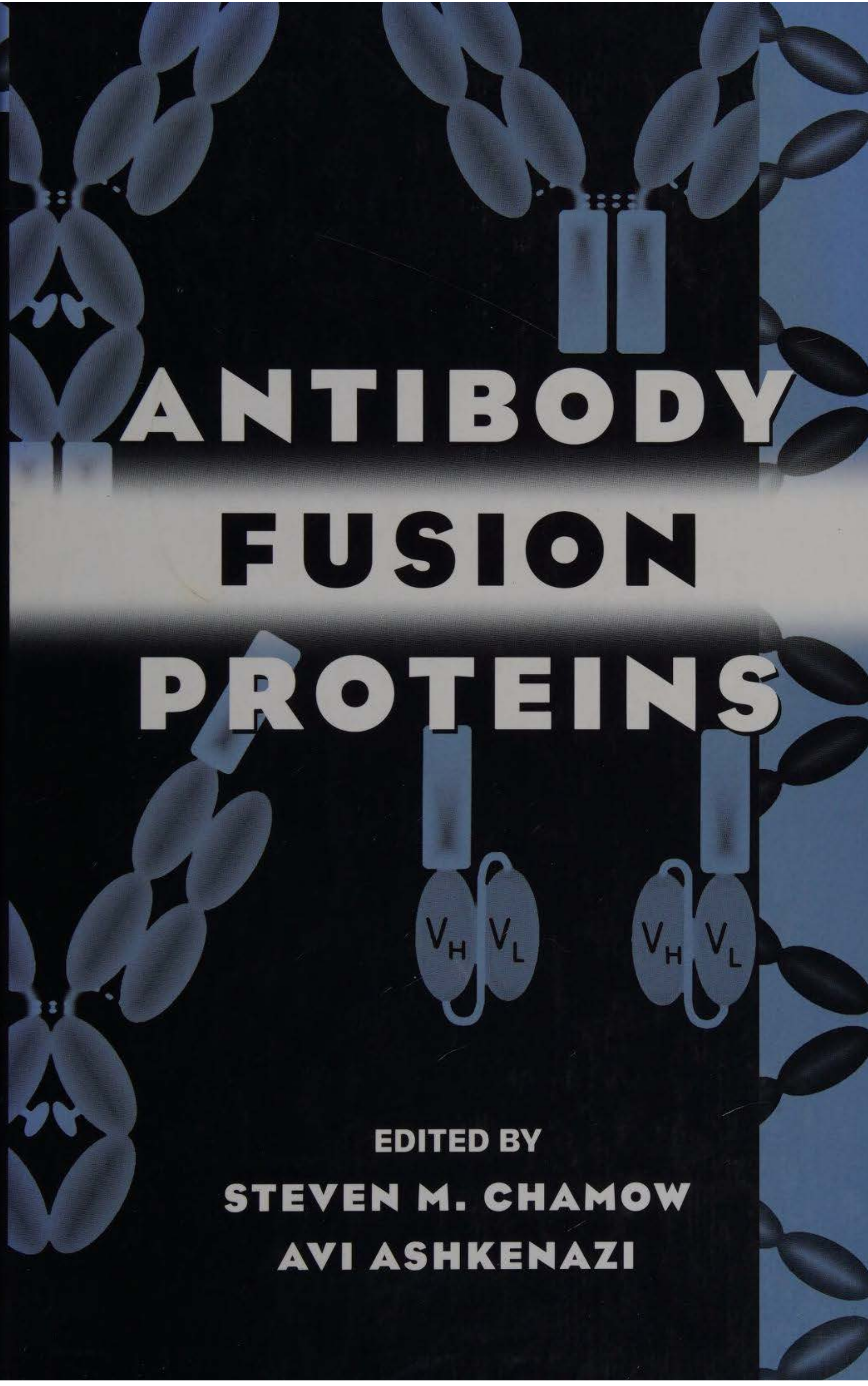


The background of the cover features a diagram of an antibody fusion protein. It consists of two heavy chain domains (represented by vertical rectangles) and two light chain domains (represented by ovals). The heavy chain domains are connected by a disulfide bond (two horizontal lines). Each heavy chain is also connected to a light chain by a disulfide bond. The light chain domains are labeled V_H and V_L. The entire diagram is rendered in a light blue color against a dark blue background with a pattern of stylized leaves.

ANTIBODY FUSION PROTEINS

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IMMUNOGLOBULIN FUSION PROTEINS

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8.1 INTRODUCTION

Rapid advances in molecular biology, protein expression, and protein purification techniques have allowed the preparation of novel chimeric polypeptides with multiple functional domains. Among the most common of these are immunoglobulin (Ig) fusion proteins. These proteins consist of the constant regions of immunoglobulin, typically mouse or human, fused to an unrelated protein or protein fragment. Ig fusion proteins have become valuable laboratory reagents for the study of protein structure and function in vitro and in vivo and in some instances might have applications in a clinical setting. In this chapter the following topics are covered: (1) design of chimeric genes encoding Ig fusion proteins, (2) expression, purification, and characterization of the Ig fusion proteins, and (3) applications of Ig fusion proteins as laboratory tools and in the clinic.

8.2 Ig FUSION PROTEINS: DESIGN OF THE CHIMERIC GENES ENCODING Ig FUSION PROTEINS

The conceptual root of Ig fusion proteins comes from the application of molecular biology techniques to the manipulation of immunoglobulin genes¹⁻³ and the finding that many cell surface receptors contain extracellular

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domains that are homologous to the domains found in the immunoglobulin proteins. It was reasoned that it should be possible to generate chimeric genes in which the DNA fragments (genomic or cDNA) encoding the variable regions of an immunoglobulin heavy chain could be replaced with a DNA fragment encoding the Ig-like domains of other cell surface proteins. Among the first such DNA constructs were those encoding CD4-Ig fusion proteins.⁴⁻⁶ In these constructs the DNA encoding the Ig heavy chain variable region was replaced with a DNA fragment encoding the extracellular domain of CD4 which is composed of four Ig-like domains. When placed in a mammalian expression vector, these chimeric genes directed the expression of CD4-Ig. It was proposed that these CD4-Ig fusion proteins could be used to slow down the progression of HIV infection by binding to virions via interactions with gp120 leading to virus neutralization. While these CD4-Ig fusion proteins have not been effective in the clinic, they have established that it is possible to replace the N-terminal Ig domain corresponding to the antigen-combining (variable) region of the antibody with an Ig-like domain from an unrelated protein and generate an Ig fusion protein in which both the receptor and Ig domains retain their biological activity. In recent years, a wide range of Ig fusion proteins have been made including Ig fusions of both type I and II cell surface proteins, with and without Ig-like domains, and Ig fusions of cytoplasmic proteins (for a review see Refs. 7 and 8). Thus, Ig fusion protein technology can be broadly applied to generate novel laboratory tools to study protein structure and function, as well as potentially novel drugs.

8.2.1 Construction of the Chimeric Gene Encoding the Ig Fusion Protein

In most instances the gene encoding the fusion protein will contain the cDNA or cDNA fragment encoding the polypeptide of interest fused in frame with either a cDNA fragment or a genomic DNA fragment encoding the hinge (H), and CH2 and CH3 domains of an immunoglobulin heavy chain. The location of the DNA fragments encoding these two regions of the fusion protein relative to one another is dependent on the characteristics of the protein under study and is discussed in a following section. While this is the most common format for the preparation of chimeric genes encoding Ig fusion proteins, it should be noted that constructs containing CH2 and CH3 or only CH3 can also be generated.

Theoretically, these chimeric genes direct the expression of covalent Ig fusion protein homodimers. The covalent nature of the homodimer is driven by the presence of the cysteine (Cys) residues that form the immunoglobulin interchain disulfide bonds in the H domain of the Ig constant region. If the fusion protein is made with an Ig domain containing a mutant H domain in which the Cys residues have been replaced by serine (Ser) residues, the Ig fusion protein is likely to be expressed as a noncovalent homodimer. Practically, it has been observed that many of these fusion proteins form higher

aggregates. For this reason, care should be taken to characterize the aggregation of the fusion protein.

8.2.2 Species, Isotype, and Functional Properties of the Ig Domain

The first consideration when designing the construction of the chimeric gene encoding an Ig fusion protein is its intended use(s). The use dictates the species and isotype of the Ig domain used in the construction of the fusion protein; it also determines whether the Ig domain should be wild-type or mutated in such a way that its own functional properties (complement and Fc-receptor binding) are altered. The choice of Ig domain also impacts the valance of the fusion protein and the type of Ig binding reagents available for purifying and manipulating the fusion protein (Box 8.1). These issues are perhaps best illustrated with a few examples:

8.2.2.1 Identification of Tissues Expressing the Ligand for a Novel Human Cell Surface Receptor. In this example, the Ig fusion protein is used to carry out immunohistology on a number of human tissues in an effort to identify tissues and cells expressing the receptor's ligand(s). In this case, the Ig domain should be derived from mouse IgG. This will ensure the fusion protein can be easily purified on a Protein A or G column and that the anti-IgG reagents used in the immunohistology study will not cross-react with tissues containing human B cells expressing Ig. Conversely, if the immunohistology is done using mouse tissues, the Ig domain for the fusion protein should be derived from human IgG. If binding is detected using the fusion proteins, care should be taken to demonstrate that this binding is not mediated by the Ig domain. This can be accomplished by preblocking Fc-receptors or using an Ig fusion protein containing an Ig domain with an isotype or point mutant that no longer binds Fc-receptors. Examples of this application include the identification of cells expressing the CD6, CD27, CD30, CD44, and CD62E ligands.⁹⁻¹³

8.2.2.2 Blocking Receptor-Ligand Interaction in vivo by Injecting Soluble Receptor. In this example, the physiologic role of the interaction between a mouse cell surface receptor and its ligand is studied in vivo by interrupting it with a receptor Ig fusion protein. In this case, the Ig domain should be derived from mouse IgG. The mouse IgG domain allows the facile purification of the fusion protein and is not antigenic when injected into mice. If a human IgG domain were used in this instance, the fusion protein would be very antigenic when injected into mice. Consequently, multiple dosing of a human IgG chimera would rapidly result in the generation of antihuman IgG antibodies that would dramatically reduce the half-life of the fusion protein in vivo and limit its efficacy. If the fusion protein is to be used in vivo, the isotype of the fusion protein should also be taken into consideration. Is it an isotype that can fix complement leading to the depletion of the cells expressing the receptor ligand? Is it an isotype that can bind Fc receptor resulting in unintended Fc

Box 8.1 Properties of the Human Immunoglobulin Fc Region.

There are five types of human immunoglobulin Fc regions with different effector and pharmacokinetic properties: IgG, IgA, IgM, IgD, and IgE. IgG is a monomeric molecule (single heterodimer) and is the most abundant immunoglobulin in serum (75–85% of serum Ig). IgG has the longest half-life in serum of any Ig (23 days) and a synthesis rate of 33 mg/kg/day. Unlike other Ig, IgG is efficiently recirculated following binding to FcRn (Fc receptor identified on neonatal intestine). There are four IgG subclasses G1, G2, G3, and G4, each of which have different effector functions. G1, G2, and G3 can bind C1q and fix complement while G4 can not. Even though G3 is able to bind C1q more efficiently than G1, G1 is more effective at mediating complement-directed cell lysis. G2 fixes complement very inefficiently. The C1q binding site in IgG is located at the carboxy terminal region of the CH2 domain. All IgG subclasses are capable of binding to Fc receptors (CD16, CD32, CD64) with G1 and G3 being more effective than G2 and G4. The FcR binding region of IgG is formed by residues located in both the hinge and the carboxy terminal region of the CH2 domain. Interactions between IgG and FcRn also allow the transfer of this immunoglobulin across the neonatal intestine and the placenta. IgA can exist both in a monomeric and dimeric form held together by a J-chain. IgA is the second most abundant Ig in serum (7–15%). IgA is the most heavily synthesized Ig (65 mg/kg/day) but is rapidly turned over in the serum where it has a half-life of 6 days. It is the most abundant Ig in secretions (tears, milk, etc.). IgA secretion is mediated by binding to pIgR, the polymeric Ig receptor that mediates the transport of polymeric Ig (IgA and IgM) across mucosal epithelium. IgA has three effector functions. It binds to an IgA specific receptor on macrophages and eosinophils, which drives phagocytosis and degranulation, respectively. It can also fix complement via an unknown alternative pathway. IgM is expressed as either a pentamer or a hexamer, both of which are held together by a J-chain. IgM is secreted during a primary immune response and represents 5–10% of serum Ig. IgM is synthesized at the rate of 7 mg/kg/day and has a serum half-life of 5 days. IgM binds weakly to C1q via a binding site located in its CH3 domain. Due to the multimeric nature of IgM, it can efficiently fix complement at low antigen densities, with the hexameric form being more efficient than the pentameric form. IgM can also bind C3b and mediate phagocytosis by binding to C3b receptors on macrophages. Like IgA, IgM binds to the pIgR and is transported across mucosal epithelium and is found in secretions. IgD is a monomeric molecule that represents 0.3% of serum Ig. It has a half-life of 3 days in serum and is synthesized at the rate of 0.4 mg/kg/day. To date, no effector functions have been ascribed to its Fc domain. IgE is a monomeric Ig and is the least abundant serum Ig (0.02%). It has a serum half-life of 2.5 days and it is synthesized at the rate of 0.016 mg/kg/day. IgE binds to two Fc receptors, the high-affinity FcεR1 and the low-affinity CD23. Binding of IgE to FcεR1 on basophils drives degranulation and results in the release of proinflammatory agents. Binding of IgE to CD23 triggers a down regulatory feedback loop that results in decreased IgE production.

receptor-mediated activities? These are important considerations which, if not taken into account, could lead to the incorrect interpretation of the *in vivo* effects of the fusion protein. For an extensive review of the functional properties of the human and murine Ig Fc region as well as detailed information on which residues are involved in Fc receptor and complement binding please see Refs. 14 and 15. It should be noted that the functional activity of the Ig domain of the fusion can be addressed by changing isotypes and/or using Ig domains with point mutations that abolish function. Examples of this application include the use of CTLA4-Ig¹⁶ to examine the effect of blocking the interaction between CD28 and CTLA4 and their ligands B7-1 and B7-2, the use of CD40-Ig to study the interaction between CD40 and its ligand (CD154) in the immune response,¹⁷ the use of CD44-Ig to examine the effect of blocking interaction between CD44 and hyaluronan on tumor cell growth,¹⁸ and the use of L-selectin-Ig to study the role of this receptor in leukocyte recruitment to sites of inflammation¹⁹ among others.

8.2.2.3 Identification of Ligand Domains and Residues Responsible for Receptor Binding. In this example, an Ig fusion of the receptor and an Ig fusion of the ligand are used to determine the ligand domains, and residues within the binding domain, which are responsible for ligand binding *in vitro*. For these studies a human IgG domain could be appended to the receptor and immobilized on plastic using an antihuman IgG antibody. The binding of wild-type or mutant (truncated or point mutant) mouse Ig fusion proteins of the ligand to the immobilized receptor could then be monitored by ELISA using antimouse IgG reagents that do not bind to human IgG. Conversely, these experiments could be done with a receptor mouse IgG and a ligand human IgG fusion protein. Examples of this application include the identification of the ALCAM domains and residues responsible for CD6 binding,²⁰ the identification of ICAM-3 domains involved in LFA-1 binding,²¹ and the identification CD31 domains involved in receptor binding,²² among others.

These three examples illustrate that while Ig fusion proteins are very useful tools, they have inherent limitations, which vary depending on the functions of the Ig domain used to prepare the fusion protein. For this reason, careful consideration should be given to the choice of Ig domain. Because these limitations have such a profound effect on the use of such fusion proteins, investigators who use this technology will usually generate more than one fusion protein, each containing a different Ig domain.

8.2.3 Placement of the Ig Domain in the Fusion Protein

Perhaps the greatest concern for an investigator using an Ig fusion protein is that the inclusion of the Ig domains would perturb the structure and function of the protein domain(s) under study. If this were to happen, observations regarding the structure and function of the protein being studied that were made with the fusion protein may not be physiologically relevant. For this

reason, it is important to consider carefully how the protein or protein fragment to be studied and the Ig domains will be joined. If the three-dimensional structure of the protein to be studied is known or can be predicted with certainty, this information should be used to generate the fusion protein. The Ig domain should be placed at either the N- or C-terminus of the protein depending on which end of the protein is free and does not participate directly in determining its three-dimensional structure. Consideration should also be given to the location of the functional regions of the protein. The Ig domain should be added to the protein in the location least likely to affect the function of the native protein. However, in most instances nothing is known about the three-dimensional structure of the protein being studied or the location of its functional domains. Indeed, in many instances the Ig fusion protein is the tool used to explore the function of a novel protein. In these instances the following guidelines should be used.

8.2.3.1 *Ig Fusions with the Extracellular Domain of Cell Surface Proteins.* If the goal is to study the extracellular domain of either a type I or type II membrane protein, the DNA sequence encoding the Ig domain should replace those sequences that encode the transmembrane and cytoplasmic domain of the cell surface protein. In this class of proteins the cytoplasmic membrane is a sharp boundary separating structural elements on the inside and outside of the cell. Thus, placing an unrelated protein domain in this location will, most likely, not perturb the structure of the extracellular domain of the protein being studied. In the case of type I and II membrane proteins, the cDNA or genomic fragment encoding the extracellular domain of the protein of interest should be fused to a cDNA or genomic fragment encoding the constant domain of Ig in place of the sequences encoding the transmembrane and cytoplasmic domain of the surface protein. Care should be taken in the design of the nucleotide sequence at the junction of the extracellular domain and the Ig domain to ensure that the sequence encoding the Ig domain will be in frame with the sequences encoding the extracellular domain of the protein of interest. In the case of Ig fusion proteins with type I membrane proteins, the N-terminal secretory signal sequence is contained within the nucleotide sequence encoding the cell surface protein of interest. Unless there is a specific reason to use a signal sequence derived from another protein (poor protein expression, etc.), the natural N-terminal secretory signal sequence should be used. Doing so will ensure that the N-terminal secretory signal sequence is clipped from the mature protein in the same location as in the native cell surface protein, ensuring that the N-terminal amino acid sequence of the cell surface receptor and its secreted Ig fusion counterpart are identical (Fig. 8.1). For type II membrane proteins, an exogenously derived N-terminal secretory signal sequence must be added to the sequences encoding the constant region of the Ig domain which will in turn be located 5' of the sequences encoding the extracellular domain of the surface protein (Fig. 8.1).²³

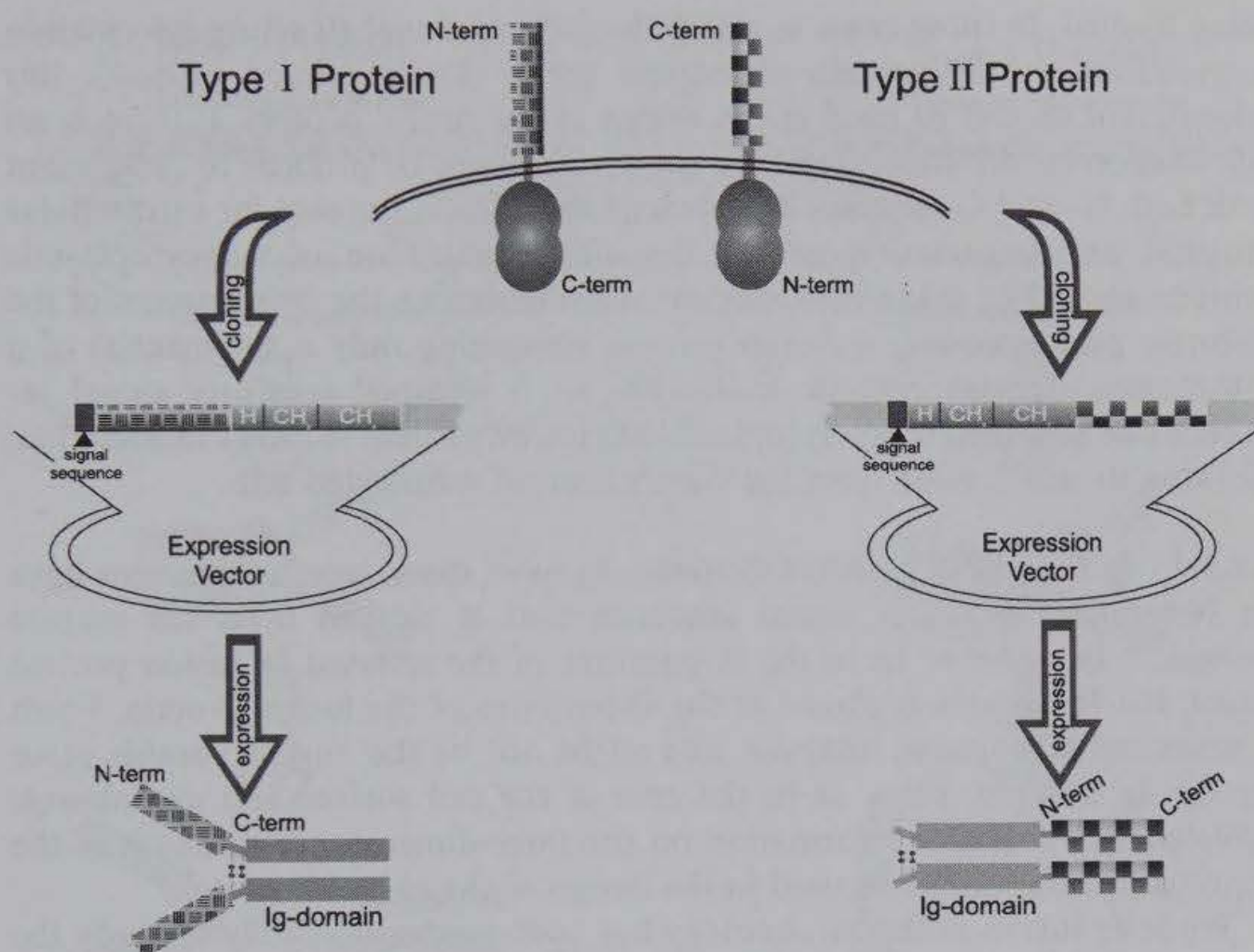


Figure 8.1 Construction of chimeric genes encoding Ig fusion proteins. Schematic representation of the chimeric genes encoding Ig fusion proteins of either a Type I or Type II cell surface protein. In both instances, the Ig constant domains are fused to the cDNA fragment encoding the extracellular domain of the protein to be studied, in place of the DNA encoding the transmembrane and extracellular domain of the cell surface protein.

If the Ig fusion protein is to be used to study a fragment of the extracellular domain of a cell surface protein, the domain organization of the extracellular region of the protein should be taken into account when designing the fusion protein. The sequences encoding the Ig region should be put in place of the sequences encoding one or more extracellular domains. Preserving the integrity of domains should minimize the possibility that inclusion of the Ig constant regions will affect the structure and function of the polypeptide fused to it. If the sequences encoding the extracellular domain fragment to be studied do not contain an N-terminal secretory signal sequence, one should be added.

8.2.3.2 Ig Fusions of Cytoplasmic Proteins. In principle, the Ig domain can be added to either the N- or C-terminus of the cytoplasmic protein to be studied;²⁴ however, placement of the Ig domain sequences should be dictated by which end of the protein is free. This will minimize the possibility that the Ig domain will perturb the structure and function of the cytoplasmic protein

being studied. In those cases in which three-dimensional structure information is available for the protein of interest (or a closely related protein), this information should be used in the design of the fusion protein. If there is no information on the structure of the protein, it might be prudent to experiment with both N- and C-terminus Ig fusion proteins. As is the case for extracellular proteins, any information on the domain organization of the cytoplasmic proteins should be taken into account when designing the construction of the chimeric gene encoding a fusion protein containing only a fragment(s) of a cytoplasmic domain protein. If desired, an N-terminal secretory signal sequence can be added to the cytoplasmic-Ig fusion protein to direct its secretion, allowing its purification from the supernatant of transfected cells.

8.2.3.3 *Ig Fusions of Secreted Proteins.* In most cases, secreted proteins have an N-terminal secretory signal sequence that is cleaved from the mature protein.²⁵ In order to leave the N-terminus of the secreted Ig fusion protein intact, the Ig domain is placed at the C-terminus of the fusion protein. From a structural standpoint, however, this might not be the most favorable place for the Ig domain. Thus, as in the case of the cell surface and cytoplasmic proteins, any available information on the three-dimensional structure of the secreted protein should be used in the design of the chimeric protein.

While Ig fusion protein technology has been used successfully to study the structure and function of many proteins (>70 different proteins to date), it is not a panacea. In many instances it is not possible to append Ig domains onto a polypeptide without affecting its structure and function. Each case is unique and investigators using this technology should carefully characterize the Ig fusion proteins that they prepare to ensure that their activity is as close to physiological as possible. In this regard, if the investigator finds that a given Ig fusion construct is unusable due to a perturbation of the structure and/or function of the protein under study, changing the location of the Ig domain (for example by adding a linker between the polypeptide being studied and the Ig domain) will sometimes ameliorate this problem.

8.2.4 Cleavable Ig Fusion Proteins

For some applications it is convenient to have an Ig domain for purification and other purposes (histology, ADCC, etc.), however, the Ig domain can also interfere with additional experiments planned for that protein. This has led to the construction of Ig fusion proteins that have an engineered protease cleavage site at the junction between the polypeptide encoding the protein of interest and the Ig domain (Fig. 8.2). Two types of cleavable Ig fusion proteins have been described: one in which the cleavage site is located at the Ig-domain junction,^{26,27} and the other in which the proteinase site is located after the hinge domain of the Ig. Proteinase digestion of the former leads to the separation of Ig domain and the polypeptide to which it has been fused. In the latter case, if the hinge sequence of the Ig domain being used has wild-type

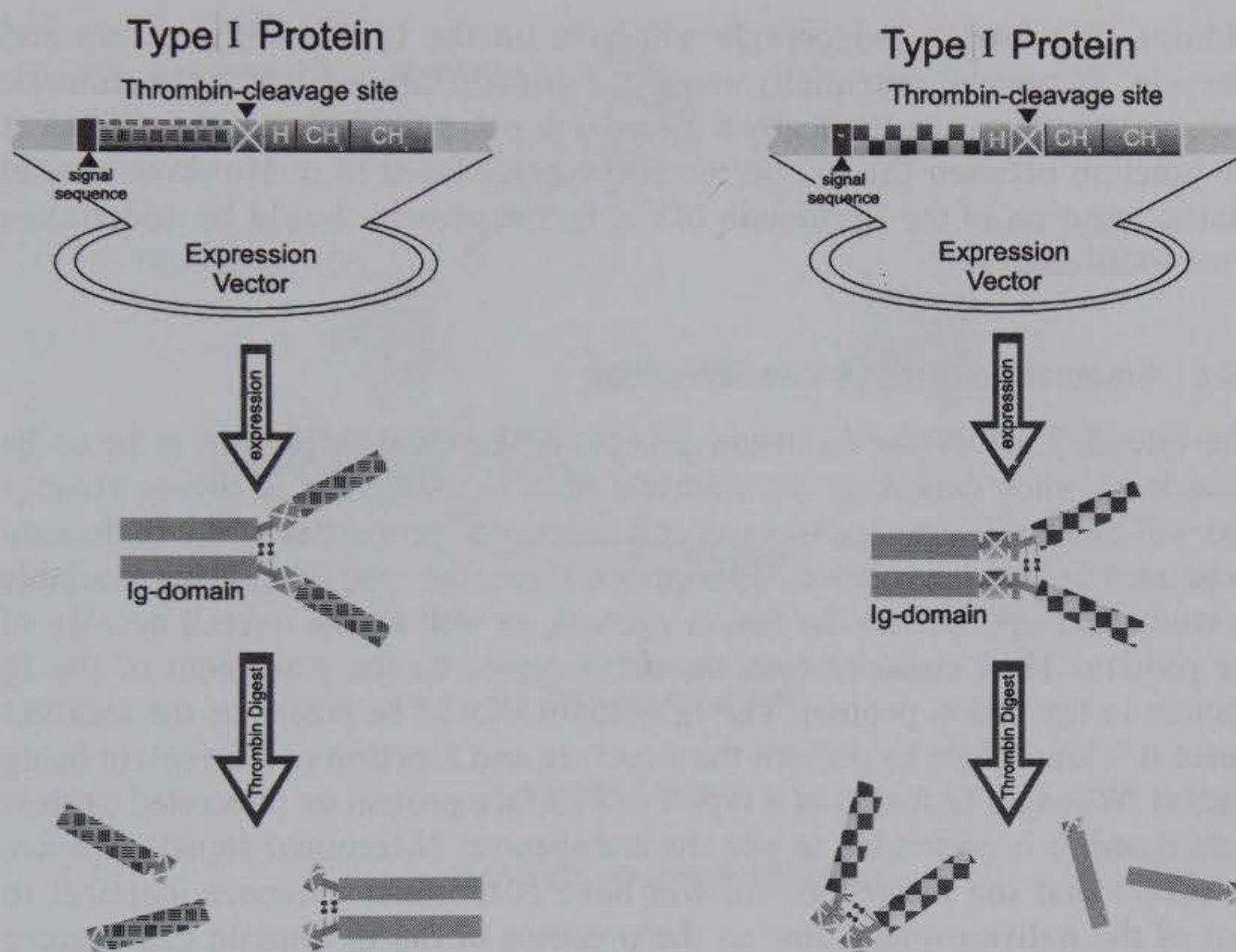


Figure 8.2 Construction of chimeric genes containing cleavable Ig domains. Schematic representation of Ig fusion proteins containing thrombin cleavage sites upstream or downstream of the hinge (H) domain. In the former case, treatment of the fusion protein with thrombin results in the release of the monomer of the polypeptide fused to the Ig constant region. In the latter, thrombin treatment results in the release of a dimer of the polypeptide fused to the Ig constant region.

sequence, proteinase cleavage results in a covalent dimer of the protein of interest that no longer has the functional activities contained within the CH2 and CH3 domains of the Ig constant domain. Examples of protease recognition sites engineered at the junction of Ig fusion proteins domains include thrombin²⁶ and Genenase I.²⁷ It should be noted that the hinge region of human IgG1 contains the sequence E-P-K, which is similar to a thrombin cleavage site in actin.²⁸ In some Ig fusion proteins this nonclassical thrombin site is susceptible to enzyme digestion,²⁹ allowing the separation of the IgG1 domains from the polypeptide to which it has been fused.

8.2.5 Additional Considerations

While the first concern with the use of the Ig fusion protein to study protein structure and function is the effect the Ig domain will have on the polypeptide being studied, consideration should also be given to the impact that the

addition of a foreign polypeptide will have on the Ig domain structure and function. To prevent potentially unwanted domain interactions in the chimeric proteins, small spacers (typically Ser-Gly rich polypeptides) can be included at the junction between the Ig and the polypeptide fused to it. However, careful characterization of the Ig domain of the fusion protein should be undertaken prior to its use.

8.2.6 Summary of Design Considerations

The intended use of the Ig fusion protein is the most important issue to be considered when designing the chimeric gene encoding the Ig fusion protein. Use will dictate the species, isotype, and functional properties of the Ig domain to be used in the construction. This choice affects the type of reagents available to study and manipulate the fusion protein, as well as the overall activity of the protein. Then consideration should be given to the placement of the Ig domain in the fusion protein. The Ig domain should be placed in the location where it is least likely to perturb the structure and function of the protein being studied. When an Ig fusion of a type I cell surface protein or a secreted protein is designed, it is preferable to use the endogenous N-terminal signal sequence to ensure that the fusion protein will have N-terminal sequence identical to that of the native protein. Just as the presence of the Ig domain can perturb the structure and function of the polypeptide to which it is fused, it is possible that the additional sequence might perturb the structure and function of the Ig domain of the fusion protein. Thus, it is important that this domain be fully characterized to ensure that the fusion protein has the expected activities (binding Protein A, complement, Fc receptors, etc.)

8.3 Ig FUSION PROTEINS: EXPRESSION, PURIFICATION, AND CHARACTERIZATION

This section reviews the methods used to produce small quantities of proteins for research use. Details of the techniques required for large-scale expression are discussed in Chapter 10.

8.3.1 Expression of the Ig Fusion Protein

The chimeric gene encoding the Ig fusion protein is typically subcloned into a mammalian expression vector designed for either transient and/or stable expression in mammalian cell lines (Fig. 8.3). Regardless of the final method of protein production, it is always prudent to test the construct by transient expression in an easily transfected cell line such as the SV40 transformed African green monkey kidney cell line (COS). The goal of this test is to ensure that the expression vector directs the expression of an Ig fusion protein of the expected molecular weight. Also, if the fusion protein is expected to be secreted,

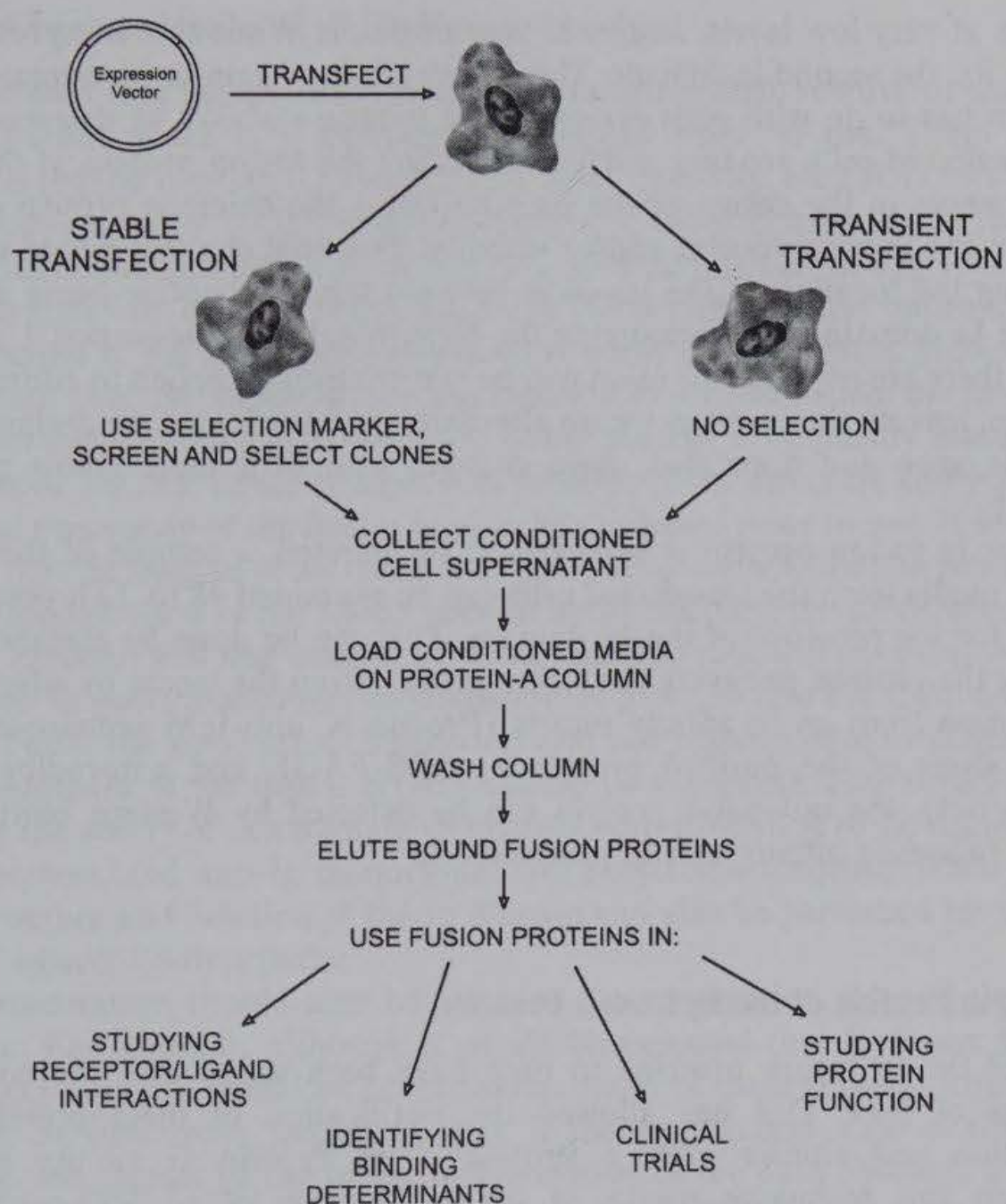


Figure 8.3 Production, purification, and use of the Ig fusion protein. Flow diagram showing how the plasmid encoding the Ig fusion protein directs either the stable or transient expression of the fusion protein. The fusion protein is purified from the spent supernatant of these cells and used as indicated.

a sample of spent tissue culture media can be tested for the presence of protein with reagents directed to the Ig moiety.³⁰

In a typical experiment, 48 h following the transfection of cells with the mammalian expression vector, the cells are fixed, permeabilized, and the intracellular expression of the Ig fusion protein examined by immunofluorescence using fluorescein conjugated anti-Ig antibodies. If the fusion protein cannot be detected intracellularly, the most likely explanation is that there is a problem with the chimeric gene, the sequence of which should be checked to ensure that the gene fragments that make up the chimeric gene are in frame and correctly located between the promoter and sequences required for mRNA polyadenylation. Less likely, but possible, is that the fusion protein

is made at very low levels, and/or is very unstable. While the first problem is easy to fix, the second is difficult. The researcher can begin to investigate if the problem has to do with gene expression or protein stability by determining if the transfected cells produce mRNAs encoding the fusion protein. If they do, then changes in the design of the gene encoding the chimeric protein can be made to enhance expression and/or stability. Potential changes would include changing the location of the junction between the polypeptide being studied and the Ig domain and/or changing the N-terminal signal sequence. Unfortunately, there are no guidelines that can be systematically applied to address this problem, leaving the investigator no alternative but to change the design of the chimeric gene and hope that these changes lead to a more stable protein product.

If the Ig fusion protein is expected to be secreted, a sample of the tissue culture media from the transfected cells can be examined 48 to 72 h posttransfection for the presence of the Ig domain. This can be done by metabolically labeling the protein, purifying the fusion protein from the media by adsorption and elution from an Ig affinity matrix (Protein A, anti-IgM sepharose, etc.), and analysis of the purified protein by SDS-PAGE and autoradiography. Alternatively, the unlabeled protein can be detected by Western blotting or ELISA following affinity matrix purification.

8.3.2 Purification of the Ig Fusion Proteins

Most of the Ig fusion proteins to date have been made with the constant domains of IgG. This has allowed the purification of these proteins by adsorption and elution from a Protein A or Protein G affinity matrix. However, this technique results in the purification of all IgG-containing proteins found in the spent supernatant of the transfected cells. Thus, if bovine serum were added to the tissue culture media, Protein A or Protein G purification would result in the isolation of both the Ig fusion protein and the bovine serum-derived Ig. If this isolation is a concern, the transfected cells should be placed in serum-free media. The method of elution from the affinity matrix should also be considered. Low or high pH buffers are often used to elute the fusion protein from the affinity matrix. However, these protocols can lead to an irreversible denaturation of the binding and/or active site of the non-Ig portion of the fusion protein, rendering the fusion protein unusable for most applications. Thus if at all possible, mild elution conditions should be employed when eluting the fusion protein. For example, imidazole-containing buffers can often be used with good results.³¹ However, a variety of purification protocols may need to be evaluated for each protein. Because of the possibility that the purification protocol might affect the activity of the non-Ig portion of the fusion protein, the investigator should examine the intended use of the fusion protein and avoid protein purification if not needed. For many applications it is not necessary to purify the fusion protein.

8.3.3 Characterization of the Ig Fusion Proteins

It is prudent that the characteristics of the fusion protein relative to the native protein from which it is derived be examined prior to use. These include function (ligand binding if a receptor, enzymatic activity, etc.), structure (ability to be recognized by mAb and in particular non-blotting mAb which recognize conformational epitopes), and its posttranslational modification (glycosylation, phosphorylation, etc.). In many instances there is little or no information on the function of the native protein, indeed in many cases Ig fusion proteins have been used as a tool to investigate the function of proteins. Also, the number of reagents available to characterize the fusion protein is frequently limited. Yet, it is important that as many aspects as possible of the structure and posttranslational processing of the fusion protein be examined prior to use. It should be noted that, at least in some cases, it is possible to manipulate the posttranslational modification of the fusion protein by transfecting the DNA encoding it into a different cell line and/or by cotransfecting plasmids encoding enzymes involved in posttranslational modification of proteins into the COS cells.

Likewise, the structure and function and posttranslational modification of the Ig domain of the fusion protein should be examined. This would include testing the ability of this domain to interact with Protein A or G, complement, Fc-receptors, and anti-Ig monoclonal and polyclonal reagents. While robust, the structure and function of the Ig domain can also be perturbed by presence of the adjacent polypeptide.

Consideration should also be given to the aggregation state of the fusion protein. For example, although it would be expected that Ig fusion proteins with IgG domains would be homodimers, many of these proteins form higher aggregates (tetramers, hexamers, etc.). If the aggregation state of the fusion protein will impact its use or the interpretation of the data obtained with it, it is important that it be examined directly before using the fusion protein. For example, if the fusion protein will be used in vivo, its aggregation state will affect its pharmacokinetics with higher aggregates having a shorter half-life than a homodimer.

8.3.4 Summary of Ig Fusion Protein Expression, Purification, and Characterization

While Ig fusion proteins can be made in a number of different expression systems, the chimeric gene can easily be tested by transient expression in a suitable mammalian cell line such as COS. The Ig domain can be used as a tag polypeptide both to examine the level of expression and for purification. The investigator should be aware that sometimes the gene encoding the chimeric protein encodes the desired protein, however this protein is made but not secreted. In this instance, changing the N-terminal signal sequence and/or the location of the junction between the two proteins may result in secretion of the protein. The fusion protein should be purified with reagents directed to the Ig

domain. Typically Protein A or G are used, however antibody columns can be used. Regardless of the affinity matrix used to purify the fusion protein, whenever possible mild elution conditions should be used to avoid denaturation of the fusion proteins. Once the protein has been prepared and purified, it is important that as many aspects of its structure and function be examined. Also, as the protein will be used over time, it is prudent to test its stability. Often Ig fusion proteins will aggregate over time affecting both their in vitro and in vivo properties.

8.4 Ig FUSION PROTEINS: APPLICATIONS

The use of the Ig fusion protein technology to study protein structure and function has been very successful and growing steadily since this technology was introduced almost 10 years ago. However, while there was early excitement about the possible application of this technique to develop novel drugs, it has only been recently that these fusion proteins are showing promise as potential drugs in the clinic. This section reviews the use of the Ig fusion protein as a laboratory tool and in the clinic.

8.4.1 Ig Fusion Proteins as Laboratory Tools

To date, the fusions of >70 different proteins with Ig constant domains have been reported in the literature. In most of these cases, the Ig fusion proteins have been used to identify novel receptor ligand interactions, map the binding determinants of proteins, and study the functional consequences of a receptor ligand interaction.

8.4.1.1 Use of Ig Fusion Proteins to Identify Novel Receptor Ligand interactions. Ig fusion protein technology has probably had its most significant scientific impact in this application. Ig fusion proteins of cell surface receptors or secreted proteins have been used to identify tissues or cells expressing molecules that can bind to the non-Ig moiety of the fusion protein, leading in many cases to its molecular characterization. The number of cases in which this technology has been successfully applied continues to grow steadily.^{7,8} The initial application of this technology for this purpose was in the identification of tissue distribution and molecular identification of the ligands for the cell adhesion molecules known as the selectins¹² and CD44.⁹ In these cases, Ig fusion proteins of the extracellular domains of E-selectin, L-selectin and P-selectin and CD44 were used to identify cells and tissues that express the ligands. These studies were then extended to identify their carbohydrate ligands as well as proteins that are decorated with these carbohydrates. These same fusion proteins were also used to determine which domains of the selectin proteins were required for ligand binding, demonstrating that this technology

could also provide important information about the activity of different domains of a protein (see Section 8.4.1.2). Other examples of the use of Ig fusion proteins to identify novel receptor ligand pairs include the identification of the Htk, REK7, CD22, and Sp,³²⁻³⁵ among others.

These early studies also showed that Ig fusion protein technology can result in unique laboratory tools. As was mentioned above, CD44-Ig was used to demonstrate that CD44 is a receptor for the glycosaminoglycan hyaluronan. Hyaluronan, due to its conserved structure across species, is nonimmunogenic.⁹ Thus antihyaluronan antibodies of good quality, which can be used to study the tissue distribution of this very important molecule, have not been available. CD44-Ig was the first antibody-like reagent that became available to study hyaluronan, and it led to the finding that CD44 is a hyaluronan receptor.

Another example of a fusion protein that was initially used in a ligand identification study but that also was found to be a reagent with unique properties is CD6. CD6-Ig was used to identify ALCAM as a CD6 ligand. Repeated attempts to obtain mouse antihuman ALCAM mAb that blocked CD6-ALCAM were unsuccessful. When the region of ALCAM responsible for CD6 binding was mapped and its amino acid sequence compared in human and mouse ALCAM it was found to be identical. This provided a molecular explanation of why it was not possible to generate mouse antihuman ALCAM antibodies that bound to the region of ALCAM responsible for CD6 binding. Therefore CD6-Ig is the only anti-ALCAM reagent currently available for blocking CD6-ALCAM binding.¹³

8.4.1.2 Use of Ig Fusion Proteins to Identify Binding Determinants. Ig fusion protein technology has also been used successfully to map regions as well as individual residues in a protein involved in binding. The utility of the Ig-fusion proteins to map binding determinants was recognized early on and was first applied to mapping the domain requirements for the ligand binding for E-selectin.¹² In these studies, Ig fusion proteins of E-selectin and L-selectin and chimeric E-/L-selectin proteins containing different E-selectin and L-selectin domain combinations were used to show that the ligand binding specificity of the selectins is mediated predominantly by the amino terminal C-type lectin domain and the adjacent EGF-like domain is needed for the selectin ligand binding. The use of Ig fusion proteins to examine the activity of the different protein domains is now well established. Other examples of the use of this technology to map functional domains include studies on CD31²² and human natriuretic peptide receptor,^{36,37} among others.

Ig fusion proteins containing single point mutations have also been used to map the location of residues involved in binding. For example, Ig fusion proteins of E- and P-selectin containing single point mutations in the lectin domain of the selectins were used to identify residues that are key to the interaction between the selectins and their carbohydrate ligands. Similarly, Ig fusion proteins of CD22, gp39, CD40, and Fas, among others, were used to

identify residues that are key to the interaction between these receptors and their ligands.³⁸⁻⁴²

8.4.1.3 Use of Ig Fusion Proteins to Study Protein Function. Ig fusion proteins have been used to study protein function. Examples include the use of CD80 and CD86 fusion proteins to characterize the signals sent by the two counter receptors for these proteins, CD28 and CTLA4, respectively.⁴³ Similarly, an Ig fusion protein of VCAM-1 was used to show that the interaction between VCAM-1 and its receptor VLA-4 results in the trigger leukocyte activation.⁴⁴ The use of Ig fusion proteins to study protein function has now become a widely implemented technique that has been applied to study the function of Heregulin, and Trk A, B, and C, among others.^{25,26}

8.4.1.4 Heterodimeric Ig Fusion Proteins. The ability of Ig fusion proteins to assemble in the cell to form a homodimer can be exploited to generate heterodimeric Ig fusion proteins. In this case plasmids encoding two different Ig fusion proteins can be cotransfected into a mammalian cell line. These cells then direct the expression of three different proteins: the two homodimeric Ig fusion proteins encoded by each of the chimeric genes and the heterodimer in which the two arms of the fusion protein are encoded by the two different transfected chimeric genes. These heterodimeric fusion proteins can be used to study the effects of cross-linking the molecules that bind to each of the two proteins fused to the Ig domains. Examples of this type of heterodimeric Ig fusion protein include LFA-3/VCAM-1-Ig and LFA-3/ICAM-1-Ig.⁴⁶ Similar techniques can be used to generate heterodimers between an Ig fusion protein and an immunoglobulin heavy and light chain. An example of such a bispecific molecule is the anti-CD3/CD4-Ig fusion protein. In this heterodimeric construct, one arm of this is derived from the heavy and light chain of a humanized anti-CD3 mAb. The other arm is derived from a CD4-Ig. This heterodimeric fusion protein was designed to redirect CD8⁺ CTLs to kill the remaining human immunodeficiency virus (HIV) infected cells in an HIV-infected patient.⁴⁷

8.4.2 Ig Fusion Proteins as Therapeutics

The use of Ig fusion proteins in a clinical setting has yet to be realized; however, there are indications that in the near future some Ig fusion proteins will become pharmaceutical agents (see, for example, Chapter 10, Table 10.1). This expectation comes from the very promising results obtained in human clinical trials of the tumor necrosis factor receptor (TNFR) and CTLA4-Ig. Recently it has been reported that an Ig fusion of the p75 subunit of the TNFR has shown significant benefit in the treatment of rheumatoid arthritis,⁴⁸ while an Ig fusion of the p55 subunit of the TNFR is being tested in patients with severe sepsis and septic shock⁴⁹ for a detailed discussion of pSSTNFR-IgG, see Chapter 9). Also, an Ig fusion of CTLA4 has shown efficacy in alleviating clinical

symptoms in psoriasis.^{50,51} These preliminary findings suggest that in the coming years, sufficient data will be available from a number of different clinical trials to determine if Ig fusion proteins will become commercially viable drugs. In addition to these clinical trials, some Ig fusion proteins are showing significant promise in preclinical animal models. For example, LFA3-Ig has been shown to prolong cardiac allograft survival in primates.⁵²

8.4.3 Summary of Ig Fusion Protein Application

The use of Ig fusion protein technology to study protein structure and function is now well established and easily accessible to most laboratories. Fusion proteins have been used successfully to identify new receptor/ligand interactions, to determine protein function(s), and domains or individual responsible for these functions. While Ig fusion proteins have yet to reach the clinic as novel therapeutic agents, promising results with at least two such proteins suggest that in the near future, Ig fusion proteins will likely become useful drugs.

8.5 CONCLUSION

Immunoglobulin fusion protein technology has proved to be a powerful tool to study the structure and function of proteins. While it is not the only technology presently available to tag proteins functionally, it is one of the most robust and accessible. A number of factors have led to the increased use of this technology. These include: (1) the very large number of research tools developed for use with antibodies that can be used with Ig fusion proteins, (2) the fact that the structural and functional characteristics of immunoglobulin constant regions are very well understood, (3) the robust nature of the Ig domain structure that is not easily perturbed by the presence of other structural motifs, and (4) the favorable in vivo properties (long half-life, etc.) of immunoglobulins retained by the Ig fusion proteins. While this technology has had an important impact in the laboratory, its clinical impact has yet to be demonstrated. However, the results of clinical and preclinical trials with four Ig fusion proteins, TNFR-p55, TNFR-p75, LFA3, and CTLA4, suggest that in the near future Ig fusion technology might yield its first drug.

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10

OPTIMIZING PRODUCTION AND RECOVERY OF IMMUNOADHESINS

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10.1 BACKGROUND

Immunoadhesins are chimeric, antibody-like molecules that combine the functional domain of a binding protein (usually a receptor or a cell-adhesion molecule) with the constant domains (usually the hinge and Fc regions) of an immunoglobulin. Immunoadhesins have many applications as research tools, for example in studies on receptor–ligand interactions. In addition, human immunoadhesins have potential applications in the clinic and provide an alternative to human monoclonal antibodies as agents that recognize and neutralize foreign or human proteins that possess pathologic functions.

The name *immunoadhesin* derives from its hybrid polypeptide structure. These molecules contain two parts: one part is derived from an immunoglobulin (Ig) molecule, and the other, from a protein that possesses an adhesion or a binding function. The Ig part of an immunoadhesin confers Ig-like properties, and the “adhesin” part determines target specificity of the chimeric molecule.

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Initial attempts to create this type of molecule employed fusions of the Ig-like V regions of the T cell receptor α and β chains with the constant region of IgG2a.¹ The V α -heavy chain fusion was assembled with light chain and secreted, when cotransfected with a normal λ light chain. In contrast, the V β -heavy chain fusion was not assembled with λ light chain and was not secreted. Attempts to reconstruct the entire T-cell receptor by coexpressing the V α -heavy chain fusion with a V β - κ light chain fusion were unsuccessful.

Subsequent attempts to create immunoadhesins were based on CD4, the primary cellular receptor of human immunodeficiency virus (HIV).² Because CD4 contains four Ig-like domains in its extracellular region, it appeared possible that part of CD4 could be substituted for the V regions of Ig without compromising the structure of either portion of the chimeric molecule. Various chimeric constructions were tested, and efficient expression and secretion of the resulting immunoadhesins by transfected mammalian cells was obtained with molecules that contained two or four V-like domains of human CD4 linked to the hinge, CH2, and CH3 domains of human IgG heavy-chain (reviewed in Ref. 3). In this chapter, we use this immunoadhesin structure as a prototype. Many other variations on this theme are possible, including molecules that contain light chains,⁴ and molecules based on other Ig subtypes, such as IgM,⁵ or IgA.⁶ In addition, many studies show that the adhesin portion of the molecule need not be derived from a protein that contains Ig-like domains. This was demonstrated first with the lymphocyte adhesion molecule L-selectin,^{7,8} and later with other molecules, including receptors for the cytokines tumor necrosis factor (TNF)⁹⁻¹¹ and interferon (IFN) γ ,^{12,13} and natriuretic peptide receptors.¹⁴ Further, the adhesin part of the molecule need not be derived from a cell surface protein such as a receptor, but may be derived from a soluble protein, e.g., the cytokines interleukin (IL) 2 or IL-10.¹⁵ Finally, the antigen-binding site of an antibody can be retained in the chimeric molecule, and the Fc region or some portion of it can be replaced with a protein possessing some desired biological function. This type of molecule has been called an antigen binding fusion protein.¹⁶ Examples of such chimeras include fusions with insulin-like growth factor 1 (IGF-1),¹⁷ TNF,¹⁸ and IL-1.¹⁹ These types of molecular constructs are detailed in Section I of this book.

In this chapter, we outline methods for the construction, expression, purification, and characterization of immunoadhesins. Additional information on immunoadhesins can be found in review articles^{3,16,20-23} and in Chapters 8 and 9 of this volume.

10.2 MOLECULAR DESIGN

The simplest immunoadhesin design combines the binding region of the adhesin protein with the hinge and Fc regions of an IgG heavy chain (Fig. 10.1). The resulting glycoprotein is a covalent homodimer, which resembles an

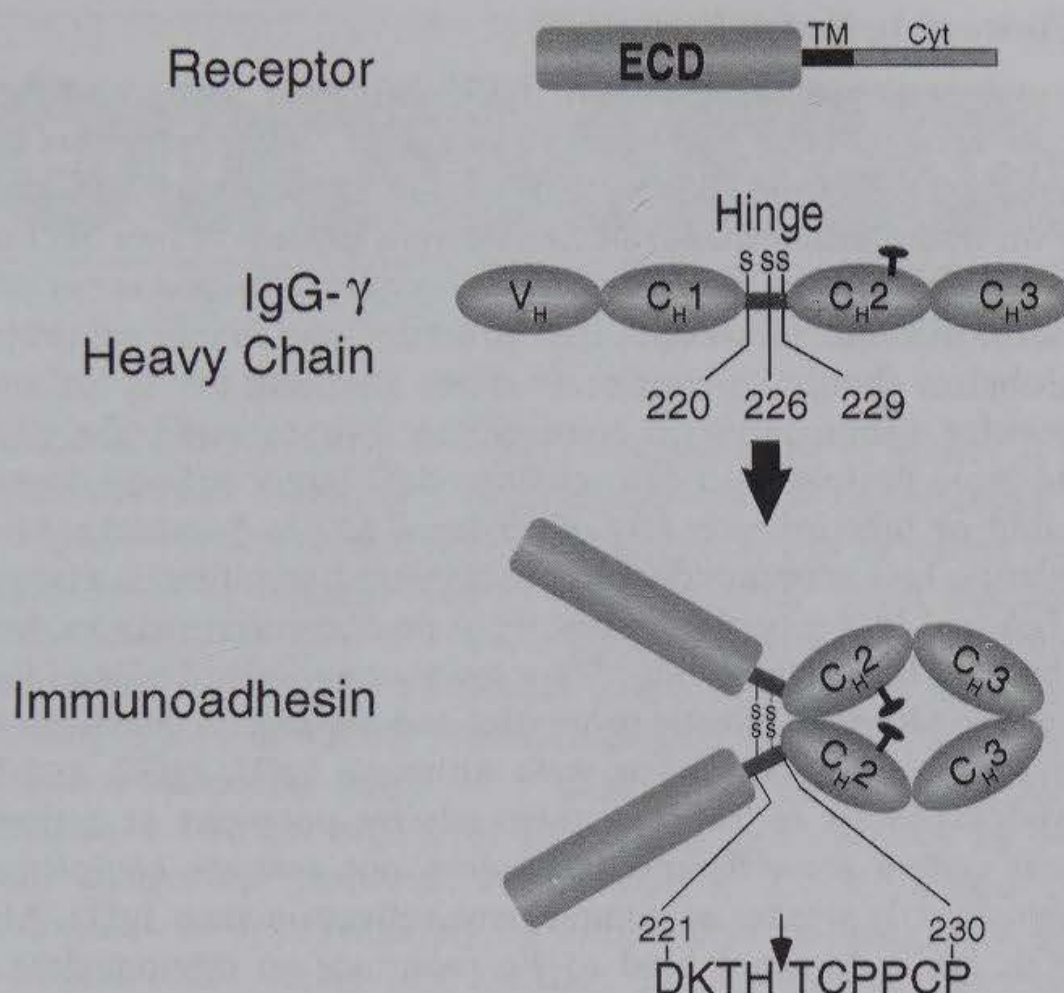


Figure 10.1 Schematic structure of a prototypic immunoadhesin. An immunoadhesin is derived from the parental ligand binding protein, in this case a type I transmembrane receptor, and an IgG1 heavy chain molecule. ECD, TM and Cyt refer to the extracellular, transmembrane and cytoplasmic domains of the receptor, respectively. The variable (V_H) and constant (C_H1, hinge, C_H2, and C_H3) regions of IgG heavy chain are shown. The domains are delimited by the following amino acid residues, according to the IgG1 Eu index³⁶: C_H1, 118–215; hinge, 216–230, C_H2, 231–344; C_H3, 345–447. The N-linked carbohydrate chain at Asn-298 within the C_H2 domain is shown. The three cysteine residues within the hinge region are involved in interchain disulfide bonds: Cys-220, H-L, Cys-226, H-H, and Cys-229, H-H. To construct the immunoadhesin, the receptor ECD is fused to the IgG hinge beginning at Asp-221, so that only Cys-226 and Cys-229 are present in the chimeric molecule (the hinge region sequence of the immunoadhesin is shown). Cys-220 is deleted, since the chimeric construct lacks a light chain. Thus, because the two hinge cysteines produce H-H chain disulfide bonds, the immunoadhesin is secreted from transfected mammalian cells as a homodimer. It can be purified using protein A affinity chromatography.

IgG antibody, but lacks CH1 domains and light chains. Deletion of the CH1 domain improves the association and secretion of heavy chains in the absence of light chains, probably because secretion is inhibited in the absence of light chain by interaction of the hydrophobic face of CH1 with the H chain-binding protein.^{5,7,24,25} In addition, joining the fusion partners at the flexible hinge usually facilitates proper folding and function of both parts of the immunoadhesin.

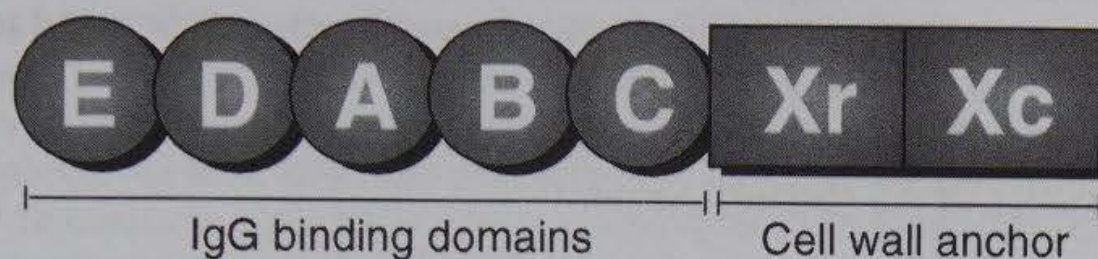
10.2.1 Choice of Ig Fusion Partner

For human immunoadhesins, human IgG1 has been used most frequently, although the IgG3 isotype,²⁶ or other Ig subtypes^{5,6} have been used as well. A major advantage of using IgG1 is that γ 1 immunoadhesins can be purified efficiently on immobilized *Staphylococcus aureus* protein A (see Section 10.6.1 and Box 10.1). In contrast, purification of γ 3 requires *Streptococcus* protein G, a less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the γ 3 hinge is longer and more flexible, so it can accommodate larger adhesin domains that may not fold or function properly when fused to γ 1. Another consideration may be valency: IgG immunoadhesins are bivalent homodimers, whereas the Ig subtypes IgA and IgM give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit.^{5,6} For immunoadhesins designed for in vivo application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG1, IgG2, and IgG4 all have in vivo half-lives of 21 days, their relative potencies at activating the complement system are different. IgG4 does not activate complement, and IgG2 is significantly weaker at complement activation than IgG1. Moreover, unlike IgG1, IgG2 does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG3 is optimal for complement activation, its in vivo half-life is approximately one-third that of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically defined allotypes are preferred. For example, IgG1 has only four such allotypic sites, two of which (G1m1 and 2) are located in the Fc region; one of these sites, G1m1, is nonimmunogenic. In contrast, there are 12 serologically defined allotypes in IgG3, all of which are in Fc; only three of these sites (G3m5, 11, and 21) have one allotype which is nonimmunogenic. Thus, the potential allotypic immunogenicity of a γ 3 immunoadhesin is much greater than that of a γ 1 immunoadhesin.

Human immunoadhesins are immunogenic in mice, and antibodies to the administered immunoadhesin can appear within 5–7 days. Therefore, if one's purpose is to carry out long-term studies in experimental animals, it is advisable to use fusion partners that are derived from the autologous species. For example, mouse IgG2a¹² and IgG1 (A. Ashkenazi, S. M. Chamow, D. Peers, and S. Marsters, unpublished results) have been used for fusion with murine adhesin partners in order to conduct long-term studies in mice. An alternative approach is to introduce the heterologous species immunoadhesin as a transgene.^{27,28}

10.2.2 Choice of Adhesin Fusion Partner

When a binding protein that interacts with the ligand of interest is known, this binding protein can be used as the adhesin fusion partner. In such cases, the



Box 10.1 Structure of Protein A.

Staphylococcal protein A (SpA) is a pathogenicity factor bound to the cell wall of the bacterium *Staphylococcus aureus*. It exhibits tight binding to IgG, IgA, and IgM from several different mammalian species including human.⁹⁶ First exploited as an immunological reagent in 1978,⁹⁷ SpA has been used extensively during the past 20 years, both in analytical and preparative applications. Its biochemical properties lend itself well to use as an antibody-binding ligand in affinity chromatography. SpA is a 524 amino acid protein with a molecular weight of 57 kDa.⁹⁸ The extracellular portion of SpA contains a tandem repeat of five highly homologous IgG-binding domains⁹⁹ designated (from the N-terminus) E, D, A, B, and C. Each domain consists of approximately 58 amino acid residues, arranged into three α -helices that overlay one another. The C-terminal portion of the sequence contains cell wall-binding and transmembrane domains, designated X_r and X_c, respectively. Domains E, D, A, B, and C all bind to the Fc fragment of IgG,¹⁰⁰ whereas domains D and E bind to Fab fragments at a site distinct from the antigen combining site.^{100,101} SpA binds to IgG multivalently—SpA and rabbit IgG have been shown to form soluble complexes in which the stoichiometry is IgG₄-SpA₂.¹⁰² SpA contains no cysteines. Due to its tandem domains, predominantly α -helical structure, and its lack of disulfide bonds, SpA is structurally resilient. Its activity is recovered quantitatively after exposure to extremes in pH, making it an excellent ligand for affinity chromatography. Moreover, its capacity for IgG is high—typically 20–30 mg/ml for human IgG. Recombinant SpA, truncated to contain only the five Ig binding domains, has been produced in *E. coli* and is used for several commercially available affinity supports. Immunoglobulins as well as Fc-containing fusion proteins can be produced in high purity and yield using SpA.

portion of the binding protein that contains the ligand-binding site (e.g., the ectodomain of a cell-surface receptor or subdomains thereof) can be used as the adhesin region. In cases where only one member of a binding pair is known, an immunoadhesin based on the known member can be used to identify the unknown binding partner.^{7,8,29–35} The adhesin domain may be derived also from a soluble ligand. For example, immunoadhesins based on the cytokines IL-2 and IL-10 have been reported.¹⁵ For most applications, if the adhesin

contains its own natural hydrophobic leader sequence, it is advisable to include that sequence in the immunoadhesin to achieve efficient secretion and processing of the recombinant polypeptide.

10.2.3 Design of the Fusion Junction

The design of the fusion junction depends on the particular fusion partners. If the adhesin is a Type I cell surface receptor, joining the extracellular sequence just upstream of the hydrophobic transmembrane region is usually suitable (Fig. 10.1). If the adhesin has a known domain structure, one can attempt to delete domains that are not required for binding; in such cases, it is important to place the junction at residues located between domains, to avoid misfolding. With respect to the parental Ig, a useful joining point is just upstream of the hinge cysteines that form the disulfide bonds between the two heavy chains. For IgG1 there are two such cysteines, located at positions 226 and 229.³⁶ A junction design that we use frequently is to place the codon for the C-terminal residue of the adhesin part of the molecule directly upstream of the codons for the sequence DKTHTCPPCP of the γ 1 hinge.^{9,13,24}

10.3 EXPRESSION

To date, all recombinant proteins from mammalian cells used for pharmaceutical purposes are based on expression vectors with constitutive promoters. Constitutive overexpression of complex recombinant proteins in mammalian cells does not pose a major metabolic burden on the cell hosts and does not significantly affect growth and other activities of genetically engineered cells. This is a major difference from products made in *E. coli*, which are mostly derived from expression vectors with inducible promoters.

Vector constructions are similar for many recombinant glycoproteins expressed in mammalian cells. SV40 promoter-based vectors have been used for immunoadhesins such as CD4-IgG²⁴ or p55 TNF receptor-IgG (TNFR-IgG).⁹ In our work on CD4-IgG and TNFR-IgG, both of which are Type I transmembrane glycoproteins, cDNA constructs were used in which the Fc-segment of the heavy chain of an IgG1-gene-cDNA was directly fused with the amino-terminal ectodomain of the respective receptor (Fig. 10.1). We found that proper folding of the fusion protein occurs if the molecule is constructed such that the two cysteines encoded within the hinge region of the Ig heavy chain (C226 and C229) serve to form two interheavy chain disulfide bonds (Fig. 10.1). Significantly, the heavy chain cysteine involved in H-L chain bonding (C220) is deleted from the fusion polypeptide.

10.3.1 Vectors for Transient Expression

Transient expression in mammalian cells is the method of choice for initial expression of a recombinant immunoadhesin. Very popular for transient expression are human embryonic kidney 293 cells which have been immortal-

ized by transfection with adenovirus DNA.³⁷ It has been shown that these cells express intracellularly the adenovirus-derived E1A protein. Subpopulations of these cells have been transfected stably with DNA sequences coding for EBNA protein from Epstein-Barr virus or the large T-antigen of SV40. These cell lines are reported to have superior characteristics as hosts for transient transfection.³⁸ In transient transfections with human embryonic kidney 293 cells, using calcium phosphate, 1–10 $\mu\text{g}/\text{ml}$ of secreted recombinant protein has been observed in cell supernatants.^{39,40} More recently, transient expression in mammalian cells has been used to produce larger quantities of desired proteins (Section 10.4.6).

10.3.2 Vectors for Stable Expression

The choice of a vector for stable expression in mammalian cells is driven mainly by the choice of the host system. The most successful and frequently used host system for mammalian expression is Chinese hamster ovary (CHO) cells; other cell lines have been used as well, including baby hamster kidney (BHK) cells and the myeloma cell line SP2/0. In this chapter, we focus on the CHO-DHFR expression system, since this is what we have used for all our immunoadhesin proteins; moreover, for many groups, it continues to be the preferred system for large-scale production of recombinant proteins.

A mutagenized cell line lacking enzyme activity for dihydrofolate reductase (DHFR), developed by Urlaub and Chasin⁴¹ for metabolic and genetic studies, can be propagated only in media supplemented with glycine, hypoxanthine, and thymidine (GHT). When these cells are transfected with a functional expression vector for DHFR, cells that have acquired a functional DHFR gene no longer require the presence of GHT, and so they can be selected and expanded in media lacking GHT. A second expression cassette for the protein of interest can be included in the DHFR plasmid or cotransfected as part of a second vector. Screening a number of clones in GHT-selective media leads to the identification of cells that coexpress the product of interest with DHFR.

Expression in CHO cells can be augmented by exposing recombinant cells to methotrexate (MTX), a folate derivative that blocks DHFR activity completely and irreversibly. Exposure to low concentrations of MTX selects cells that express DHFR at an elevated level. Frequently, the elevated DHFR expression is the result of an increased copy number of the recombinant sequence. Stepwise selection with elevated concentrations of MTX can be repeated many times, and may result in the isolation of cells that contain dramatically increased copy numbers of the transferred genes. The same principles as those discussed previously for the CHO system apply also to other systems with stable integration of exogenous DNA.

One can choose from two vector strategies to generate recombinant CHO cells via the DHFR selection route. To introduce DNA sequences for both the protein of interest and the protein that is the selective marker, either (1) a single vector, or (2) a set of two or more vectors, is assembled. A two vector system offers increased flexibility to improve expression of oligomeric proteins (e.g.,

immunoglobulins). With a two-vector system, one can optimize the molar ratio of vectors for individual polypeptide chains within the transfection cocktail, assuring that appropriate amounts of each polypeptide are produced.

10.4 LARGE-SCALE PRODUCTION

Immunoconjugates are usually designed to be very effective antagonists, inhibiting the interaction of a receptor with its ligand. When considered for *in vivo* applications, they are needed in relatively large doses—the annual requirement for production can be in excess of one kilogram. Large-scale production requires an efficient process. Productivity from mammalian cell-based processes for recombinant proteins has changed dramatically during the last decade. While a concentration of about 50 mg of recombinant human tissue plasminogen activator³⁹ per liter of cell culture supernatant was regarded as good productivity in 1985, today's cell culture processes have been shown to provide 500 mg/L or more of recombinant antibody in a batch process. This improvement in volumetric productivity is the result of several advances in cell culture technology: (1) a more profound understanding of the physiology of cells, (2) better control of process parameters, and (3) richer and more sophisticated media formulations to support higher culture densities in bioreactors. As an example, $1\text{--}2 \times 10^6$ cells per ml of culture medium were considered peak densities for CHO cells in 1985. Now, with some CHO cell lines in optimized processes for batch cultures, cell densities 4–5 times higher have been obtained.

10.4.1 Laboratory Scale Cell Culture

Here we focus on process development, using “deep tank technology,” for manufacture of human therapeutic proteins. During the early phase of development, progress depends on regular production of small quantities of the new protein to supply efforts in recovery and assay development.

One strategy to speed early progress is to use parallel cell culture systems—one for production of R&D material and the other for cell culture process development. For example, early in the development of CD4-IgG, an extended perfusion culture with cells in suspension was operated in a 2 L bioreactor for a period of 3 months. With very high perfusion rates (up to 6 reactor volumes/day), supporting very high cell densities (up to 1.4×10^8 cells/ml, corresponding to 35% packed cell volume), this culture provided gram quantities of CD4-IgG. With this material—much more abundant than might have been expected during this early phase of development—useful insights were gained, both of the biology of the molecule and of available recovery options. In cell culture, meanwhile, we were able to assess different media compositions and the effect of these on product quality and cell productivity.

10.4.2 Transfection, Amplification, and Clonal Selection

The most frequently used method for introduction of DNA into mammalian cells is the calcium phosphate DNA coprecipitation method. This method was developed about 20 years ago by Graham and van der Eb.⁴² It is a reliable method for stable and for transient expression of recombinant proteins from mammalian cells. Recent insights in the physicochemical parameters for the generation of calcium-phosphate DNA coprecipitates have improved its reliability and efficiency.^{40,43}

Following transfection of the DHFR-deficient CHO host cell line with a vector or vector combination containing a functional DHFR cassette, cell lines were established from colonies emerging under selective conditions. We used an approach that provided an increased frequency of high expressor cell lines among all emerging cell colonies (Fig. 10.2). Two days after transfection using the calcium-phosphate method, cells were plated into media lacking glycine, hypoxanthine, and thymidine (GHT medium). For the GHT selection, only a portion of the transfected cell population was used. The majority of transfected cells was plated into media containing various concentrations of MTX (see Section 10.3.2). In most cases, concentrations of 30, 100, and 300 nM were used. We preferred to isolate, expand, and analyze cell colonies that emerged under selection from the highest concentration of MTX, since these colonies often show the highest level of coexpression for the protein of interest. This approach allowed us to reduce the number of cell lines for expansion and analysis to not more than 100 (24 clones from GHT medium, and 24 each at 30, 100, and 300 nM MTX).

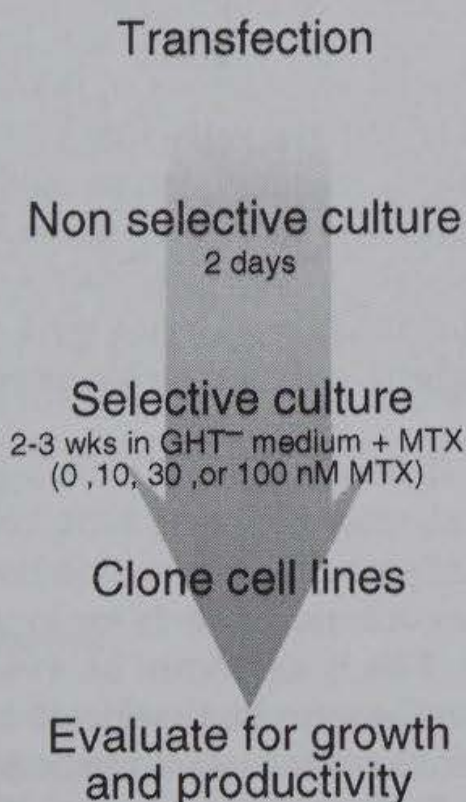


Figure 10.2 Strategy for transfection of DHFR vector cocktails into CHO cells and identification of high producer cell lines.

As many as 10 cell lines were carried through to the final evaluation phase. Growth behavior, adaptability to suspension conditions, robustness for serum-free culture, and scalability were the criteria used for evaluation of cell lines. Also, responsiveness to medium additives that enhance productivity and the impact of MTX removal were assessed. Growth for extended periods of time in non-selective medium and assessment of specific productivity allowed us to develop confidence in the long-term stability of candidate cell lines as good production hosts.

Two to three cell lines were finally considered acceptable for scale up. These candidate cell lines were transferred into good manufacturing practice (GMP) facilities for cell banking, while further process development continued. We chose to use robust methods for the final process. As a robust process engineering method, a batch cell culture process was developed in which product derived from a fixed-period production phase was harvested in one step. In processes developed more recently, extended batch approaches with feeding of culture additives have been used.

10.4.3 Cell Banking

Cells contained in a master cell bank are stored frozen in liquid nitrogen. The freezing of cells for banking was done according to standard cell culture techniques,⁴⁴ utilizing 10–20% dimethyl sulfoxide (DMSO) in fresh cell culture medium, into which cells were taken up, after gentle centrifugation and removal of spent medium. In most cases, cryovials with 1 ml of cell suspension were immersed directly into the vapor phase of liquid nitrogen storage tanks.

Selection of a cell line from which to establish the master cell bank is a critical decision. The generation of a master cell bank, usually 50–500 vials containing highly concentrated suspensions of mammalian cells, requires the use of facilities meeting GMP requirements. Cell genetics, cytogenetics, production stability, growth pattern, viability status, and absence of adventitious agents all must be evaluated. We minimized the risk inherent in this process by generating multiple prebanks from which vials were thawed and cultivated. Cells derived from these individual seed trains were used both for comparison in process development efforts and for stable productivity assessment in a standardized assay.

It is important to note that cells derived from a prebank and master-bank are not necessarily identical, since they may have been treated differently. For example, cells that eventually go into production have to endure two freezing and thawing cycles, whereas cells under study for long-term stability experience only one freeze-thaw cycle. This is significant because, unlike prokaryotic cells, mammalian cell populations have to be considered heterogenetic. Exposure to variations in culture conditions—the most drastic being freezing and thawing of cells in specialized media formulation—can often result in a change in overall performance of the cell line under study.

10.4.4 Growth of the Seed Train

To begin development of a seed train, ampules containing viable cells are thawed and the cells are expanded. The quantity of cells in the vial may vary, according to procedure. If, for example, the cell line had been previously adapted to growth in suspension culture, the number of viable cells should be large enough to inoculate a small spinner flask. To seed a spinner at a minimum volume of 50 ml with a seed density of 3×10^5 cells/ml, the thawed vial should contain at least 15×10^6 cells.

Adaptation to growth in suspension could take anywhere from 3 weeks to 3 months, depending on the particular cell line. Several subcultivations of cells grown on plastic cell culture substrates (splits) were performed, to produce a homogeneous population in 850 cm² roller bottles. Confluent cultures of these cells in roller bottles were trypsinized, and the cells were suspended in media suitable for growth in spinner flasks. One roller bottle would contain enough cells to prepare about 200 ml of a suspension culture. Cell suspensions established in this manner were then processed as described above, until a robust cell seed train was established.

Growth of the seed train can be accomplished in medium with or without added fetal bovine serum (FBS). Most cell lines required 5% or less FBS and, during the last few years, FBS-free culture media have been developed and have proved to be reliable.

After an initial lag phase of 24–48 h, cells began to grow in spinners, reaching a final cell density of $0.8\text{--}2 \times 10^6$ cells/ml within 4–5 days. However after 5–6 days, a subcultivation was initiated, even when growth was poor. The protocol for the subcultivation was adapted to the performance of the cell line. In the case of poor growth, e.g., a cell density of $0.8\text{--}1 \times 10^6$ cells/ml, the medium was removed by gentle centrifugation and replaced with fresh, prewarmed medium, utilizing a volume of medium that adjusted cell density to about 0.3×10^6 cells/ml. In the case of excellent growth, e.g., a density of $1.5\text{--}2 \times 10^6$ cells/ml, a subcultivation method was used which simply diluted out the medium, adjusting the cell density again to about 0.3×10^6 cells/ml. Thus, the seed train was kept at a constant volume, by repetitive subcultivation of spinners or bioreactors with a smaller working volume.

In summary, the goal of the seed train development is to establish a robust procedure and a reliable growth performance of the cells under study. Critical for success is the availability of media formulations that have been specifically developed for this purpose.

10.4.5 Cell Culture Production

We have preferred to do process development work using bioreactors of nominal 2 L volume, with a maximal working volume of 1.5 L. To optimize process parameters, a battery of bioreactors was used. To compensate for reactor variability, duplicates or triplicates of the same condition were employed.

One key parameter was the need to create sufficient cell mass (cell number) for the inoculation of the production vessel at large scale. Therefore, factors such as media composition, stirring rate, oxygenation, pH- and osmolarity control—all of which allow for high growth rates and a high final density of the cell suspension prior to transfer to a larger tank—were optimized. If the cell line had not been adapted to growth in serum-free media, a reduction of the required serum to less than 5% was another development goal. Finally, since high cell viability at the start of production phase was found to be essential for high productivity, much effort went into developing an optimum procedure for transferring cells between vessels.

Production phase is defined as the final phase of the cell culture process, which is optimized for product accumulation in a large-volume bioreactor. For the inoculum train, a complete exchange of the culture medium prior to the start of production phase can result in improved performance and productivity. We evaluated various methods for this purpose. Initially, exchange of medium by centrifugation was used; however, this method was not suitable for large-scale operation. Therefore, other methods, such as tangential flow filtration, were evaluated. Tangential flow filtration⁴⁵ was found to be an excellent means by which to achieve medium exchange, insofar as cell viability remained high during the operation. Consequently, this method of medium exchange was adopted for large-scale use.

We found that achievement of five objectives was necessary for optimal productivity during production phase: (1) rapid attainment of high cell density, (2) maintenance of high cell density and viability for the entire production run, (3) manipulation of cellular metabolism to promote high specific productivity, (4) maintenance of consistent product quality, and (5) selection of an efficient cell harvest technology capable of sustaining cell integrity and product quality.

10.4.6 Rapid Manufacturing of Non-clinical Grade Proteins for Evaluation and Analysis

Transient gene expression in animal cells, when performed at the 1–10 L scale, represents a fast approach for the synthesis of tens to hundreds of milligrams of recombinant proteins. In addition to virus-mediated nucleic acid transfer, calcium-phosphate, polyethylenimine, cationic lipids, and electroporation have been reported to be useful in promoting efficient DNA transfer. Efforts to increase the scale of such production processes to 100 L are underway in many laboratories, since the classical approach for recombinant protein production in animal cells using stable integration of exogenous DNA is costly, very time consuming, and requires substantial know-how in many areas of process development and process engineering.

Human embryonic kidney 293 cells seem to be the most popular host system for these efforts. Attempts to use CHO cells in transient expression approaches have not been successful. Expression levels for secreted proteins like CD4-IgG, TNFR-IgG, or rtPA are only 1-10% of those observed in human embryonic

kidney 293 cells (F.M. Wurm, unpublished). For virus mediated transfer of genetic information to animal cells, Sindbis virus-based RNA transfer to BHK, CHO, or Raji cells^{46,47} has become popular recently. Lastly, baculovirus-mediated DNA transfer to insect cells is used successfully in many laboratories (for example, see Ref. 48). Following is a brief discussion of the various transient expression technologies that are currently in use.

Calcium phosphate-mediated DNA transfer. Jordan et al.⁴⁹ reported the use of calcium phosphate-mediated DNA transfer to suspended 293 cells, grown in a bioreactor or in spinners. A most critical factor in these transfections is the removal of DNA-calcium phosphate precipitates 3–6 h after transfer of the DNA to the reactor. Persistent precipitates are toxic to mammalian cells, most pronounced in the absence of serum and in cultures with low cell densities. In small-scale experiments (e.g., cell culture plates), DNA-calcium phosphate coprecipitates are removed or solubilized by a medium exchange; in reactors, solubilization is mediated by a deliberate change of the culture pH, coupled with the addition of fresh, low-calcium growth medium.

Up to 3 mg of an rtPA variant has been obtained within 5 days upon transfection in 2 L mini-bioreactors with 1 mg of purified plasmid DNA transferred.⁴⁹

Polyethyleneimine as carrier for DNA transfer. Using cationic polyethyleneimine⁵⁰ or polyethyleneimine in combination with dioleoyl-melittin as carrier complexes for plasmid DNA, Schlaeger and coworkers achieved transient production of up to 1.4 mg/L of TNFR55-IgG with human embryonic kidney 293 (EBNA) cells grown in stirred tanks at 10 L scale.⁵¹

Lipid- and liposome-based DNA transfer systems. A number of lipid- and liposome-based DNA transfer systems have been developed over the last 5 years and are commercially available. Among the most popular ones are LipofectAMINE (GIBCO-BRL), DOTAP and DOSPER (Boehringer Mannheim), Transfectam (Promega), and CLONfectin (Clontech). These are intended for small-scale experiments and usually for attached cells. They work well in multiwell plates or in single 100 mm dishes, and they provide similar product concentrations for secreted proteins as the ones seen with calcium phosphate. None of these have been reported to be used at a scale beyond 100 ml of cell culture medium—most likely because they are very expensive. So far, the only nonviral systems that may allow scale-up beyond the 1 L scale are calcium phosphate and polyethyleneimine.

Electroporation for DNA transfer. Blasey and coworkers⁵² have pioneered the use of electroporation at 10 L scale. Using a commercial electroporator, COS cells, derived from a CellCube for expansion of cell mass, were electroporated and transferred subsequently to a 10 L bioreactor. Other cell lines, like CHO and human embryonic kidney 293 cells were tested

as well. Milligram quantities of product were harvested from the culture 7–12 days later. This technique appears to require some further improvement in order to achieve robustness and reproducibility.

Virus-mediated protein expression. Baculovirus mediated protein expression is popular in many laboratories.⁵³ The possibility that susceptible insect cells (*Spodoptera frugiperda* Sf9 cells, *Drosophila* Schneider cells) can be grown in suspension in stirred bioreactors or spinners allows for scale-up, so far reported up to the 100 L scale.^{54,55}

Using mammalian cells, semliki forest virus (SFV) has been used for rapid production of recombinant protein at 15 L scale with BHK cells.⁵⁶ The powerful promoter of this virus system allows for very high protein synthesis by infected cells. However, this results in increased lysis of cells,—a disadvantage for recovery. In CHO cells, high levels of the serotonin 5-HT receptor were shown following SFV-mediated gene transfer.⁵⁷

10.5 IMMUNOADHESIN STRUCTURE: IMPLICATIONS FOR PURIFICATION

The prototypic IgG1-based immunoadhesin has a disulfide-bonded homodimeric structure that resembles an antibody but lacks CH1 domains and light chains (Fig. 10.1). The subunit structure of the immunoadhesin can be analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). One technique is to label the transfected cells with ³⁵S-methionine and/or ³⁵S-cysteine and to precipitate the secreted immunoadhesin with protein A or protein G; then, the labeled immunoadhesin can be analyzed by SDS-PAGE and autoradiography.⁵⁸ Alternatively, serum-free supernatants from transfected cells can be subjected directly to SDS-PAGE, electroblotted onto nitrocellulose, and the immunoadhesin can be visualized on the blot with labeled protein A or G, with antibodies, or with labeled ligand.

Fig. 10.3 shows an example of this type of analysis; in this case, with a protein A-purified preparation of human interferon γ receptor immunoadhesin (IFN γ R-IgG). The purified immunoadhesin was visualized directly by staining with coomassie blue (A), or transferred onto nitrocellulose and visualized with anti-Fc antibody (B), which confirms the presence of the Fc region, or with labeled ligand (C), which confirms the presence of the adhesin region in functional form. Under nonreducing conditions, the protein migrates with an apparent molecular weight approximately twice that of the reduced protein, confirming the molecule's disulfide-linked homodimeric structure.

Results with many such immunoadhesins show that fusion of the adhesin and the Fc regions usually does not perturb the folding of each domain. Comparisons of the binding function of the adhesin portion with those of the soluble or cell-surface counterparts generally show similar properties (e.g., CD4-IgG,⁵⁹ IFN γ R-IgG,^{12,13} or IL-1R-IgG⁶⁰). The Fc region also seems to

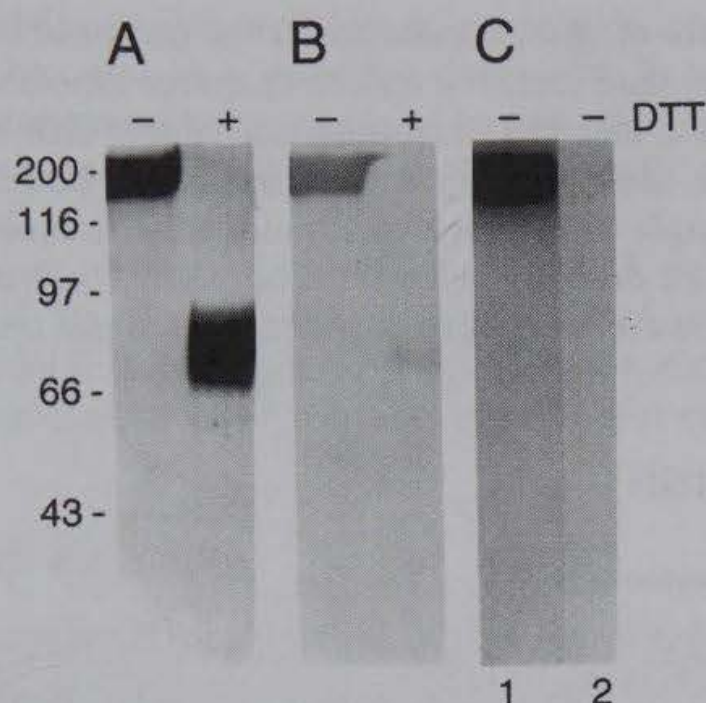


Figure 10.3 Subunit structure of an immunoadhesin as analyzed by SDS-PAGE and immunoblotting and ligand blotting techniques. Human embryonic kidney 293 cells were transfected with a vector directing transient expression of human interferon- γ receptor immunoadhesin (hIFN- γ R-IgG). The protein was recovered from culture supernatants and purified on protein A agarose. SDS-PAGE was carried out without (–) or with (+) reduction by 10 mM dithiothreitol (DTT). The protein was stained with coomassie blue (A) electroblotted onto nitrocellulose and incubated with (B) antibodies to IgG-Fc or with (C) ^{125} I-labeled hIFN- γ (10 mM) alone (lane 1), or with 1 mM unlabeled hIFN- γ (lane 2). Blots were developed with horseradish peroxidase-conjugated second antibody and 4-chloronaphthol (B) or by autoradiography (C). (Reprinted with permission from A. Ashkenazi and S. M. Chamow, *Immunoadhesins: An alternative to human monoclonal antibodies*, *Methods: A Companion to Meth. Enzymol.* vol. 8, 104–115. Copyright © 1995 by Academic Press, Inc.).

fold correctly, because it is recognized by antibodies and by protein A or protein G, and because it retains many of the effector functions characteristic of antibodies, such as binding to Fc receptors.²⁴

IgG1-based immunoadhesins are produced in mammalian cells as glycoproteins, due to the presence of a single N-linked glycosylation site within the CH2 domain (see Fig. 10.1). Additional glycosylation sites (both N- and O-linked) can be present within the adhesin portion of the molecule, as in the TNF and IFN γ receptor immunoadhesins.^{9,13} Heterogeneity of oligosaccharide sequences (particularly sialic acid residues) can produce overall molecular charge heterogeneity.^{61,62} In immunoadhesins as in antibodies, the mature heavy-chain polypeptide contains no C-terminal lysine, notwithstanding a C-terminal lysine predicted by the DNA coding sequence.⁶³ Rather, the C-terminal residue in the mature polypeptide is the penultimate glycine.³⁶ Presumably, the C-terminal lysine is removed during cellular processing of the nascent polypeptide.⁶⁴

One unique feature of immunoadhesins is the potential to display a charge dipole, resulting from their chimeric nature. A charge dipole will exist if the pI's of the adhesin domain and the Fc domain are widely different. Chimeras like this will behave non-ideally in separation techniques based on ionic charge. CD4-IgG is an example of this type of immunoadhesin molecule. Because the pI of the CD4 and Fc domains differ by more than 2 pH units, the behavior of this molecule in ion exchange chromatographic systems is difficult to predict.

10.6 PURIFICATION

10.6.1 Affinity Chromatography

A significant advantage of expressing adhesins as immunoadhesins is that they can be purified easily by affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of the immunoglobulin Fc domain used in the chimera. Protein A can be used to purify immunoadhesins that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains.⁶⁵ Protein G is recommended for mouse $\gamma 1$ and for human $\gamma 3$.⁶⁶ The matrix to which the affinity ligand is attached is most often agarose, but other matrices, are available. Mechanically stable matrices such as controlled pore glass⁶⁷ or poly(styrene-divinylbenzene)⁶⁸ allow faster flow rates and shorter processing times than can be achieved with agarose.⁶⁹

The conditions for binding an immunoadhesin to the protein A or G affinity column are dictated entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing feature of immunoadhesins is that, for human $\gamma 1$ -containing molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type.⁷⁰ Bound immunoadhesin can be efficiently eluted either at acidic pH (≤ 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. For cases in which the adhesin portion of the molecule is sensitive to treatment with acid, the latter method of elution is preferred.^{14,71} This affinity chromatography step can result in an immunoadhesin preparation that is $>95\%$ pure.

10.6.2 Other Chromatographic Methods

Other methods can be used in place of, or in addition to, affinity chromatography on protein A or G (see, for example, Refs. 72, 73). In our experience, immunoadhesins behave similarly to antibodies in thiophilic gel chromatography⁷⁴ and immobilized metal affinity chromatography.^{75,76} In addition, partitioning via aqueous two-phase extraction⁷⁷ or chromatographic recovery in expanded beds⁷⁸ have both been applied to antibodies and should be directly applicable to immunoadhesins. In contrast to antibodies, however, the behav-

ior of immunoadhesins on ion exchange columns is dictated not only by their isoelectric points, but also by a potential charge dipole in the molecules (see Section 10.5). Microheterogeneity of charge can also be a factor for immunoadhesins in which the adhesin portion of the molecule is glycosylated and contains sialic acid.

We designed our recovery process for CD4-IgG so that it was easily scalable. In general, we sought to link successive operations in a logical flow, combining tangential flow filtration with affinity, ion exchange and hydrophobic interaction chromatography operations in series (for example, see Ref. 79).

10.6.3 Large-Scale Recovery

Several immunoadhesins have been advanced as candidates for human therapy (Table 10.1). In the case of CD4-IgG, which was advanced to clinical trials as an HIV antiviral, we designed a recovery process that was intended to produce 0.5–1 kg of purified product per batch, using cell culture operation in a 12 kL bioreactor. Initially the process was developed at laboratory scale, using 2 L feedstocks provided by the cell culture development lab. As larger fluid volumes of feedstocks became available, we scaled the recovery process stepwise in three 10–20 fold size increments, checking performance at each step as new production equipment was introduced.

Guidelines for scaling column operations were relatively simple (Table 10.2). In making the transition from laboratory to pilot to manufacturing scale, parameters such as sample load volume, volumetric flow rate, and media volume were increased, while the column bed height, linear flow rate, sample concentration, and ratio of sample to gel were kept constant. Since a gradient was used in operation of one of the columns, the ratio of gradient volume to gel volume was held constant. Thus, the time required for the gradient to develop was approximately the same on the larger column.

10.6.4 Nonprotein Contaminants

Pharmaceutical production requires removal of nonprotein as well as protein contaminants from the product. Among the nonprotein contaminants that must be removed are host cell DNA, bacterial cells (e.g., bioburden) and components such as endotoxin and viruses. DNA can be present in the feedstock as a consequence of cell lysis during production. As much as 1–5 mg/ml of DNA can be present in harvested cell culture fluid that must be removed. Removal of DNA can be obtained in many types of chromatography operations, but since it is a polyanion, removal is best achieved using anion exchange methods. The potential for bioburden in a process always exists; we have dealt with this by using filtration (0.22 μ m) of process buffers and pools. Efficient removal of bacterial endotoxin can occur in anion exchange and hydrophobic interaction chromatography steps.

TABLE 10.1 Immunoadhesins Studies for Clinical Application

	CD4-IgG	TFN Receptor (p55)-IgG	TFN Receptor (p75)-IgG	LFA3-IgG	CTLA4-IgG
IgG subclass	IgG1	IgG1	IgG1	IgG1	IgG1
Target ligand	HIV gp 120	TNF α , β	TNF α , β	CD2	B7
Product designation		lenercept Tenefuse	TNFR:Fc Enbrel	LFA3TIP	
Company	Genentech, Inc. (S. San Francisco, CA)	Hoffmann-LaRoche Basel, Switzerland/ Nutley, NJ	Immunex (Seattle, WA) Wyeth-Ayerst Laboratories (Philadelphia, PA)	Biogen (Cambridge, MA)	Bristol-Myers Squibb, New York, NY)
Main clinical indication	HIV infection	Severe sepsis	Rheumatoid	Psoriasis	Psoriasis
Reference	24, 90	9, 10, 91	92, 93	61, 94	30, 95

*see also chapter 9.
†see also chapter 8.

TABLE 10.2 Guidelines for Scaling Chromatographic Operations from Laboratory to Plant

Maintain	Type of chromatographic media (including particle size) Column bed height Linear flow rate (cm/h) Sample load concentration Gradient volume: media volume
Increase	Sample load volume Volumetric flow rate (L/min) Column diameter
Check System Factors:	Distribution system Wall effects Piping (linear flow in whole system)

Source: Modified from Ref. 86.

As outlined previously, all rodent cells that are used for protein expression contain viruslike C-particles. Although these particles are noninfectious, they warrant concern and should be removed. Since our production method utilized cultured hamster (CHO) cells, we had to validate that any viral particles present in the feedstock were removed or inactivated during the recovery process. Typically the log titer reduction (LTR) values ($LTR = \log \text{ of challenge titer to recovery titer ratio}$) for affinity, ion exchange, hydrophobic and size exclusion chromatography range between 2 and 4.⁸⁰ This means that a purification scheme that uses four chromatography steps can be expected to yield an LTR value between 8 and 16. The most effective methods for removal of retroviruses were affinity, anion exchange, and hydrophobic interaction chromatography. In order to increase process robustness, we sought to combine removal steps with additional steps in which virus inactivation occurred. We used several methods for inactivation of viruses, including exposure to low pH, mild chaotropic agents, and nonionic detergents.⁸⁰

10.6.5 Protein Quality, Purity and Consistency

The required purity of a product is, of course, dictated by its final use. For recombinant protein products, purity can be compromised by the presence of contaminating host cell proteins, but also by variant forms of the desired product. Aggregated product is often of concern with immunoadhesins, since these proteins are nonnatural and so it is difficult to predict their solution behavior. If the recovery process includes affinity chromatography on protein A, then care must be taken that leached protein A is not detectable in the product. The recovery process should be designed to remove both of these potential contaminants.

Protein consistency can be an issue of concern with immunoadhesins, since these are intended as recombinant glycoproteins with long in vivo half-lives. Often the adhesin portion of the molecule contains sialylated complex oligosaccharides through which clearance of the glycoprotein can occur.⁸¹⁻⁸³ In situations where batch-to-batch consistency varies, the serum stability of the product can be affected due to the variable glycosylation in these oligosaccharides.^{62,84,85}

10.6.6 Large-Scale Process Integration

To develop a robust process, integration of the cell culture and recovery processes must begin early in the development effort. The goal of all activities is to develop a reliable process, capable of delivering a protein of consistent quality (at high yield and low cost).

In our experience, three issues are important for successful integration of cell culture and recovery development in scale-up.^{79,86} First, equipment used for development and production will likely be different. Piping, vessels, pumps, and other equipment that come in contact with product streams can have an impact on product quality and recoverability. Second, changes in the composition of the production medium can have a major influence on recovery steps, sometimes even at downstream steps in the recovery train. Finally, differences in handling cell culture fluid streams during production and harvest can have a major impact on the amount and type of contaminants (proteins, nucleic acids, particulate cell debris).

10.7 CONCLUSIONS

Immunoadhesins have an established practical utility as research tools, and are beginning to show efficacy as human therapeutic agents. As research tools, they offer many of the advantages of monoclonal antibodies and are useful especially in studies on ligand-receptor interactions and in identification or isolation of unknown ligands for known receptors. An immunoadhesin approach, if applicable, also should be considered when it is difficult to obtain antibodies to a given antigen, for example, when the antigen is available in limited quantity or is poorly immunogenic. As human therapeutics, immunoadhesins share many of the properties of monoclonal antibodies, including target specificity and Fc effector functions. As compared with chimeric antibodies, which contain nonhuman sequences, immunoadhesins are potentially less immunogenic in humans. In addition, immunoadhesins can be easier to obtain than "humanized" antibodies, because they do not require CDR grafting. Immunoadhesins also may provide a useful alternative to human monoclonal antibodies, for which successful production has lagged far behind that of mouse antibodies, due to several factors including human tolerance to human anti-

gens, ethical limitations to experimental immunization of humans, and instability of human antibody-producing hybrid cell lines. These obstacles are just now being overcome, as advances in genetic engineering techniques⁸⁷ for producing human antibodies from combinatorial libraries⁸⁸ or by genetic manipulation of laboratory animals⁸⁹ continue to develop. Finally, as compared with soluble receptors, immunoadhesins can have advantages in areas such as ease of purification, increased binding affinity or avidity, added effector functions, and extended in vivo half-life. Immunoadhesins generally are straightforward to produce at large scale. To date, several immunoadhesins have been produced using mammalian cell culture at large scale for human therapeutic use.

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