions. Almost all five isotypes, IgA (dimer), IgD (monomers), IgE (monomer), IgG (monomer), and IgM (pentamer), are composed of the same basic immunoglobulin unit with some modifications [3]. However, all the members of *Camelidae*

family have a heavy-chain antibody, which consists of two heavy chains (one variable region and two constant regions per chain), and lacks the two light chains. There are three subclasses of IgG in camels and llamas, i.e., the conventional IgG1 (~160 kDa, with full-length light and heavy chains), IgG2 (~92 kDa; with a long hinge; *see* Fig. 1b) and IgG3 (~86 kDa; with a short hinge; *see* Fig. 1c), which lack both light chains and C_H1 [4]. Heavy-chain antibodies are also found in cartilaginous fish. They are called immunoglobulin new antigen receptors (IgNARs; ~175 kDa; *see* Fig. 1d). An IgNAR contains only two heavy chains and each chain has one variable region and five constant regions (C_H1–5) [5].

Avian immunoglobulins are of three principal classes, IgA, IgM, and IgY (the 180 kDa homologue of mammal IgG). Unlike the heavy chain of mammalian immunoglobulins, the heavy chain of IgY consists of four constant Ig domains (*see* Fig. 1e). Female chickens (hens) are favored for producing large amounts of IgY, as this can be harvested from egg yolk. The process is more convenient than isolation of antibodies from blood and other organs (e.g., spleen and bone marrow). IgG contains regions between C_H1 and C_H2, while in IgY two regions (one between C_H1 and C_H2 and the other between C_H2 and C_H3), containing proline and glycine residues, enable limited flexibility [6].

2 Structure of Recombinant Antibodies

A recombinant antibody does not exist naturally but is assembled from DNA by combining antibody heavy-chain and light-chain gene sequences. The single-chain variable fragment (scFv) and fragment antigen-binding (Fab) region are the most popular recombinant antibody formats used due to their short generation time and high antigen affinity and structural stability [7].

A scFv consists of variable (binding) regions of the antibody heavy (V_H) and light (V_L) chains, with a flexible linker [e.g., $(GGGGS)_3$ linker] joining the terminal ends of either the V_H to V_L (or V_L to V_H) (*see* Fig. 2a). It is popular and effective to use a disease-specific scFv for targeted therapy through fusing to therapeutic proteins or genes.

The Fab fragment, which is double the size of the scFv, is formed by one variable and one constant domain of both light and heavy chains, and is linked by a disulfide bridge (*see* Fig. 2b) [8]. There are many medicines, which have been derived from Fabs that are now approved by the Food and Drug Administration (FDA).

Moreover, various kinds of scFv and/or Fab-derived antibodies have been generated for clinical applications. Bivalent or trivalent scFvs consist of two or three scFvs linked with a short amino acid

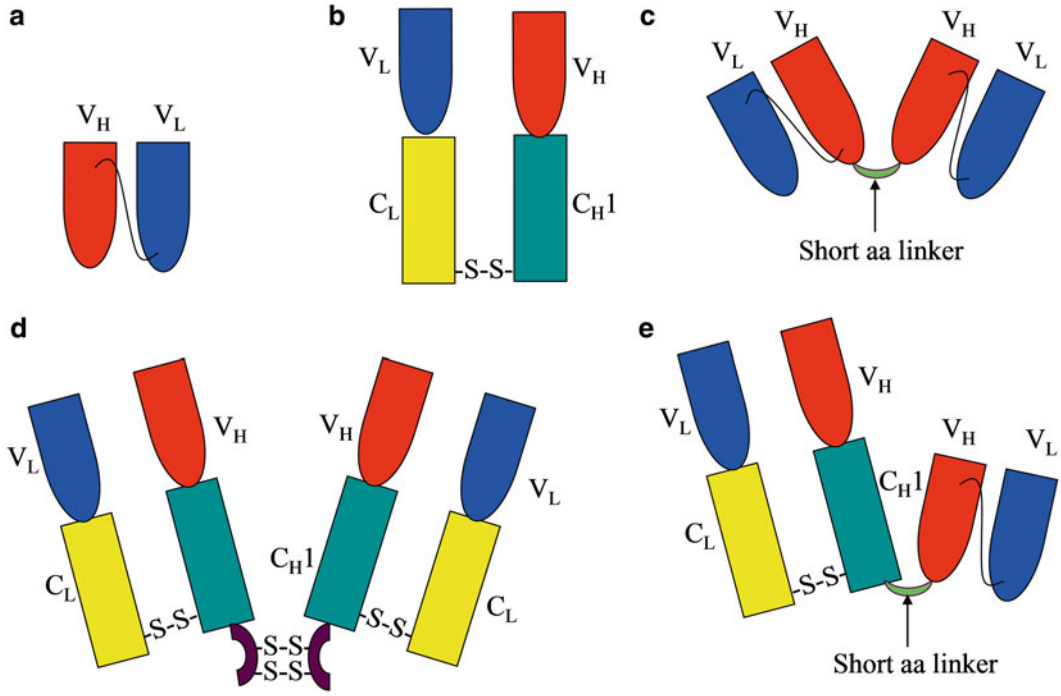

Fig. 2 Structures of a scFv (**a**), a Fab fragment (**b**), a $(scFv)_2$ (**c**), a $F(ab')_2$ (**d**), and a Fab-scFv (**e**). V_H = variable region of antibody heavy chain; V_L = variable region of antibody light chain; C_L = constant region of antibody light chain; C_H1 = constant domain one of antibody heavy chain; S–S = disulfide bond; and aa = amino acid

linker [9] (*see* Fig. 2c). A $F(ab')_2$ fragment contains two Fab fragments linked by disulfide bonds. It can be obtained by cleaving whole immunoglobulins using the enzyme pepsin below the hinge region (*see* Fig. 2d). The Fab-scFv fusion antibody is formed by a Fab and an scFv via a short amino acid linker (*see* Fig. 2e).

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