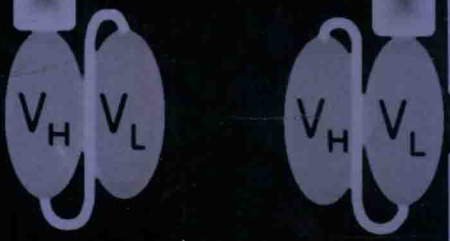
ANTIBODY FUSION PROTEINS



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ANTIBODY FUSION PROTEINS

FOREWORD

The generation of specific antibodies in the immune system has been the prototype for protein engineering. Antibody molecules can be made to specifically bind just about anything, and for the most part, they have quite reasonable physical and chemical properties. Yet, the success of natural monoclonal antibodies in different fields of therapy has been far less than spectacular.

In this book, a number of leading scientists in the field outline that man-made molecules will be required not only to overcome the limitations of monoclonal antibodies, but also to extend the principle of selective targeting. The antibody molecule is designed primarily to fight viruses and bacteria, and does so quite effectively. The immunoglobulin is actually a specific adapter molecule. It mediates the contact between a target surface and an effector mechanism, which may be based on either activating whole cells or a cascade of enzymes, such as the complement system. It follows that specific binding to the target is essential, but is only half the story.

Taking the natural antibody as a source of inspiration, a variety of biological responses can be elicited by fusing other types of molecules to the antigen-binding site of the antibody. Many different and elegant ideas are discussed in the first part of this book, such as making fusion proteins with toxins, cytokines, or enzymes, which can activate a prodrug. A further strategy is to create a bispecific molecule by linking a second antigen binding site to the first one in order to activate an effector mechanism.

Another set of fusion proteins has been constructed which retain only the Fc part of the antibody. The Fc portion has two important features; not only does it dimerize the proteins fused to it, but it also extends their serum half-life.

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The exploitation of this principle, giving rise to so-called immunoadhesins or Fc-fusion proteins, has led to a wealth of unique molecules with extremely interesting therapeutic potential, which is summarized in the second part of the book.

As elegant and promising as all of these approaches are, they are far from perfect. Studying the detailed in vitro and in vivo mechanisms of action of a number of these molecules, especially those that perform differently than expected while designed according to a plausible idea, will help develop the knowledge necessary for making even better second generation molecules. Such studies will advance the whole field of immunotargeting much more than serendipitous success.

Rapid progress in this field has become possible since many production issues have been solved, using either bacterial or eukaryotic expression systems. For example, Chapter 10 gives an outline of the state of the art in producing clincial grade material of immunoadhesins. Furthermore, the generation of specific anti-binding sites with reasonable affinity is a problem that is now largely solved through the use of antibody libraries and elegant selection tools such as phage display, to which another chapter is devoted. Whereas the tools for selection of binding molecules have come a long way, the biophysical understanding of what exactly makes a stable, high-affinity molecule is lagging far behind. Folding, expression, and stability remain challenging issues for the future and may ultimately decide the utility of a particular molecule in practical applications.

The great successes that have already been achieved with the strategies described in this book invite speculations on how the field might move ahead. In the beginning, the recombinant antibody technology strove to imitate nature in its combinatorial diversity and its pragmatic selection principles. At the next stage, the adapter principle of the antibody was taken to more abstract terms, leading to the variety of fusion proteins described in this volume. The third stage of abstraction may then be to combine the two, and fuse unnatural, optimized targeting domains to equally reengineered artificial effector functions in an approach merely inspired by nature's concepts. Although we now have many of the tools to achieve this, we still lack much of the required biological and molecular understanding.

This, however, is delightful for the researcher.

ANDREAS PLÜCKTHUN

ACKNOWLEDGMENTS

This book represents the culmination of more than a decade of work, during which the tools of protein engineering forged a new field in antibody fusion proteins. The work on immunoadhesins was begun at Genentech in the 1980s. We wish to acknowledge Dan Capon, who initiated this work, and Doug Smith, who helped Dan with the early experiments. In addition, we are grateful to Larry Lasky, Scot Marsters, and David Peers for their help in bringing the immunoadhesin technology to fruition. Moreover, we thank our wives, Judy and Chris, for their continued support and encouragement.

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OVERVIEW

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1.1 ANTIBODIES

Immunoglobulins are the most critical components of the immune system. As proteins that bind to preselected molecular targets, immunoglobulins arise in response to foreign substances introduced into the body. The immunoglobulins comprise a heterogeneous group of proteins that account for approximately 20% of the total plasma protein in humans. In addition, different populations of immunoglobulins are found in extravascular fluids, in exocrine secretions, and on the surface of some lymphocytes. The biologic activity of immunoglobulins is best understood in the context of their structure.

The basic three-dimensional structure of antibodies was first elucidated as early as 1973,[1] when the crystal structure of a Fab fragment was solved. An antibody is a Y-shaped molecule, composed of two identical light chains and two identical heavy chains (Fig. 1.1). Both light and heavy chains contain variable and constant regions. The four chains are held together by disulfide bonds, which are located in a flexible region of the heavy chain known as the hinge. Variable regions of both heavy and light chains combine to form two identical antigen-binding sites, one on each arm of the Y. Heavy chain constant regions define five classes of antibodies: IgA, IgD, IgE, IgG, and IgM, each with its own class of heavy chain— α, δ, ε, γ, and μ, respectively. Each antibody class (termed an *isotype*) has distinct structural and functional characteristics. In isotypes such as IgM or IgA, multimeric assemblies of four-chain units

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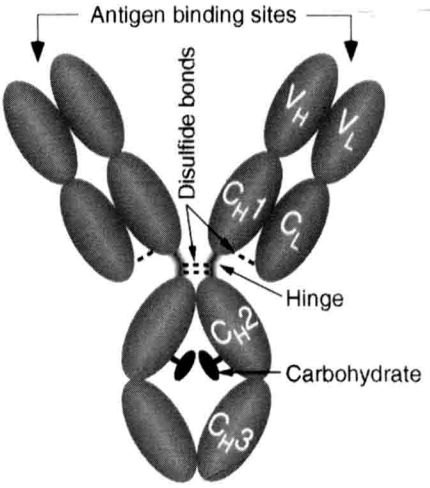

Figure 1.1 Structure of the antibody molecule. A schematic model of human IgG1, showing the basic four-chain structure and domains. V indicates variable region; C, constant region; V_L and C_L are domains of the light (L) chain; V_H, C_H1, C_H2, and C_H3 are domains of the heavy (H) chain. The hinge region, containing two inter-H chain disulfide bonds (dotted lines) and disulfide bonds connecting H and L chains, is indicated. The antigen-binding sites, of which there are two in each antibody, are comprised of unique segments contained within the V_H and V_L domains. A single asparagine-linked carbohydrate is encoded within the C_H2 domain of each H chain. X-ray structural studies confirm that these carbohydrates are sequestered within a pocket formed by the C_H2 and C_H3 domains of the Fc.

produce antibody molecules with ten or four antigen binding sites, respectively. In addition, there are a number of subclasses of IgG and IgA immunoglobulins; for example, within the human IgG isotype, there are four subclasses (IgG1, IgG2, IgG3, and IgG4) having $\gamma 1$, $\gamma 2$, $\gamma 3$, and $\gamma 4$ heavy chains, respectively. Effector functions of antibodies, such as complement activation, binding to phagocyte Fc receptors, antigen-dependent cellular cytotoxicity, and transport across the placenta, are mediated by structural determinants within the Fc region (the tail of the Y shape).

Two major discoveries in the 1960s ushered in the period of detailed structural study of antibodies. The first was the finding that enzymes and reducing agents could be used to digest or dissociate immunoglobulin molecules into smaller components. The second was the realization that the electrophoretically homogeneous proteins found abundantly in serum and urine of patients with a disease called multiple myeloma were related to normal immunoglobulins. These myeloma proteins were found to be structurally homogeneous. They are also called monoclonal proteins, since they are synthesized by single clones of malignant plasma cells.

To help the reader understand the information presented in this book, we have compiled a list of concepts and definitions that lay the groundwork for grasping the structural elements of immunoglobulins. Because most of the protein engineering approaches described in this book are based on the structural framework of human IgG, the list below defines concepts that apply specifically to the IgG molecule.

Structural Elements of Immunoglobulins

Basic unit (monomer): Each immunoglobulin contains at least one basic unit or monomer comprising four polypeptide chains (Fig. 1.1). The oligomeric structure is stabilized by interchain disulfide bonds that connect all four chains together.

H and L chains: Immunoglobulins contain two types of polypeptide chains. One pair of identical polypeptide chains contains approximately twice the number of amino acids, or is approximately twice the molecular weight, of the other pair of identical polypeptide chains. The chains of higher molecular weight are designated heavy (H) chains, and those of lower molecular weight, light (L) chains.

V and C regions: Each polypeptide contains an amino terminal portion, the variable (V) region; and a carboxyl terminal portion, the constant (C) region. These terms denote the considerable heterogeneity or variability in the amino acid residues in the V region compared to the C region. Heavy and light chains each have a single V region, and light chains possess a single C region. Heavy chains contain three C regions.

Antigen binding site: The part of the antibody molecule that binds to antigen is formed only by small numbers of amino acids in the V regions of H and L chains. These amino acids, contained within six complementarity-determining regions (CDRs), are brought into close proximity by the folding of the V regions.

Domains: The polypeptide chains are folded by disulfide bonds into globular regions called domains. The domains in H chains are designated as V_H, C_H1, C_H2 and C_H3; those in L chains are designated V_L and C_L. An IgG monomer contains two Asn-linked oligosaccharides, one attached to each H chain within the C_H2 domain. These oligosaccharides are oriented into a pocket formed by the bowing outward of the C_H2 and C_H3 domains of the Fc (Fig. 1.1).

Hinge region: The area of the H chains in the region between C_H1 and C_H2 domains. This region of approximately 12 amino acid residues is quite flexible and is more exposed than are other regions of the molecule to enzymatic and chemical cleavage. Thus, papain acts here to produce Fab and Fc fragments (see following).

1.1.1 Immunoglobulin Fragments

Fragmentation of antibodies to produce segments that retain biological function (Fig. 1.2) has been important to elucidation of antibody structure. Several examples of antibody fragments that are themselves useful reagents are presented in this book. Initially, these fragments were made by proteolytic treatment of purified whole antibodies. More recently, fragments have been produced directly by recombinant means. The possibility of generating antibody Fab fragments as recombinant proteins in *Escherichia coli*[2] has been a major breakthrough in the field of antibody engineering. Some definitions of fragments follow:

Fab and Fc fragments: Digestion of an IgG molecule by the enzyme papain cleaves the molecule within the hinge region at a site upstream of the inter-H chain disulfide bonds, producing two Fab (ab = antigen binding) fragments and one Fc (c = crystallizable) fragment[3] (Fig. 1.2A).

F(ab′)$_2$ fragment: Digestion of an IgG molecule by the enzyme pepsin cleaves the molecule also within the hinge region, but at a site downstream of the inter-H chain disulfide bonds (Fig. 1.2B). In addition, pepsin cleaves the Fc fragment into several peptide fragments. Thus, pepsin cleavage results in production of one F(ab′)$_2$ molecule.[4] The F(ab′)$_2$ molecule is composed of two Fab units and the hinge (hence the nomenclature "Fab′" to distinguish it from the "Fab" produced by papain). It contains two antigen-binding sites and can therefore bind bivalently to antigen.

Fv fragment: A fragment consisting only of the two V regions, V_L and V_H (Fig. 1.2C). Capable of binding to antigen, this fragment is unstable due to noncovalent association of its two polypeptide chains. Fv fragments were originally produced enzymatically by cleavage of IgG[5].

Single-chain Fv fragment (*scFv*): A stable variant Fv fragment in which the two V region polypeptides are covalently attached via a linker peptide (Fig. 1.2C).[6] This can be done in either orientation, so that the linker peptide attaches the C-terminus of V_H to the N-terminus of V_L, or vice versa. In either molecular construct, antigen-binding activity can be retained. Single-chain Fv fragments are most commonly produced by direct expression of recombinant fragments in bacteria.[7]

Fd fragment: The N-terminal half of the H chain comprising V_H and C_H1 (Fig. 1.2C).[8] The receptor domain of an Fc fusion protein is sometimes referred to as an "Fd-like" fragment, by analogy to this H chain nomenclature.

1.1.2 Genetically Engineered Immunoglobulins

For monoclonal antibodies to be maximally useful as human therapeutics, they must possess several qualities: (1) high affinity binding to antigen, (2) an ability

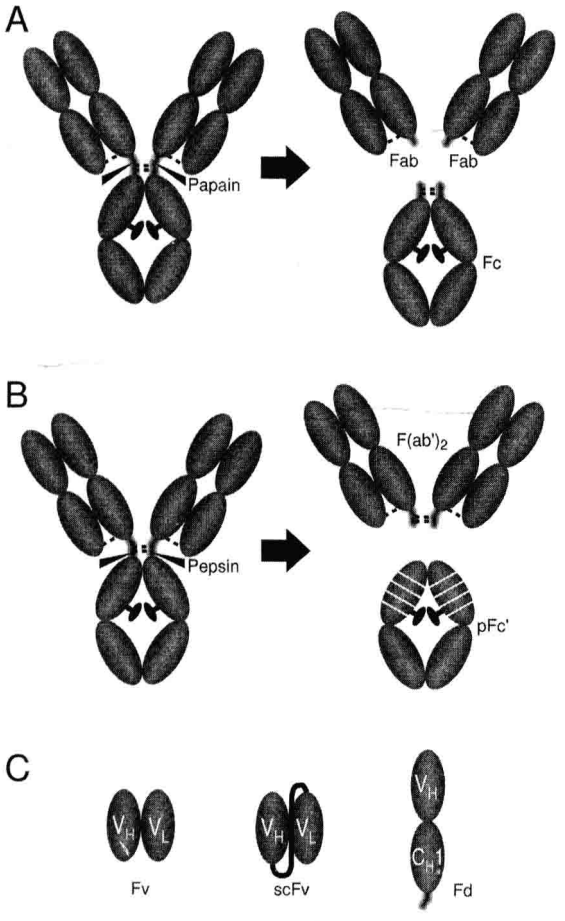

Figure 1.2 Immunoglobulins can be fragmented by partial digestion with proteases. The resulting fragments retain full antigen-binding activity. *A*. Treatment with papain cleaves the antibody molecule at a unique site in the hinge region, upstream of the interheavy chain disulfide bonds. This results in generation of three fragments: two identical Fab fragments that each bind monovalently to antigen, and the Fc fragment that retains its ability to bind to Fc receptors, including *Staphylococcus aureus* protein A. *B*. Treatment with pepsin cleaves the molecule at sites within the hinge region and also in the C_H2 domain, generating many peptides. The Fc (pFc′) is largely destroyed by this treatment. The cleavage site within the hinge region is downstream of the interheavy chains disulfide bonds, resulting in production of a bivalent antigen-binding fragment, $F(ab')_2$. *C*. Additional fragments to which the text refers are Fv, single-chain Fv (scFv), and Fd. Fv and scFv represent the smallest antigen-binding fragments yet produced. Fd fragments do not bind to antigen, but when this region is replaced in an Fc fusion protein, the ligand-binding domain is sometimes referred to as an "Fd-like" fragment. Fab, $F(ab')_2$, and scFv fragments are versatile alternatives to full-length antibodies that have been produced in recombinant form by direct expression in bacteria.

to neutralize antigen activity, (3) long serum half-life, and (4) low immunogenicity. Antibodies from different animal species can have some of these qualities, but only human antibodies are nonimmunogenic when injected into patients. Thus, the goal of therapeutic antibody research during the past 20 years has been to create specific antibodies that are increasingly human in sequence, in order to reduce immunogenicity of the molecule.

The application of genetic engineering to antibodies was not possible without creation of a source of a single, homogeneous antibody of defined antigen specificity. This was achieved in 1975 with the advent of hybridoma technology to produce murine monoclonal antibodies[9] (Fig. 1.3). Despite the success of this method for production of mouse antibodies, however, hybridoma technology has not been successful in production of human antibodies.

To overcome this limitation, investigators in the 1980s sought to use the emerging tools of protein engineering to convert murine antibodies into human forms. A first step toward this goal was the development of genetically engineered antibodies that contained some human constant region sequence but retained the mouse V regions—mouse–human chimeric antibodies.[10] Mouse–human chimeric antibodies are less immunogenic than are mouse antibodies in human patients. A further refinement was the construction of engineered antibodies with even more human sequence—humanized antibodies.[11] These were mouse antibodies in which all portions of the molecule were replaced by human sequence except the six CDRs within the V_H and V_L domains—the amino acid sequences responsible for antigen binding. With further advances that have been made more recently—specifically, the development of mice that have been genetically reconstituted with parts of the human immune system[12] and antibodies generated from phage libraries[13]—production of fully human antibodies is a goal that is just now beginning to be realized.

1.2 ANTIBODY FUSION PROTEINS

1.2.1 Fab Fusions

Antibody fusion proteins fall generally into two classes that are distinguished structurally by the molecular entity that provides the targeting function. Molecules in the first class are termed *Fab fusions* (Fig 1.4A–F): proteins in which the variable regions of the antibody molecule, which are responsible for antigen recognition, are retained. The non-immunoglobulin fusion partner is added to (Fig. 1.4A) or replaces (Fig. 1.4B–C), the Fc domain. Additionally, the nonimmunoglobulin fusion partner can be attached to the Fab portion of an intact IgG (Fig. 1.4D) or to a single chain Fv (Fig. 1.4E–F). Fab fusion proteins are the focus of Part I of this book.

Part I comprises Chapters 2 through 7. In Chapter 2, "Fab Fusion Proteins: Immunoligands," Manuel Penichet, Seung-Uon Shin, and Sherie Morrison of

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