Development of recombinant antibodies for diagnostic applications by protein engineering

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Abstract

Since the advent of hybridoma technology the exquisite specificity of monoclonal antibodies has been exploited for a number of applications in diagnostics, medicine and research. Today, monoclonal antibodies and binding fragments can also be produced by recombinant methods and this offers, in addition to efficient production, the means for convenient and rapid engineering of antibody fragments for different specific applications. In this work antibody engineering was used to increase the available lysine content of an anti-human alpha-fetoprotein (hAFP) Fab fragment and to improve its labelling properties for applications in which sensitive detection is essential. Monoclonal antibody derivatives binding testosterone (TES) with affinity and specificity sufficient for their use as reagents in immunoassays designed for the quantitative measurement of TES in clinical samples were also developed and characterised.

Europium (Eu^{3+}) chelates provide a nonradioactive alternative for sensitive labelling of antibodies for diagnostic immunoassays. Lysine residues at antibody surfaces are suitable targets for labelling by an isothiocyanate derivative of the chelate. In this work the labelling efficiency of a recombinant anti-hAFP Fab fragment was improved by increasing its lysine content by protein engineering. Molecular modelling was used to identify three light chain constant domain surface arginine residues which were mutated to lysine residues. The mutations did not influence the affinity of the lysine-enriched Fab fragment, and its labelling efficiency was found to be about 40 percent higher than that of the wild-type Fab fragment. With a low degree of labelling, the affinities of the two Fab fragments were identical and comparable to that of the original monoclonal anti-hAFP IgG. With higher degrees of labelling the affinities of both Fab fragments decreased more than that of the intact IgG since more lysine residues are available for labelling in the additional heavy chain constant domains of the larger molecule.

A great number of small, rigid and hydrophobic steroid hormones, with very similar structures and containing only a few functional groups capable of direct

interactions with antibodies, have remained as a major challenge for immunodiagnostics. It has been very difficult or impossible to develop monoclonal antibodies by hybridoma technology with sufficient specificity or affinity to distinguish between the closely related steroids in routine clinical assays. The majority of commercially available diagnostic test kits for steroid hormones utilise polyclonal antibodies, not because of optimal performance and product quality, but because of the apparent lack of monoclonal antibodies fulfilling the clinical requirements. From a production and quality control standpoint monospecific, non-serum derived reagents would be highly beneficial.

Semirandom mutagenesis of an existing monoclonal antibody combined with different phage display selection strategies was employed in the development of highly specified TES-binding recombinant antibodies. The genes encoding the Fab fragment of a monoclonal anti-testosterone antibody, 3-C_4F_5, were subjected to several rounds of mutagenesis, selection by phage display technique and characterisation. During the project all the complementarity determining regions (CDRs) of the 3-C_4F_5 antibody were randomised using synthetic oligonucleotides. To improve the affinity, the mutant libraries were selected by using limiting, decreasing concentrations of biotinylated TES in solution and capturing the binders on streptavidin coated microtiter plates. The specificity was improved by preincubating the mutant libraries in solution with a high concentration of the cross-reacting steroid, dehydroepiandrosterone sulfate (DHEAS), and then isolating the TES binders using microtiter plate wells coated with a TES conjugate. A combined selection procedure allowing simultaneous selection with respect to affinity and specificity was also used. Highly improved variants of 3-C_4F_5 were screened from new CDR combinations created from optimised CDR loop sequences. Finally, new further improved light chain CDR3 sequences were created by combining mutations from different light chain CDR3 mutant clones. The optimisation of the CDR sequences and the CDR combination resulted in clones having an excellent overall binding profile. It was shown that many variant sequences of the original 3-C_4F_5 antibody can confer an antibody TES-binding specificity and affinity comparable to those of a rabbit polyclonal anti-testosterone antiserum currently used in a diagnostic immunoassay. To the best knowledge of this author the developed mutant Fab fragments of the 3-C_4F_5 antibody are the first steroid hormone-binding recombinant antibodies shown to work accurately over the whole physiological concentration range of clinical samples.

Preface

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List of publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals (I - IV).

I   Hemminki, A., Hoffrén, A.-M., Takkinen, K., Vehniäinen, M., Mäkinen, M.-L., Pettersson, K., Teleman, O., Söderlund, H., and Teeri T. 1995. Introduction of Lysine Residues on the Light Chain Constant Domain Improves the Labeling Properties of a Recombinant Fab fragment. *Protein Eng*. **8**, 185-191.

II   Hemminki, A., Niemi, S., Hoffrén, A.-M., Hakalahti, L., Söderlund, H. and Takkinen, K. 1998. Specificity Improvement of a Recombinant Anti-Testosterone Fab Fragment by CDRIII Mutagenesis and Phage Display Selection. *Protein Eng*. **11**, 311-319.

III   Hemminki, A., Niemi, S., Hautoniemi, L., Söderlund, H. and Takkinen, K. 1998. Fine Tuning of an Anti-Testosterone Antibody Binding Site by Stepwise Optimisation of the CDRs. *Immunotechnology* **4**, 59-69.

IV   Hemminki, A., Teerinen, T., Niemi, S., Söderlund, H., and Takkinen, K. Development of Recombinant Anti-Testosterone Antibodies with Clinical Performance by Phage Display. S*ubmitted for publication*.

Contents

Appendices of this bublication are not included in the PDF version.
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(http://www.inf.vtt.fi/pdf/tiedotteet/1998/)

Abbreviations

BSA	bovine serum albumin
C_H1	first constant domain of the Ig heavy chain
C_L	constant domain of the Ig light chain
cDNA	complementary deoxyribonucleic acid
CDR	complementarity determining region
CMO	carboxymethyloxime
DHEAS	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
ED	effective dose
ELISA	enzyme-linked immunosorbent assay
Eu^{3+}	europium
hAFP	human alpha-fetoprotein
Ig	immunoglobulin
Kd	dissociation constant
kDa	kilodalton
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RIA	radioimmunoassay
scFv	single chain antibody
SCR	structurally conserved region
TES	testosterone
V_H	variable domain of the Ig heavy chain
V_L	variable domain of the Ig light chain

1. Introduction

1.1 Immune response

Multicellular organisms have many defence mechanisms against invasions by microorganisms or foreign substances, e.g. an impermeable outer skin or the antiviral agent interferon, both of which are relatively non-specific. Vertebrates have evolved a sophisticated immune system that provides specific defences against almost any microorganism or other foreign substance that enters the body, while ignoring all the molecules that are 'self'. Foreign substances that elicit an immune response are called antigens. The immune system has two types of defence mechanisms against antigens, humoral and cellular immunity. The agents of humoral immunity are known as B-lymphocytes, or B-cells. B-cells produce circulating proteins, called antibodies or immunoglobulins (Ig), which bind to antigens and inactivate them or mediate their destruction. Cellular immunity is carried out by a different group of immune cells, termed T-lymphocytes, or T-cells. In contrast to B-cells, T-cells do not produce antibodies; rather they recognise foreign antigens in association with a specific set of proteins on different cells. For this purpose, they are equipped with a specialised class of molecules, called T-cell receptors. Typical manifestations of T-cells at work include such diverse phenomena as the rejection of foreign skin grafts and the killing of tumor cells. Antibodies and T-cell receptors are the primary means by which the body recognises specific antigens. Although humoral and cellular immunity have basically different functions and purposes, they interact during an immune response. T-cells, for example, help to regulate the function of B-cells.

1.1.1 Humoral immunity

When challenged by infection or immunisation, efficient humoral responses to the majority of antigens require the concerted action of T- and B-cells (Armitage and Alderson, 1995; Pierce and Margoliash, 1988). T-cell dependent activation of B-cells occurs via two steps. First, antigens are recognised by the antibody molecules expressed on the surface of B-cells that continually circulate in the bloodstream. Antigens are taken up and processed into fragments for presentation to T-cells by MHC class II molecules (Parker, 1993). T-cells

expressing a particular T-cell receptor-CD3 complex that recognises a fragment of the processed antigen then become activated. This event sets forth a complex chain of responses, involving expression of many B- and T-cell surface adhesion molecules and soluble cytokines, that results in the stimulation of B-cells (Callard and Turner, 1990; Clark and Ledbetter, 1994). These B-cells, in turn, proliferate and differentiate to produce large quantities of soluble antibodies which bind to the foreign invader. The binding event between antibody and antigen marks the foreign invader for destruction via phagocytosis or activation of the complement system.

A striking characteristic of antibodies is their binding diversity. For example, humans can produce up to 10^{10} antibodies with different specificities. The diversity is so great that virtually any foreign macromolecule can be recognised.

1.1.2 Antibody structure

The antibody molecule is assembled from two copies of each of two protein chains: the light chain (~25 kDa) and the heavy chain (~50 kDa) (Edelman *et al*., 1969). Each light chain and the N-terminal half of each heavy chain pair to form the two Fab parts of the molecule, while the two carboxyl ends of the heavy chains pair to form the Fc domain. The heavy and light chains are covalently linked by disulfide bonds, the number and position of which vary with antibody class and subclass (Porter, 1973). The resulting antibody is a large, flexible, Y- or T-shaped molecule, which has antigen-binding sites located at the ends of each Fab module. Recently, the first X-ray crystallographic structure of an intact monoclonal antibody was published (Harris *et al*., 1997). In mammals the light chains are of two types, designated kappa and lambda. There are five classes of heavy chains: mu, delta, gamma, epsilon and alpha. Different heavy chain classes have different roles and functions in the immune response. For example, the membrane-bound antibodies that serve as B-cell receptors incorporate mu or delta chains, and most of the antibodies secreted in the response to an antigen include gamma or alpha chains. The binding and effector functions of the antibody can usually be separated by proteolytic enzymes, such as papain or pepsin, to yield three fragments: two antigen-binding Fabs and one Fc. The Fab or Fv fragment

(containing only the variable domains) can also be expressed from a cloned sequence.

The Fab fragment comprises four copies of 'immunoglobulin domains' (Poljak *et al*., 1973), two-layered, seven- or nine-stranded β-sandwiches linked across the centre by disulfide bonds, termed the variable light (V_L), variable heavy (V_H), constant light (C_L), and constant heavy (C_H1) domains. The C_L and C_H1 domains associate to form the constant region, which has a highly conserved amino acid sequence and three-dimensional structure. The V_L and V_H domains associate to form the variable region containing the antigen binding site. The variable region also has a high degree of sequence and structure conservation in the strands of the β-sheets that form a stable framework. The binding site is formed by six loops (three from each chain), called the complementarity determining regions (CDRs), joining the conserved β-strands at the end of each Fab fragment. These CDRs, also called hypervariable loops, are highly variable in length and amino acid sequence (Kabat and Wu, 1971; Wu and Kabat, 1970). Thus, by varying the length and amino acid composition of the CDRs, antigen-binding sites with substantially different shapes (Wilson and Stanfield, 1994) and specificities can be created.

Furthermore, antibodies can exist in multiple conformational states, although whether the binding of antigen induces the change, or whether the antigen binds to an already existing antibody conformation, is not clear (Stanfield and Wilson, 1994). In one study several structures of the anti-progesterone Fab fragment DB3 in complex with different progesterone-related antigens showed that the Fab fragment underwent subtle changes to accommodate different antigens (Arevalo *et al*., 1993a and b, 1994). The conformational changes seen in Fabs and Fvs range from very small overall changes to significant side-chain movements, small movements of CDR loops as rigid bodies, large rearrangements of CDR loops, V_L-V_H domain rearrangements and to combinations of some or all of the above (Stanfield and Wilson, 1994). These structural changes can alter dramatically the size, shape and charge distribution in the antigen-binding pocket, and present problems for the prediction, design and engineering of antibody combining sites. Even if an X-ray structure is available for the unliganded form of an antibody, its binding site may differ substantially from that of its antigen-bound counterpart (Stanfield *et al*., 1993).
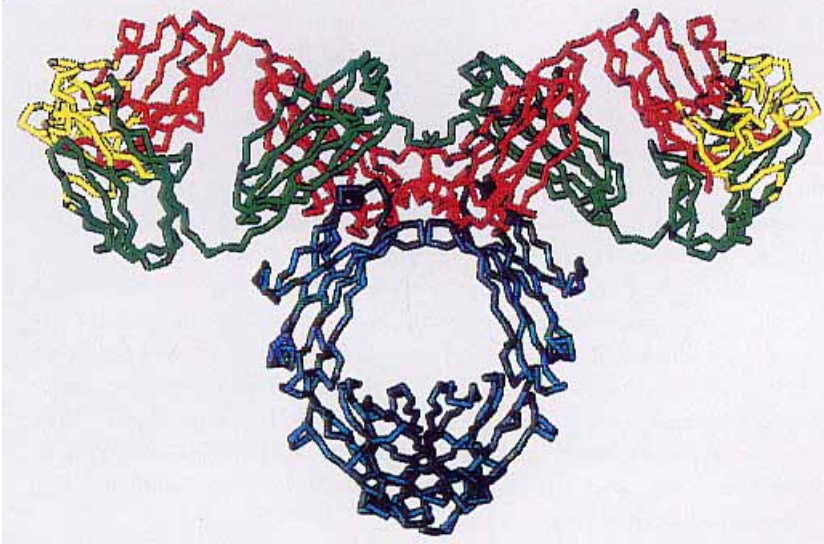
Figure 1. Representation of an intact antibody molecule (IgG). The Fc fragment is coloured blue. In Fab fragments heavy chains are coloured red and light chains green. CDRs in the variable domains of the Fab fragments are coloured yellow.

1.2 Generation of antibody diversity and specificity *in vivo*

The ability of antibody molecules and T-cell receptors to interact specifically with an almost limitless variety of foreign antigens is a consequence of their particular amino acid sequences. These proteins that recognise foreign invaders are the most diverse proteins known. The body synthesises millions of antibody molecules and T-cell receptors with slightly different amino acid sequences even before it meets with an antigen. Among this vast repertoire of molecules are invariably some that can bind to any incoming antigen.

1.2.1 Clonal selection

The clonal selection theory is an established model used to explain the function of B-cells in immune response (Ada and Nossal, 1987; Burnet, 1969). As a B-cell matures in the bone marrow, it becomes committed to the synthesis of antibodies that are capable of recognising only one specific antigen. The antibodies made by the B-cell remain bound to the cell membrane, where they are arranged on the surface as receptor molecules. When an antigen binds to an antibody in the membrane a complex chain of responses, mediated by helper T-cells, is set forth that results in the proliferation of antigen-specific B-cells (Pierce and Margoliash, 1988). All the descendants of each such cell retain the same specificity, forming a clone of immunologically identical cells. Self-reactive antibody receptors generated during the process can be eliminated in the bone marrow by continued recombination or by deletion (Nemazee and Bürki, 1989; Radic and Zouali, 1996; Tiegs *et al*., 1993). Some of the progeny of the selected clones remain as circulating B-lymphocytes providing a faster response to any subsequent exposure to the same antigen. These memory cells are responsible for the immunity that develops following many infections or as a result of vaccination. Other members of the selected B-cell clones differentiate to plasma cells synthesising and secreting large quantities of antibodies with the same specificity as that on the surface of the parent lymphocyte.

1.2.2 Antibody diversity - somatic recombination

Instead of being encoded by an enormous number of genes, a vast repertoire of antibodies are generated by a combinatorial system of diversification. Genes for antibodies exist as small segments scattered widely throughout the genome and huge numbers of antibodies are made by reshuffling a much smaller set of gene fragments. The cutting and joining of gene sequences is an essential feature in the synthesis of antibodies (Tonegava, 1983; Winter and Milstein, 1991). During the development of B-lymphocytes in the bone marrow a special recombinase machinery shuffles the gene segments that encode antibody chains (Oettinger *et al*., 1990; Van Gent *et al*., 1995). Heavy chains are encoded by four gene segments, V, D, J, and C, and light chains by three, V, J, and C. For human heavy chain, for example, there are 51 functional V-segments,

approximately 30 functional D-segments, and six functional J-segments (Cook and Tomlinson, 1995). The C-segment is constant for a particular antibody class and does not contribute to antibody diversity. The assembly of a functioning antibody gene takes place in two stages. First the particular V- and J-segments in a light chain or the V-, D- and J-segments in a heavy chain are brought together within the DNA. Then the intron that separates the V-J- or V-D-J-complex and the C-segment is excised from the messenger RNA which is then translated into protein. In principle, heavy chains can be brought together in almost 10 000 combinations. Combining heavy chains randomly with similarly constructed light chains can probably yield more than 10 million distinct antigen binding sites.

1.2.3 Antibody diversity - junctional variability

The many possible combinations that can be formed from several hundred gene segments are the key to antibody diversity, but there are at least two additional sources of variety. One of these is junctional diversity introduced during the DNA-rearrangement that fuses V-, (D-), and J-segments (Feeney, 1990; Meek, 1990; Victor and Capra, 1994). Addition and deletion of nucleotides produces very heterogeneous CDR3 sequences. As a result, even if two antibodies are specified by the same collection of gene segments, they may still have slightly different antigen-binding sites.

1.2.4 Antibody diversity - somatic mutation

Another source of diversity is somatic hypermutation (Berek and Milstein, 1987). The immune system creates high-affinity antibodies in two stages. A diverse primary repertoire of antibodies is produced by the combinatorial rearrangement of germline gene segments and specific antibodies are selected from this repertoire by binding to the antigen. Their affinities are then improved by somatic hypermutation and further rounds of selection. The molecular mechanism of the antigen-driven somatic hypermutation process inducing mutations is still unclear (Steele *et al*., 1997; Storb, 1996; Storb *et al*., 1998). Mutations are seen in the variable regions and in the immediately adjacent regions but not in the constant regions. Estimates of the rate of mutation suggest

that there should be one change in the V-region for every three to 30 cell divisions (Tonegawa, 1983). The DNA-joining variability and somatic mutation mechanisms have been estimated to increase antibody diversity by perhaps a factor of 100. Thus, the total number of different antibodies could well exceed 10^9 (Tonegawa, 1983).

Mutations do not occur randomly within V-genes but are preferentially targeted to certain positions (hot spots) and away from others (cold spots) (Jolly *et al.*, 1996). Cold spots often coincide with residues essential for V-domain folding. Hot spots, which appear to be strategically located to favour affinity maturation, are most frequently located in the CDRs, particularly CDR1. Recently, Tomlinson *et al.* (1996) compiled a database of 1181 rearranged human V_H and 736 rearranged Vκ sequences, and identified the location of somatic mutations in each sequence. They concluded that generally the number of amino acid differences introduced by somatic hypermutation is less than the number of amino acid differences between germline V-segments. In addition, they noted that somatic hypermutation is primarily a point mutation process and rarely results in codon insertions or deletions, whereas the CDR lengths do differ between germline V-segments (Chothia *et al.*, 1992; Tomlinson *et al.*, 1995). By locating sequence diversity in the antibody structure Tomlinson *et al.* (1996) showed that in the primary repertoire diversity is focused at the centre of the binding site. With somatic hypermutation, diversity spreads to regions at the periphery of the binding site that are conserved in the primary repertoire.

1.3 Antibody engineering

Antibodies are widely used as research reagents and as biospecific recognition molecules in immunoassays and immunoaffinity materials for chromatographic separations. The use of antibodies for *in vivo* diagnosis, imaging and therapy currently attract considerable interest within the pharmaceutical industry, and in the future catalytic antibodies (Smithrud and Benkovic, 1997) may be important tools for different industrial applications. Protein engineering is commonly used to study structure/function relationships and to modify the properties of industrial enzymes and therapeutically important proteins (Buckel, 1996; Murphy, 1996; Shao and Arnold, 1996). Three technological advancements have greatly facilitated the successful engineering of antibodies. Production of

soluble, functionally active antibody fragments in *Escherichia coli* (Better *et al*., 1988; Skerra and Plückthun, 1988; Plückthun *et al*., 1996) has been a major breakthrough in the field, since rapid and reliable expression of a target protein is essential for efficient protein engineering. Antibody phage display is without doubt another major breakthrough in the field, providing rapid and efficient selection of genetically engineered antibodies (McCafferty *et al*., 1990; Hoogenboom *et al*., 1998; Rader and Barbas, 1997). The third important technical advance has been the development of rapid methods of cloning and manipulating antibody gene fragments by the polymerase chain reaction (PCR) (Saiki *et al*., 1985, 1988) and the application of PCR to create very large antibody gene repertoires (Griffiths *et al*., 1994; Hoogenboom, 1997; Sheets *et al*., 1998; Vaughan *et al*., 1996).

Today, it is possible to mimic the key features of the humoral immune system *in vitro* (Griffiths and Duncan, 1998; Hoogenboom *et al*., 1998; Winter *et al*., 1994). As a result, high-affinity antibodies can be produced without the use of conventional hybridoma-based monoclonal antibody technology (Köhler and Milstein, 1975) and even without immunisation of laboratory animals. Antibody engineering technology offers possibilities to tailor-make immunoreagents to fit exactly a particular application and thereby improve the performance characteristics of the various applications. Efficient expression systems allow reliable and economical production of engineered antibodies (Plückthun *et al*., 1996).

The antibody structure has been successfully engineered to produce antibody variants with lower immunogenicity (Güssow and Seemann, 1991), higher affinity (e.g. Barbas and Burton, 1996; Low *et al*., 1996; Schier *et al*., 1996c; Yang *et al*., 1995; Yelton *et al*., 1995), altered specificity (Ohlin *et al*., 1996), or enhanced stability (Glockshuber *et al*., 1990; Reiter *et al*., 1994). Genetic fusions to effector proteins and toxins are potential tools in the fields of medicine and diagnostics (Huston *et al*., 1993a and b). Furthermore, naive antibody gene libraries can be used to obtain antibodies against 'new' antigens, e.g. self and tumor antigens (Griffiths *et al*., 1994; Vaughan *et al*., 1996) or MHC-peptide complexes (Andersen *et al*., 1996).

1.3.1 Cloning of antibody genes

Cloning and sequencing of antibody genes and cDNAs form the basis of antibody engineering. Today, PCR is commonly used to clone the genes of individual monoclonal antibodies as well as whole antibody gene repertoires of immunised or non-immunised animals, including humans. Cloning and sequencing retains and immortalises unique monoclonal antibodies, which can be crucial for the rescue of unstable hybridoma cell lines. By antibody gene repertoire cloning it is possible to by-pass the hybridoma technology and even the need for immunisation of laboratory animals.

Amplification of a specific gene by PCR uses 5' and 3' oligonucleotide primers complementary to the respective ends of the gene. Design of primers for the 3' end of antibody genes is straightforward, since primers can be based on the constant regions, all of which have been sequenced (Kabat et al., 1991). Design of primers for the 5' end of the V-genes is less straightforward due to the sequence variability. Previously, N-terminal protein sequencing of purified antibodies from hybridomas was often used to deduce the V_H and V_L gene families. Relatively conserved 5' terminal framework sequences in the V-gene families were then used to design degenerate primers (Larrick et al., 1989). A generally applicable approach was developed by Orlandi et al. (1989). The nucleotide sequences of murine V_H and V_L genes were extracted from the Kabat database, aligned, and the frequency of the most common nucleotide was plotted for each position. Conserved regions were identified at the 5' and 3' regions of the V_H and V_L genes, and the sequences of these regions were used to design oligonucleotide primers. Since then a number of authors have evaluated different primer sets for murine (Dattamajumdar et al., 1996; Krebber, A. et al., 1997; Orum et al., 1993) and human (Campbell et al., 1992; De Boer et al., 1994; Marks et al., 1991a and b; Welschof et al., 1995) V-genes. Databases of antibody V-region sequences have enlarged rapidly and e.g. all the human germline V_H and $V\kappa$ segments have now been sequenced (Tomlinson et al., 1995; Cook and Tomlinson, 1995). The more complete on-line sequence databases permit more rapid and reliable cloning of antibody genes.

PCR with antibody-specific primers can be used to amplify V_H and V_L gene repertoires from rearranged genomic DNA or total RNA prepared from mouse spleens or peripheral lymphocytes (Gram et al., 1992; Huse et al., 1989; Ward et

al., 1989) or from human lymphocytes (Marks *et al*., 1991b; Sheets *et al*., 1998; Vaughan *et al*., 1996). To maximise gene repertoire diversity and the efficiency of amplification, PCR primers designed on the basis of the consensus sequence of each V_H and V_L gene family are commonly used. Sequence analysis of the amplified V_H and V_L genes has indicated that diverse gene repertoires could be produced (Güssow *et al*., 1989).

1.3.2 Recombinant antibody fragments

E. coli is well characterised with regard to its genetics, physiology and fermentation and its genetic modification by recombinant DNA technology is far developed. Therefore, *E. coli* has become a useful host for expression of many recombinant proteins and steady progress in our understanding of protein folding, solubility, stability, and membrane transport has expanded its utility (Hannig and Makrides, 1998; Makrides, 1996; Thomas *et al*., 1997). High expression levels up to 30% of total bacterial protein can often be reached. The possibility of producing small, antigen-binding antibody fragments in active form in *E. coli* (Better *et al*., 1988; Skerra and Plückthun, 1988) was a major breakthrough in the field of antibody engineering. In addition to Fab fragments (Better *et al*., 1988), individual V_H (Ward *et al*., 1989) and Fv (Skerra and Plückthun, 1988) fragments, single chain antibodies (scFv)(Glockshuber *et al*., 1990; Skerra *et al*., 1991), F(ab')$_2$ dimers (Carter *et al*., 1992), 'miniantibodies' (Pack and Plückthun, 1992; Pack *et al*., 1993), 'diabodies' (Holliger *et al*., 1993) as well as different antibody fusion proteins (Huston *et al*., 1993a,b) have also been successfully produced in *E. coli* (Plückthun *et al*., 1996; Plückthun and Pack, 1997). The overall simplicity of cloning and the rapid generation time of *E. coli* facilitate the production optimisation and engineering of antibody structure.
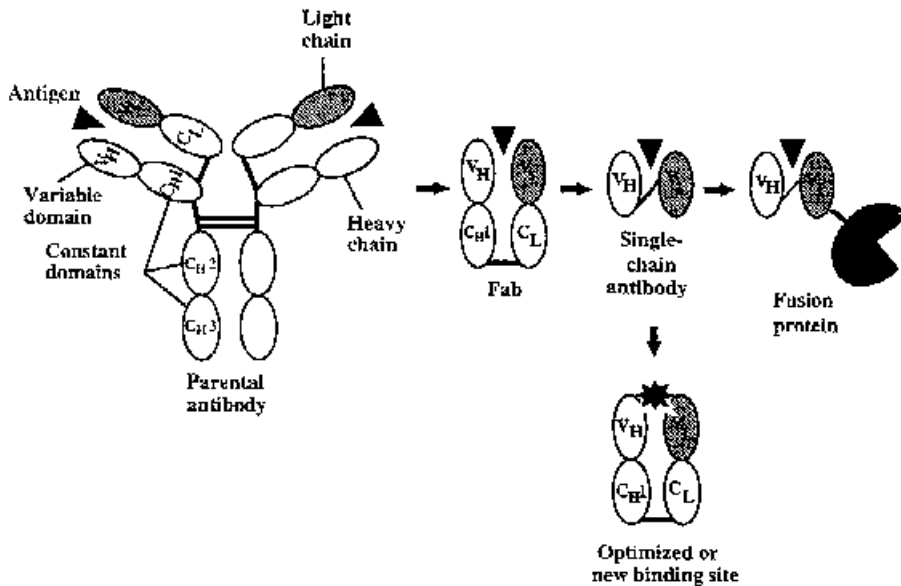
Figure 2. Schematic representation of some of the different antibody fragments which can be produced in E. coli.

In order to produce correctly folded, functional antibodies with correctly formed disulfide bonds, the individual antibody chains must be transported to the oxidising environment of the bacterial periplasm (Better *et al*., 1988; Skerra and Plückthun, 1988). This is similar to the production of antibodies in eukaryotic cells, in which the two chains are transported from the cytoplasm to the lumen of the endoplasmic reticulum (Rapoport, 1992). The periplasm also contains proteins such as disulfide isomerases and chaperonins (Bardwell *et al*., 1991; Landry and Gierasch, 1994), which aid in the refolding of the newly synthesised proteins. Antibody chains are directed to the periplasmic space by fusion with prokaryotic signal peptide sequences. In the first reports of bacterial expression, antibody fragments were harvested from the bacterial periplasm and very high yields, more than 3 g/l, in optimised high-cell-density fermentations have now been obtained from periplasmic extracts with correctly folded chains (Carter *et al*., 1992; Horn *et al*., 1996; Pack *et al*., 1993). Antibody fragments can also be

released in the culture medium as a consequence of leakage of the outer membrane of some strains during prolonged inductions (Hoogenboom *et al*., 1991; Ward *et al*., 1989), and hence unpurified bacterial supernatants can be screened directly for antigen binding activity. Another strategy to obtain antibody fragments from *E. coli* is the *in vitro* refolding of protein obtained from inclusion bodies, either from the cytoplasm or from the periplasm (Huston *et al*., 1995).
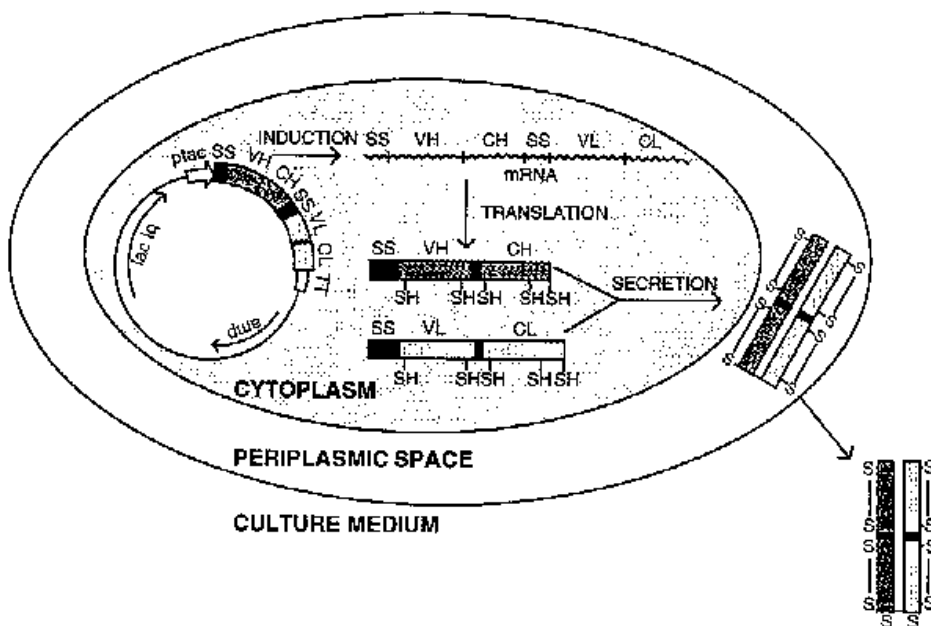
Figure 3. Expression of a soluble secreted Fab fragment in E. coli. After induction dicistronic mRNA is synthesised and subsequently translated into preproteins in the cytoplasm. Secretion to the periplasm is facilitated by the use of signal peptides which are removed during the translocation process. The secreted chains fold and assemble in the oxidising environment of the periplasm and the active Fab fragment leaks into the culture medium.

The production yields of different antibodies in *E. coli* differ significantly and low yields can in some cases be improved by protein engineering (Knappik and Plückthun, 1995; Nieba *et al*., 1997; Plückthun *et al*., 1996). These studies have demonstrated that it is possible to engineer improved frameworks for antibodies and that some limitations in functional folding can be overcome by single amino acid substitutions. The yield of functional antibody fragments in *E. coli* has also been improved significantly by optimising expression vectors and fermentation conditions (Horn *et al*., 1996). It is important to note that for antibody fragments produced from phage display libraries (see below), the selection process eliminates those fragments which do not express or fold properly in *E. coli*, thereby improving the likelihood of successful bacterial expression of library isolated fragments. To facilitate the purification, detection and immobilisation of recombinant antibody fragments, different affinity domain 'tags' (Nilsson *et al*., 1997) are commonly fused to either the N- or C-terminus of the recombinant antibody. Frequently used affinity tags include a C-terminal oligo-his (His6) (Skerra *et al*.,1991), N-terminal FLAG (Knappik and Plückthun, 1994) and c-myc (Ward *et al*., 1989). These affinity tags do not normally interfere with the antigen binding function and provide a practical alternative for specific antigen-affinity columns.

Functional antibody fragments have also been produced in alternative heterologous systems, including yeast (Horwitz *et al*., 1988), filamentous fungi (Nyyssönen *et al*., 1993), insect cells (Bei *et al*., 1995; Kirkpatrick *et al*., 1995), plant cells (Ma *et al*., 1995), and mammalian cells (Trill *et al*., 1995). Each of these hosts has advantages and disadvantages for the production of secreted functional antibodies or their domains, but at least for engineering of the binding domains *E. coli* will be the system of choice due to its versatility and simplicity.

In addition to production as soluble secreted molecules, recent applications of antibody engineering involve intracellular production of engineered antibody fragments within a target (mammalian) cell (Beerli *et al*., 1996; Biocca *et al*., 1994; Richardson and Marasco, 1995). By including specific intracellular trafficking signals in the antibody genes, the expressed antibody fragments can be directed to the specific cellular compartment where the antibody binding is desired. These recombinant antibody molecules, called intrabodies (Chen *et al*., 1994), can block

or modify the normal function of the target antigen and thereby alter the cellular activities or phenotype, as shown e.g. by Deshane *et al*. (1994, 1996) using adenovirus-mediated expression of an endoplasmic reticulum targeted anti-erbB-2 scFv in erbB-2 expressing tumor cells.

1.3.3 Rational design & molecular modelling

Genetic engineering has provided the possibility to plan and make deliberate changes in protein sequences. It is possible to test hypotheses about structure, folding and function and to attempt to alter structures in order to impart desired properties; for example, increased affinity or altered specificity for ligands, or increased thermostability. Specific mutations are easily introduced into the DNA sequence of a given protein through oligonucleotide-directed mutagenesis, in which an oligonucleotide containing the desired alteration is synthesised and used as a primer to synthesise a gene containing the mutated nucleotide(s). However, rational protein design requires detailed knowledge of the structure and function of the target protein. Recently, great advancement in the understanding of protein structures, folding and structure-function relationships have been made through assembly of large sequence databases and structural analysis, including X-ray crystallography, nuclear magnetic resonance (NMR), computer modelling, and site-directed mutagenesis. Methods for determination of three dimensional structures of proteins have improved rapidly and, additionally, molecular modelling has improved to the point where the effects of single-point mutations can be predicted with better and better reliability.

The overall structure of antibodies is well known, highly conserved, and much is known about binding interactions between antibodies and different kinds of antigens, making antibodies good targets for protein engineering. Rational site-directed mutagenesis guided by molecular modelling has been successfully used in antibody engineering, e.g. to improve the affinity of antibodies against lysozyme (Roberts *et al*., 1987), phosphotyrosine (Ruff-Jamison and Glenney, 1993) and 2-phenyloxazolone (Riechmann *et al*., 1992). Rational design has also been used to engineer properties other than the binding characteristics of antibodies, e.g. to humanise rodent antibodies (Roguska *et al*., 1994, 1996) and enhance labelling of antibody fragments (Lyons *et al*., 1990). However, current methods for the prediction of fine atomic details of a binding site and mutational

effects on antibody-antigen binding are not often precise enough for the rational design of fine-tuned changes in affinity or specificity. As a result, rational design by site-directed mutagenesis has been restricted to a few well-studied cases. It seems that the best strategy is still often to use semirandom mutagenesis methods to produce large mutant libraries from which the improved variants can be searched using powerful selection/screening techniques.

1.4 Phage display technology

Smith (1985) first demonstrated that foreign DNA fragments can be cloned into filamentous phage vectors and expressed as part of a fusion protein on the surface of virions without destroying the infectivity of the particles. The inherent advantage of phage display is its direct link of DNA sequence to the properties of the corresponding polypeptide. Expression of the foreign gene fused to a phage coat protein gene results in the display of foreign polypeptide on the phage surface with the cognate DNA within the phage particle.

1.4.1 Filamentous phage

The filamentous phages *fd*, *f1* and M13 are structurally related members of the *Ff* class of bacterial viruses which infect Gram-negative bacteria by specifically adsorbing to the tip of F pili on male cells (Rasched and Oberer, 1986). They are long (1 - 2 μm), thin (6 - 7 nm) and flexible particles consisting of a single-stranded closed circular DNA molecule, approximately 6 400 nucleotides in length, sheathed in a tube of protein (Makowski, 1994; Russel *et al.*, 1997). The major coat protein pVIII is present in about 2 700 copies, forming a tubular array of subunits. The minor coat proteins are present in about 5 copies per phage particle; pIII and pVI are located at one tip of the phage and pVII and pIX at the other end (Makowski, 1994). In addition to these 5 structural proteins, the phage genome encodes 3 proteins which are required for phage DNA synthesis and 3 proteins serving assembly functions (Vieira and Messing, 1987). An intragenic region does not code for proteins but contains signals for initiation of synthesis of both the (+) and (-) strand of DNA (see below), initiation of capsid formation and termination of RNA synthesis.
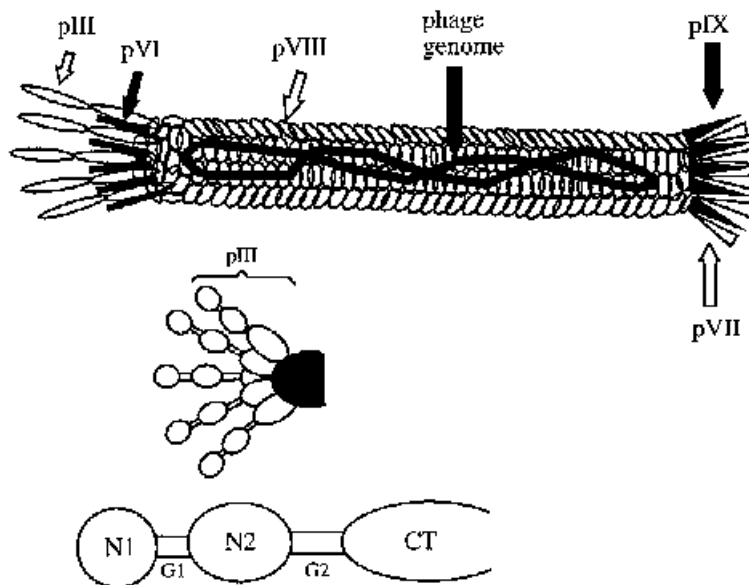
Figure 4. Schematic figure showing filamentous bacteriophage fd and pIII minor coat protein. See text for details.

The filamentous phages are only able to infect bacterial strains that express sex pili encoded by an F factor. Infection begins with attachment of pIII to the F-pilus protruding a long distance from the cell surface. From there the phage transfers to its co-receptor, the C-terminal domain of the periplasmic protein TolA, which mediates membrane penetration of the phage particle (Riechmann and Holliger, 1997). The pIII has three functional domains; N1 (68 amino acids), N2 (131 aa) and CT (150 aa), connected by glycine-rich linkers of 18 (G1) and 39 (G2) amino acids (Krebber, C. *et al.*, 1997; Stengele *et al.*, 1990). The N-terminal domains of pIII (N1 and N2) are exposed and are required to initiate the infection (Riechmann and Holliger, 1997; Stengele *et al.*, 1990), whereas the C-terminal domain (CT) is buried and is required for the assembly of phage particles (Crissman and Smith, 1984). On entering the cell, the virus is stripped of its protein coat. The single-stranded viral DNA, the (+) strand, is then converted by cellular enzymes to the double-stranded replicative form (Fulford and Model, 1988a and b). Amplification of the viral genome begins when the pII protein, encoded by the phage genome,

cuts specifically the (+) strand of the double stranded DNA. *E. coli* DNA polymerase I then synthesises new (+) strand, progressively displacing the original (+) strand from the circular (-) strand template. Finally, the displaced (+) strand is cleaved by the pII to generate a unit-length viral genome that is then circularised, converted to the double-stranded replicative form and serves as a template for further rounds of replication. By the time 100 - 200 copies of double stranded DNA molecules have accumulated in the infected cell (15 - 20 min), there is sufficient single-stranded DNA-binding pV protein to repress synthesis of pII and bind to the newly synthesised (+) strands, preventing their further conversion to double stranded form. The pV-DNA complexes move to the bacterial membrane, where the pV is stripped from the (+) strand and the viral genome is extruded through the inner membrane, concomitantly acquiring the coat proteins. This process does not lyse the infected cells and they continue to grow, although at a slower rate, producing several hundred phage particles per cell generation. This leads to the accumulation of vast numbers of particles in the medium; the amount of infectious bacteriophages from a culture of infected bacteria frequently exceeds 10^{12} pfu per ml.

Because the viral genome is not inserted into a preformed structure, there is no strict limit to the size of the single-stranded DNA that can be packaged. Cloning of foreign DNA into the genome results in the formation of a longer particle. Therefore, filamentous bacteriophages have the unique advantage of generating large quantities of DNA molecules that carry the foreign sequence in single-stranded form and they have been widely used as cloning vectors for different applications, e.g. site-directed mutagenesis, DNA sequencing and generation of DNA probes.

1.4.2 Phage display

Smith (1985) first introduced small antigenic determinants on phage by fusion in the middle of pIII and developed the concept of affinity selection from phage libraries displaying random peptides. Parmley and Smith (1988) developed the phage display system further by moving the cloning site to the N-terminal of pIII and demonstrated the biopanning protocol with biotinylated antibodies selecting specific peptide-displaying phage from a background of 10^8 wild-type phage.

Since then numerous random peptide libraries have been constructed, displaying peptides of different lengths (Smith and Petrenko, 1997). Importantly, the phage has not been limited to the display of small peptide inserts. Active, correctly folded proteins, including human growth hormone (Bass *et al*., 1990), alkaline phosphatase (McCafferty *et al*., 1991), ricin B chain (Swimmer *et al*., 1992), extracellular domains of human IgE receptor (Scarselli *et al*., 1993), cellulose-binding domain of cellobiohydrolase I (Smith et al., 1998), low-molecular-mass forms of human plasminogen (Lasters *et al*., 1997), Z domain of staphylococcal protein A (Nord *et al*., 1997), and different antibody fragments (Griffiths and Duncan, 1998; McCafferty *et al*., 1990) have also successfully been displayed on filamentous phage. In addition to pIII, pVIII (Greenwood *et* al., 1991; Iannolo *et al*., 1995, 1997; Kang *et al*., 1991a) and pVI (Jespers *et al*., 1995, 1996) have also been used for displaying on phage. Phage vector fusions result in multivalent display, i.e. every copy of the structural protein is a fusion protein which gives great avidity of binding and might help retain lower affinity phage during affinity purification. However, discrimination between phage with different affinities may be difficult with multivalent display and larger inserts appear to interfere with the phage assembly and the infection process, especially in pVIII (Smith, 1993).

The essential functions of the phage proteins can be preserved either by including a second, wild-type gene in the phage vector construction or by using a phagemid vector to carry the fusion gene. In both cases the resulting virion is a hybrid in which recombinant pIII or pVIII coat proteins are supplemented by wild-type molecules allowing rather large foreign peptides to be displayed even as pVIII fusions. Phagemid vectors contain the origin of replication from a filamentous bacteriophage in addition to the plasmid origin (Cleary and Ray, 1980). The phage origin of replication contains signals for rolling circle DNA replication, as well as packaging of single-stranded DNA into viral particles, but is inactive until the cell is infected with the 'helper' phage. Production of single-stranded phagemid (and helper phage) DNA is initiated by superinfecting a growing phagemid-containing culture with the helper phage. Both helper phage and phagemid single-stranded DNAs are then packaged and secreted into the culture medium without lysis of the host cell. Preferential packaging of phagemid can be achieved using special helper phages with impaired replication or packaging signals.
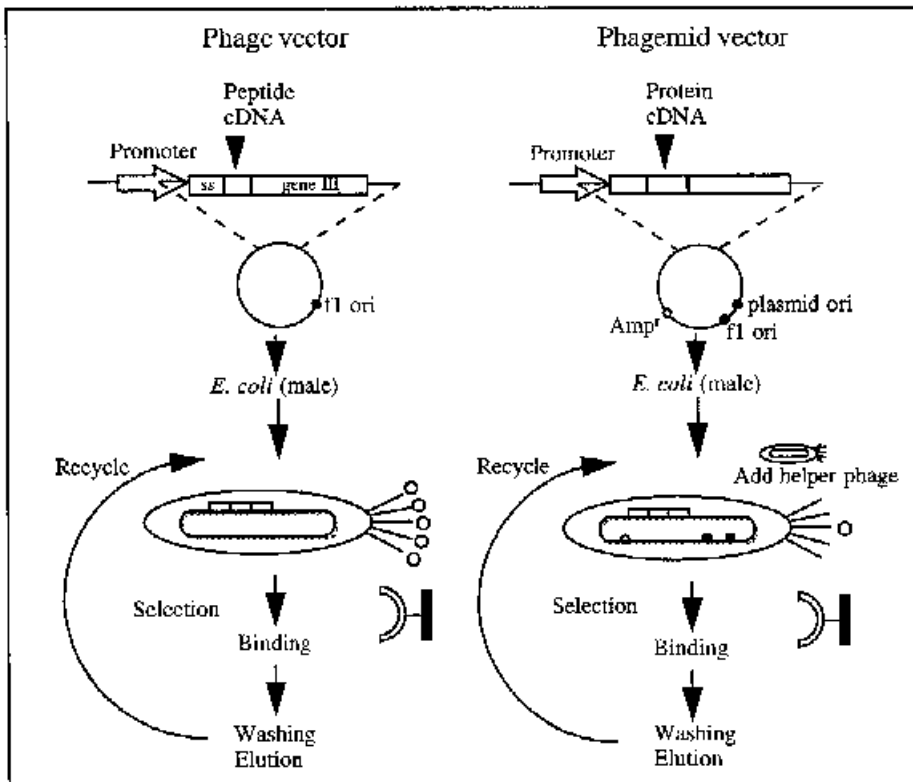
Figure 5. Schematic representation of mono- and multivalent display on phage using a phage and phagemid vector, respectively (modified from Clackson and Wells, 1994). See text for details.

The pIII phagemid expression system, on average, produces phage displaying less than one copy of the fusion protein and enhances the discrimination between phage with different affinities. The pIII encoded by phagemid can be either intact as in the case of the vector pHEN1 (Hoogenboom *et al*., 1991), or without the N-terminal domains N1 and N2 (Barbas *et al*., 1991; Krebber, A. *et al*., 1997). The truncated form is commonly used since cells expressing the N-terminal domains are resistant to superinfection by helper phage. Phagemids have high transformation efficiencies and are therefore ideally suited to generating very large antibody gene repertoires.

1.5 Antibody phage display

New ways of making antibodies using gene technology were developed in the late 1980s. Immunoglobulin genes were amplified from hybridomas or B-cells by PCR, cloned into expression vectors, and soluble antibody fragments secreted from bacteria were then screened for binding activities (for review, Winter and Milstein, 1991). Especially the development of immense combinatorial antibody libraries made up of millions or billions of different clones necessitated a reliable high-throughput technique for a successful library screening. The first combinatorial antibody gene libraries were constructed into bacteriophage λ and antigen binding antibody fragments were identified by replica plaque lifts onto hybridisation filters (Huse *et al*., 1989; Mullinax *et al*., 1990; Sastry *et al*., 1989). It was soon realised that antibody screening would be revolutionised if they could be expressed on the surface of bacteriophage. Phage carrying antibody fragments encoding a specific binding activity could then be selected directly with antigen.

In 1990 McCafferty and co-workers first published a successful antibody phage display (McCafferty *et al*., 1990) in which the V_H and V_L genes from an anti-lysozyme antibody were connected as an scFv form to the gene encoding the phage pIII coat protein. The fusion protein was incorporated into the phage, allowing the phage to bind specifically to the antigen and the fusion did not affect the infectivity of the phage. Subsequently, two groups demonstrated that heterodimeric Fab fragments could also be displayed on the surface as pIII fusions by linking either the heavy or light chain to the coat protein and secreting the other chain into the bacterial periplasm where the two chains associated (Hoogenboom *et al*., 1991; Kang *et al*., 1991a). Phage bearing antigen-binding antibody fragments could be separated from non-binding phage by an antigen affinity enrichment procedure called biopanning (Kang *et al*., 1991a; McCafferty *et al*., 1990). The affinity selection procedure couples antigen binding with phage replication and by successive rounds of selection and replication it is possible to isolate originally extremely rare binders from a vast excess of weaker binders.
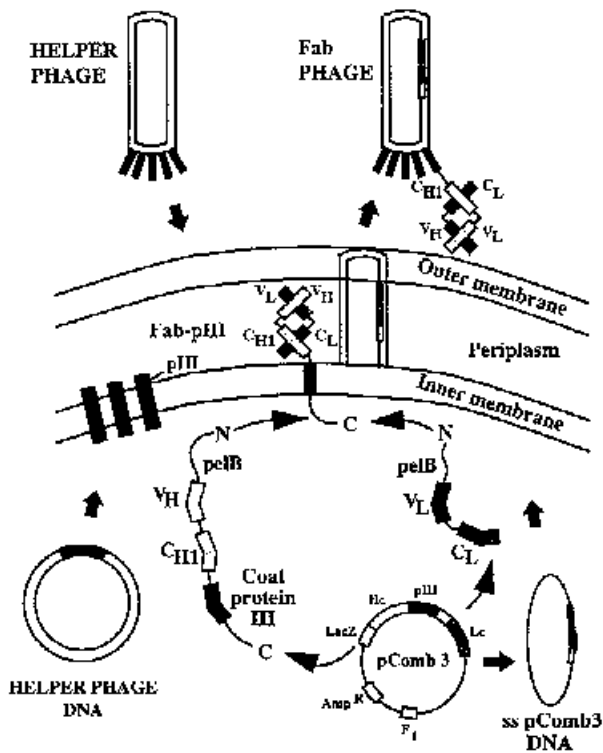
Figure 6. Schematic view showing Fab assembly and incorporation into phage particle by using phagemid vector (modified from Burton and Barbas, 1994).

Antibody phage display provides a much more practical and faster method than any screening technique to isolate the desired antibody and its gene (Hoogenboom *et al*., 1998; Winter *et al*., 1994) and will gradually take the place of conventional hybridoma technology for the production of monoclonal antibodies. This is possible by transferring the antibody gene repertoire of the B-cells of an immunised animal to a phage display vector by cDNA cloning (1.5.1). Alternatively, phage display can be used to make antibodies without prior immunisation by displaying very large and diverse non-immunised ('naive', 1.5.2) or synthetic (1.5.3) antibody gene repertoires on the surface of

phage. If necessary, the binding characteristics of antibodies can be engineered for specific applications (1.6). A commonly used method to improve the binding properties of recombinant antibodies is the generation of random point mutations directed either to a set of defined residues or to the whole variable regions of light and heavy chains and the selection of improved variants from the mutant library by phage display (Hawkins *et al.*, 1992).

In the future, the ribosome display technology (Hanes and Plückthun, 1997; Mattheakis *et al.*, 1994), in which proteins are translated, displayed and selected totally *in vitro* on ribosomes, will probably provide access to repertoires of potentially unlimited diversity. This technology may also be an ideal method for the selection and engineering of antibodies.

1.5.1 Antibody gene libraries from immune source

As described earlier (1.3.1), PCR with antibody-specific primers can be used to amplify V_H and V_L gene repertoires from cDNA prepared from spleen B-cells or peripheral lymphocytes. Large combinatorial libraries are generated by combining the heavy and light chain gene repertoires cloned by PCR. Prior immunisation leads to a high representation of chains arising from *in vivo* binders (differentiated plasma cells), since stimulated B-cells typically have mRNA levels up to 1000-fold higher than unstimulated B-cells (Schibler *et al.*, 1978). Thus, amplification through PCR generates a high proportion of antigen specific antibodies (Ward *et al.*, 1989), although the random pairing of heavy and light chains in such combinatorial libraries mimics nature's random pairing. As only a relatively small number of monoclonal antibodies can be produced and screened by immunising mice and preparing hybridomas, antibody gene libraries, when combined with the phage display technology, enable more thorough characterisation of natural immune response than conventional hybridoma technology (Burton, 1991; Chester *et al.*, 1994; Clackson *et al.*, 1991; Kettleborough *et al.*, 1994). Antibody gene libraries have also been used to isolate specific antibody clones from naturally infected human (Barbas and Burton, 1996; Barbas *et al.*, 1993b; Burton *et al.*, 1991; Williamson *et al.*, 1993). An interesting application is the isolation virus neutralising human antibodies (Barbas *et al.*, 1992b; Burioni *et al.*, 1994; Ping *et al.*, 1996).

1.5.2 Antibody gene libraries from non-immune source

By providing a much more sensitive and faster method than any screening technique, antibody phage display has enabled the isolation of specific antibodies even from naive combinatorial gene libraries constructed without prior immunisation (Hoogenboom, 1997; Marks *et al*., 1991b; Winter *et al*., 1994). For creation of naive antibody gene repertoires, repertoires present in peripheral lymphocytes, bone marrow, tonsil or spleen B-cells are isolated from non-immunised source by PCR amplification of the V_H genes from IgM and the V_L genes from κ and λ mRNA. The IgM V_H gene repertoire is generally more diverse than the IgG repertoire since it is more likely to present B-cells that have not been selected and amplified by antigen. A complete antibody gene library can theoretically be amplified from the mRNA of a large pool of non-immunised donors. This library could be repeatedly selected for antigen binding with a variety of different antigens, obviating the need to immunise with the appropriate antigen each time an antibody with a certain specificity is desired. Importantly, the genes of self-reactive antibodies can also be amplified from a naive source and expressed in antibody gene libraries.

The most promising application of the naive gene library approach is the generation of a complete human antibody gene expression library that would enable direct production of human monoclonal antibody fragments. It has been extremely difficult to make human monoclonal antibodies using conventional hybridoma technology (Larrick *et al*., 1989) and it is normally not possible to immunise humans with a desired antigen, e.g. virus or toxin. However, human antibodies are preferred for therapy because murine antibodies are immunogenic when administered to humans, resulting in decreased efficacy over time and the risk of allergic reactions (Shawler *et al*., 1985; Dillman, 1990). The naive antibody gene libraries can also be used to apply antibodies to new applications, e.g. by selecting specific antibodies against human self-antigens (Griffiths *et al*., 1993; Vaughan *et al*., 1996).

The affinities of antibody fragments selected from naive libraries are likely to be lower than those from the immunised libraries, since naive libraries by definition lack the increased affinity that occurs as a result of somatic hypermutation during an antigen-driven response. However, the ability to generate large, diverse libraries improves the chances of finding a high-affinity antibody to a given

target. Perhaps the best example of the power of extremely large antibody libraries was described by Vaughan *et al*. (1996), who aimed to generate a single stable resource from which specific high-affinity human antibodies to any given antigen could rapidly be isolated. The authors used functional V-gene segments from 43 non-immunised human donors to construct a repertoire of 1.4 x 10^{10} scFv fragments displayed on the surface of phage. The great diversity was achieved by performing several hundred electroporations into *E. coli*. Several of the antibodies selected from this repertoire, directed against both protein and hapten antigens, have binding affinities of Kd less than 10 nM. The best of these, an anti-fluorescein antibody (0.3 nM) and another directed against the hapten DTPA (0.8 nM), demonstrated for the first time that antibodies with subnanomolar binding affinities can be isolated from a naive library. Many of the isolated antibodies had sufficiently high affinity for their intended application without further manipulation. It was also possible to isolate antibodies to antigens that have proved difficult using traditional hybridoma techniques, including antibodies to doxorubicin which is both immunosuppressive and toxic, and a high-affinity, high-specificity antibody to the steroid hormone estradiol. This work shows that very large naive antibody gene libraries may potentially replace conventional hybridoma technology as a direct source of high-affinity antibodies if stability of the libraries can be achieved.

1.5.3 Synthetic antibody gene libraries

Ideally, antibodies to any chosen antigen can be selected from universal, antigen-unbiased libraries. Antibody gene repertoires cloned from naive sources depend on *in vivo* rearranged antibody gene sequences present in B-cells, as described above. An alternative approach is to use synthetically assembled variable regions to construct semisynthetic and ultimately totally synthetic combinatorial antibody gene libraries (Barbas *et al*., 1992a, 1993a and c; de Kruif *et al*., 1995a and b; Garrard and Henner, 1993; Griffiths *et al*., 1994; Hoogenboom and Winter, 1992; Iba *et al*., 1997; Söderlind *et al*., 1995). In contrast to antibodies cloned from a donor animal, synthetic antibodies can have CDRs of any size with any sequence, providing access to sequences not present in nature. Very high structural diversity can be obtained, which is important for the generation of different specificities and, as pointed out by Barbas *et al*. (1993a), completeness of a library is in many cases not as important as

structural diversity. According to Hoogenboom (1997), the advantages of using synthetic antibody libraries will eventually reduce primary naive libraries to useful but transient sources of V-genes and most novel features in antibody engineering will be based on the synthetic antibody concept. Recently, Hoogenboom *et al.* (1998) suggested that the total genetic diversity accessed by the phage display selection procedure would be at the most 10^{11} clones.

The first completely synthetic human antibody gene library was created in MorphoSys (MorphoSys, Germany, unpublished) and termed HuCAL (Human Combinatorial Antibody Library). It comprises a fully synthetic 49 gene (7 V_H genes combined with 7 V_L genes) 'master library' which forms the basis for an unlimited number of daughter libraries. The 14 master genes were derived from the analysis of all known human antibody sequences, and represent all sub-classes and the repertoire of structural framework variations. By condensing the human antibody sequences down to a small set of 14 master genes, total chemical synthesis was made possible. This enabled incorporation of special features to achieve efficient *in vitro* affinity maturation and high yield production of antibodies derived from HuCAL. The aim was to achieve complete control over the composition of the CDR and framework 'cassettes' which are built into the HuCAL genes. Thus, a fully modular system of vectors and gene cassettes with unique restriction sites for rapid exchange and randomisation of all CDRs and frameworks was designed and constructed. Additionally, optimised codon usage for bacterial expression was used and amino acids causing bottlenecks in the folding pathway were replaced.

As a first application of HuCAL, one out of 49 V_H/V_L combinations, cloned as an scFv fragment, was randomised in the heavy chain CDR3 using the trinucleotide-directed mutagenesis (TRIM) method (1.6.1; Virnekäs *et al.*, 1994). The library (3 x 10^7 members) was panned against a variety of novel antigens such as haptens, steroids, glycoproteins and oligosaccharides. In all cases, a high number of binders were obtained after few rounds of panning that recognised the antigens specifically with affinities between 1 and 60 nM. Randomly picked members of the library were all produced in *E. coli* in the >10 mg/l range of soluble, functional antibody in conventional shake flask cultures (Plückthun, personal communication). Thus, a small number of fully synthetic antibody genes, covering the structural diversity of the human immune

repertoire and designed for *in vitro* combinatorial biology approaches, may represent the next generation of human antibody libraries.

1.6 Antibody mutant libraries

The binding characteristics of the antibodies selected from gene libraries, as well as those of many hybridoma-based antibodies, are not sufficient for many applications. Generally, an alternative to rational engineering of proteins is to use efficient selection/screening methods to isolate desired mutants with the anticipated properties from a large collection of randomly or semirandomly generated variants of the original protein. The procedure in random engineering goes through the generation of variation, selection/screening and characterisation in a recursive manner, so that each new cycle results in variants which better meet the defined requirements.

Different mutagenesis approaches can be used to generate structural variation into an antibody combining site. Mutant libraries combined to efficient *in vitro* selection methods have been successfully used to yield improved antibodies without requiring detailed structural information or devising special screening procedures (Barbas *et al*., 1994; Deng *et al*., 1994; Gram *et al*., 1992; Jackson *et al*., 1995; Low *et al*., 1996; Schier *et al*., 1996b and c; Yang *et al*., 1995; Yelton *et al*., 1995).

1.6.1 Oligonucleotide-directed CDR mutagenesis

During the *in vivo* immune response, antibodies of increasing affinity and specificity for the antigen emerge with time in an affinity maturation process (1.2.4; Berek and Milstein, 1987). This progressive refinement for antigen binding is a result of somatic hypermutation events primarily localised to the six hypervariable regions and their flanking sequences (Berek and Milstein, 1988; Jolly *et al*., 1996; Tomlinson *et al*., 1996). These regions are also reasonable targets for *in vitro* mutagenesis, especially when antibodies with nanomolar affinities are used as starting leads (Hoogenboom *et al*., 1998). The first step in constructing antibody mutant libraries is identifying all CDRs of the antibody and,

if structural information is available, only those CDRs or residues predicted to contact an antigen can be selected for mutagenesis.

Typically, the selected CDR(s) are randomised by an oligonucleotide-directed mutagenesis approach (Barbas *et al*., 1994; Riechmann and Weill, 1993; Schier *et al*., 1996b and c; Yang *et al*., 1995; Yelton *et al*., 1995). Complete randomisation at the amino acid level can be achieved by having an equal mixture of A, C, G, and T in each position of the respective codons in the oligonucleotide synthesis (NNN randomisation). By using an NN(C/G) or NN(C/G/T) mixture it is possible to get all 20 amino acids while eliminating all but one stop codon. This restriction is also useful because it reduces the overall DNA sequence complexity and the coding discrepancy between different amino acid residues. However, in multiple codon randomisations the number of amino acids that can be simultaneously scanned completely and collected into a library is limited to five (3.3×10^6 different amino acid sequences), due to the transformation efficiency of *E. coli*.

Semirandom mutagenesis exploits information regarding a primary (wild-type) clone(s) to limit sequence complexity. A doping scheme for oligonucleotide synthesis is used to create limited amino acid subgroups, such as hydrophobic or neutral, at particular sites (Arkin and Youvan, 1992). For example, a randomisation using NT(C/G) or NA(C/G) would be restricted to five hydrophobic amino acids or to seven relatively hydrophilic side chains, respectively. Another way to limit the sequence complexity is by biasing the randomisation towards the wild-type residue. In this case oligonucleotides are synthesised by including low concentrations of the three non-wild-type nucleotide at each step of the synthesis (Hermes *et al*., 1989; 1990). This mimics natural mutagenesis by taking advantage of the natural tendency of the genetic code to favour chemically and/or sterically conservative amino acid substitutions. The level of mutagenesis can be adjusted by varying the proportions of nucleotides during the synthesis. A great number of amino acids can be scanned by a combined mutagenesis approach, called the parsimonious mutagenesis method (Balint and Larrick, 1993), using oligos designed both to minimise coding sequence redundancy and also to limit the number of amino acids which do not retain wild-type structural features. Codons in which degeneracy is equal to or only slightly greater than the subsets of amino acids encoded are used (Arkin and Youvan, 1992; Goldman and Youvan, 1992). Simultaneously, non-viable

structures are minimised by using wild-type-biased nucleotide mixtures (Hermes *et al*., 1989, 1990).
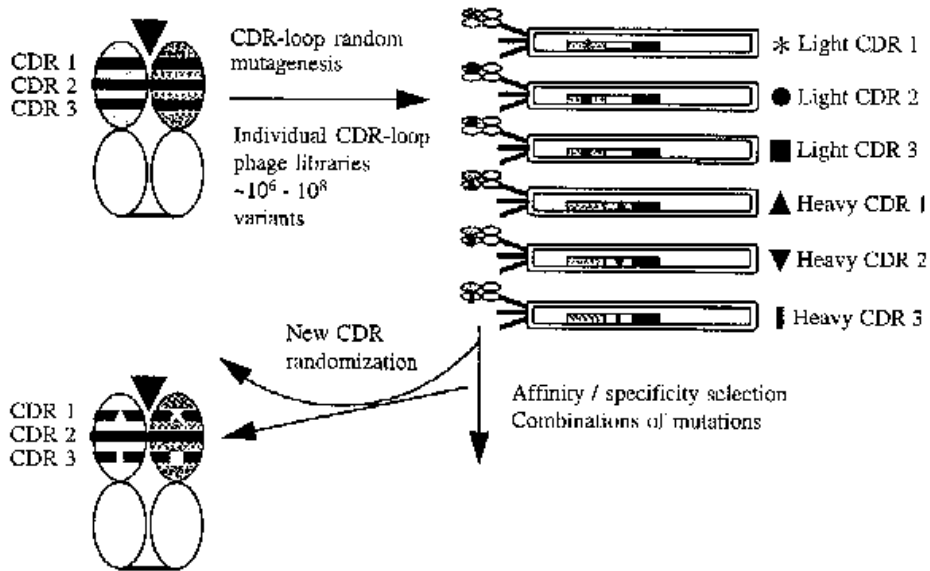
Figure 7. Construction of CDR mutant libraries into Fab phage display vector for selection of improved binders.

To obtain full control over the distribution of amino acids in targeted sequences, Virnekäs and co-workers described the synthesis of 20 different trinucleotide phosphoramidites, representing all the necessary codons (Virnekäs *et al*., 1994). During standard DNA synthesis trinucleotide phosphoramidites or any kind of desired subset thereof can be directly coupled in >99% yield per trimer as a building block. Using trinucleotide-directed mutagenesis, if technically practicable, all 20 amino acid residues can be introduced in an even distribution with a representation efficiency independent of the number of randomised sites. A codon-based oligonucleotide-directed mutagenesis approach (Glaser *et al*., 1992; Huse *et al*., 1993; Yelton *et al*., 1995) replaces entire nucleotide triplets rather than individual nucleotides.

1.6.2 Parallel and sequential CDR mutagenesis strategies

One strategy for optimising the antibody combining site is to target each CDR separately for mutagenesis, identify clones with improved characteristics, and then combine the mutated CDRs into a single antibody (Barbas *et al*., 1992a). This approach assumes that each of the amino acid substitutions independently influences antigen binding and that the combined effect will be an improvement over the precursor clones (Lowman and Wells, 1993; Wells, 1990). In many cases additivity has been observed. Yang *et al*. (1995) increased the affinity of an anti-HIV gp120 Fab 420-fold (Kd 1.5 x 10^{-11} M) by mutating four CDRs in five libraries and combining independently selected mutations. The greatest increases were from sequential mutation of two separate regions of V_H CDR3, resulting in a 63-fold increase in affinity over the wild-type. Mutation of V_L CDR3 resulted in the next greatest increase in affinity.

Another optimisation approach is to stepwise accumulate CDR mutations (Schier *et al*., 1996c; Yang *et al*., 1995). The CDRs are mutated and selected sequentially one by one and after every round the highest affinity mutant is used as a template for the subsequent round of mutagenesis and selection. Sequential optimisation takes into account the facts that additivity may not always be observed and that optimal binding may result from the interdependence of loops. Yang *et al*. (1995) successfully applied several rounds of CDR-directed mutagenesis and selection (CDR-walking) to prepare anti-HIV gp120 Fab fragments with very high affinities. Four different optimisation sequences were examined and variants with improved affinity were isolated from all of them. By mutating only two CDRs Schier *et al*. (1996c) achieved a 1230-fold increase in affinity of an antibody binding the glycoprotein tumor antigen c-*erb*B-2, suggesting that focusing mutations in CDR3s may be the most efficient means to increase affinity. First, nine amino acid residues in the V_L CDR3 were partially randomised (the frequency of wild-type amino acids was 49%). After four rounds of selection using decreasing concentrations of biotinylated antigen, improved variants with affinities up to 1.0 x 10^{-9} M (16-fold increase) were achieved. To further increase the affinity the V_H CDR3 of the highest affinity mutant was randomised four residues at a time in four separate libraries and selected on biotinylated antigen. By combining mutations from the two successfully mutated V_H CDR3 libraries, a significant increase in affinity was obtained yielding an scFv that had a 1230-fold lower Kd than the wild-type (Shier *et al*. 1996c).

1.6.3 Mutagenesis of the whole V_L/V_H regions

One procedure for the generation of random point mutations into wide regions is the propagation of the vector carrying the gene of interest in special *E. coli* mutator strains (*E. coli* mutD5) having a significantly higher frequency of spontaneous mutations than the wild-type strain (Fowler *et al*., 1986; Greener *et al*., 1996). By combining the capacity of *E. coli* mutator strains to introduce point mutations into a target DNA sequence together with the affinity selection procedures of phage display, Irving *et al*. (1996) successfully increased the apparent binding affinity of a human scFv fragment over 100-fold. They constructed a strain of the mutD5 mutator *E. coli* to be competent for M13-based phagemid transfections and rescues. This strain generated primarily point mutations in over 90% of the scFv DNAs. The point mutations which resulted in single amino acid substitutions generally brought about increases in affinity in 1% of the selected scFvs. These improvements occurred as a result of mutations in framework regions adjacent to the CDRs and were generally up to a 10-fold increase. However, one increase in affinity greater than 100-fold was produced, which was shown by BIAcore analysis to have an affinity in the order of 10^{-7} M. Low *et al*. (1996) also reported successful improvement of the binding affinity of an scFv fragment by multiple rounds of growth in the mutator strain and increasingly stringent selection. Four sequentially acquired mutations together improved the binding affinity of the antibody by a factor of 100-fold. The distributions of mutations and nucleotide substitution preferences strongly resembled those of somatic hypermutation.

Random point mutations can also be introduced into cloned genes through an error-prone PCR method, which is based on the low intrinsic fidelity of the *Taq* polymerase and conditions that favour the misincorporation of incorrect nucleotides (Cadwell and Joyce, 1992; Leung *et al*., 1989). Hawkins *et al*. (1992) mutated an anti-4-hydroxy-5-iodo-3-nitrophenacetyl-(NIP)-caproic acid scFv by error-prone PCR and used phage display selection to isolate a mutant with a 4-fold improved affinity. Gram *et al*. (1992) isolated clones from a naive antibody library and used error-prone PCR for *in vitro* affinity maturation of the selected antibodies. Error-prone PCR provides a good method to introduce random point mutations simultaneously into wide regions, e.g. whole variable domains, but introduction of multiple mutations into restricted regions such as individual CDRs is highly unlikely and the method is relatively difficult to control. Recently,

Saviranta *et al*. (1998) described increased reproducibility of the error-prone PCR mutagenesis method by adjusting the number of effective duplications and they employed the method to improve the steroid-specificity of an anti-estradiol Fab fragment up to 20-fold.

Pairing a heavy chain segment from an antigen-binding antibody with a library of light chains, or vice versa, through a combinatorial construct may reveal new chain combinations that exhibit improved binding characteristics. This process is called chain shuffling, and its utility was first demonstrated by Clackson *et al*. (1991) and Kang *et al*. (1991b). Chain-shuffling has been successfully used e.g. to increase the affinity of a non-immune human anti-phenyloxazolone scFv 300-fold to 1.0 x 10^{-9} M by sequentially shuffling the rearranged V_L gene and the V_H gene segment (the wild-type V_H CDR3 was retained) (Marks *et al*., 1992a). The V_H CDR3 was conserved because it results from the splicing of three segments (V_H, D, and J), being the most genetically diverse part of the rearranged V_H gene. Since V_H CDR3 contributes a disproportionate number of amino acid residues which contact antigen, shuffling the rearranged V_H gene might result in a library containing few binders. Schier *et al*. (1996a) used the same strategy to increase the affinity of a human scFv which binds to the extracellular domain of the tumor antigen c-erbB-2 with a Kd of 1.6 x 10^{-8} M up to 6-fold, although the suitability of chain shuffling for increasing the affinity of protein-binding antibody fragments has been questioned. Compared to antibodies which bind haptens, there are a great number of contacts between protein and antibody and a large surface area is buried upon binding. Thus the chances of disrupting multiple favourable contacts by shuffling are greater, but could be compensated by the loss of unfavourable contacts or generation of new contacts.

DNA shuffling is a new technique for directed evolution of proteins, which generates diversity by recombination, combining useful mutations from individual genes *in vitro* (Crameri *et al*., 1998; Stemmer, 1994). The method is based on random fragmentation of a pool of related (mutant) genes, followed by reassembly of the fragments in a self-priming PCR reaction. DNA shuffling has an intrinsic mutagenesis rate similar to error-prone PCR and, additionally, synthetic (mutagenic) oligonucleotides and PCR fragments can be added to the reassembly mixture and incorporated into the gene. A pool of related naturally occurring genes, such as antibodies, or homologous genes from different species can also be used as a source of diversity. Combination of DNA shuffling and high throughput

selection/screening is a powerful tool for the optimisation of proteins (Crameri *et al.*, 1996a, 1997; Harayama, 1998; Moore et al., 1997; Patten *et al.*, 1997), including antibodies (Crameri *et al.*, 1996b). Another approach to *in vitro* recombination, staggered extension process (StEP), was recently developed by Zhao *et al.* (1998).

1.7 Selection strategies for antibody phages

Recent research has shown that filamentous phage is able to display on its surface peptides and proteins allowing the construction of libraries containing millions of different clones. In an affinity enrichment process commonly known as biopanning a library displayed on filamentous phage is selected for binding to a target (Hoogenboom *et al.*, 1998; Winter *et al.*, 1994). Biopanning of antibody libraries mimics the *in vivo* selection of B-lymphocytes bearing antigen-responding IgM receptors on their surface by specifically enriching phage particles displaying antibodies with desired binding characteristics on their surface. Biopanning is a repetitive process that enriches the amount of the desired sequence with each panning step until only a small number of sequences remain. Specific clones binding to the target can then be characterised and used for production of the desired protein or peptide in *E. coli* or other suitable host.

1.7.1 Basic panning procedure

The outcome of biopanning selection is profoundly dependent on the selection conditions. In its simplest form, biopanning is carried out by incubating the mixture of phage-displayed protein variants with an immobilised target. Several formats have been used, including the target coupled to microtiter plate wells, columns or tubes (Hoogenboom *et al.*, 1998). The phage particles that do not bind to the immobilised target, or that are weakly bound, are washed away, followed by elution of the phage particles that do bind to the target. Elution can be performed e.g. by treatment with acid, alkali, chaotropic agents, or a soluble antigen (Hoogenboom *et al.*, 1998). After neutralisation, the eluted phage particles are used to infect *E. coli*, in order to reproduce the affinity-selected phage particles for the next round of enrichment. The panning process is normally repeated two or more times to eliminate proteins that bind only weakly

or non-specifically. After 4 - 5 rounds, individual clones are usually analysed and interesting ones are identified by DNA sequencing.

1.7.2 Affinity selection

Alternatively, phage can be biopanned against the target in solution, followed by affinity capture of the target-phage complexes (Barbas *et al.*, 1994; Hawkins *et al.*, 1992; Marks *et al.*, 1992b; Schier *et al.*, 1996a, b and c). One widely used method is to make the binding reaction in solution by using a biotinylated antigen and then to capture the antigen-bound phages by immobilised streptavidin. This allows more specific control of the selection procedure. For example, for the initial rounds of selection an antigen concentration greater than the Kd of the desired or wild-type clone can be used in order to capture rare or poorly expressed phage antibodies. To select on the basis of affinity an antigen concentration significantly less than the desired Kd, and less than the phage concentration, is used in the later rounds of selection (Schier *et al.*, 1996a).

Kinetic properties of the antibodies are important in many applications. Thus, different selection strategies have been devised that rely on either the dissociation or association rate of the antibody phage and the capturing antigen (Duenas *et al.*, 1996; Hawkins *et al.*, 1992; Low *et al.*, 1996; Malmborg *et al.*, 1996; Marks *et al.*, 1992b). The method based on the use of limiting concentration of biotinylated antigen for affinity selection was described by Hawkins *et al.* (1992) and has been successfully applied (e.g. Barbas *et al.*, 1992a; Schier *et al.*, 1996a, b and c; Thompson *et al.*, 1996). Hawkins *et al.* (1992) also described a dissociation rate selection method in which antibodies are preloaded with biotinylated antigen and then diluted into an excess of unlabelled antigen for variable times prior to capture on immobilised streptavidin (Low *et al.*, 1996; Duenas *et al.*, 1996). Later, a competing antibody-based dissociation rate selection was developed (Yang *et al.*, 1995). Duenas *et al.* (1996), and Low *et al.* (1996) demonstrated that by limiting the concentration of antigen and the contact time between antigen and antibody it is possible to select higher affinities and faster association rates. Malmborg *et al.* (1996) successfully used BIAcore to select phage-displayed antibodies on the basis of dissociation rate constants and showed that the time of elution was directly proportional to the affinity, due to decreased dissociation rate constants. BIAcore has also been successfully used for optimising phage selection conditions

and for ranking isolated clones after panning by measuring the k_{off} of antibody fragments from unpurified periplasm samples (Schier *et al*., 1996a). A decreased dissociation rate is typically the major kinetic mechanism resulting in higher affinity, both *in vivo* (Foote and Milstein, 1991) and *in vitro* (Marks *et al*., 1992b) and this approach should generally result in the identification of higher affinity clones.

MUTANT LIBRARY ~10¹¹ PHAGES

+ BIOTINYLATED ANTIGEN

+ STREPTAVIDIN

WASH, ELUTE

INFECT *E. COLI* CELLS
PHAGE STOCK
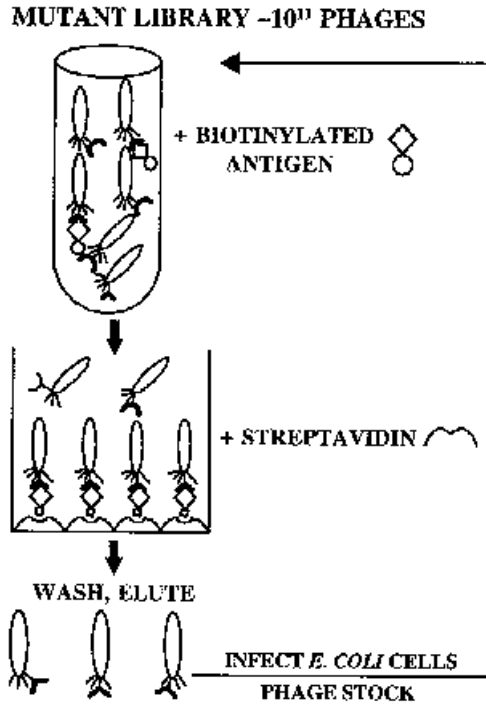
Figure 8. Principle of an affinity selection procedure.

1.7.3 Specificity selection

Several biopanning procedures have been developed for directing the selection towards a specific epitope. By using 'competitive deselection' (de Kruif *et al*., 1995b; Parsons *et al*., 1996) it is possible to compete out cross-reactive clones during selection. This is achieved by providing an excess of cross-reactive antigen in solution, and then isolating binders not blocked by the soluble cross-reactive antigen using either an immobilised antigen or a biotinylated antigen followed by capturing on immobilised streptavidin. Parsons *et al*. (1996) used competitive deselection to isolate antibodies specific for foetal haemoglobin, despite the presence of a cross-reactive clone with a 600-fold higher affinity.

Figure 9. Principle of a specificity selection procedure.

Specific elution with a soluble antigen or a related molecule has widely been used to isolate phage expressing specificity directed against a defined antigen (Clackson *et al*., 1991; Hawkins *et al*., 1992; Riechmann and Weill, 1993; Schier *et al*., 1996a). Meulemans *et al*. (1995) utilised an existing monoclonal antibody to competitively elute phage antibodies having the same binding specificity. Jespers *et al*. (1994) developed a chain shuffling-based strategy for guiding the selection of human antibody fragments to a single epitope of an antigen, using rodent monoclonal antibodies as a template. Ditzel *et al*. (1995), Sanna *et al*. (1995) and Ping *et al*. (1996) used an existing monoclonal antibody to block the binding of phage antibodies to one epitope in an antigen, directing the selection procedure to other epitopes of the same antigen.

1.7.4 New applications

The versatility of phage display selection technology is demonstrated by some recent applications. An increasing number of antibodies have now been selected by panning on cell surface expressed antigens (Andersen *et al*., 1996; Cai and Garen, 1996; Chowdhury *et al*., 1997; Eggena *et al*., 1996; Watters *et al*., 1997) and this has become an important method for the selection of antibodies with therapeutic potential. In contrast to panning on purified immobilised antigen, cell panning is applicable to antigens in their native environment on cell surfaces, enabling the selection of antibodies that bind to epitopes accessible *in vivo*. Cell panning, as well as panning on tissue sections (Hoogenboom *et al*., 1998; Tordsson *et al*., 1997; Van Ewijk et al., 1997), can also be used when the antigen is not available in purified form or when the exact target antigen is not even known. For example, the majority of antigens expressed on the surface of human red blood cells have been defined only serologically and are not available in purified form. Cell panning of combinatorial antibody libraries derived from auto- and alloimmunised individuals can now be used to select for these antigens (Marks *et al*., 1993; Siegel, 1995). The specificity of selection on cells or other complex, unpurified targets can be improved e.g. by using subtractive selection procedures (Cai and Garen, 1995; de Kruif *et al*., 1995a; Siegel *et al*., 1997), competitive elution (1.7.3) or by alternating between different sources (cell types) of antigen (Andersen *et al*., 1996).

Recently, Osbourn *et al*. (1998) introduced a novel 'pathfinder' selection method for targeted recovery of binding molecules from phage libraries. In this approach an existing binding specificity (a pathfinder molecule) is used as a tool to select phage libraries *in situ*, obviating the need to purify or clone the target. Natural ligands, as well as existing mono- and polyclonal antibodies, can serve as pathfinders when conjugated directly or indirectly to horseradish peroxidase. In the presence of biotin tyramine these molecules catalyse the biotinylation of phage binding to the same target as the pathfinder, or in its close proximity (within 25 nm radius), allowing specific recovery of biotin-tagged phage from the total population using immobilised streptavidin. Recovery of antibodies to specific cell surface antigens and immobilised purified antigens from a naive human scFv library has been demonstrated using different specific pathfinder molecules.

Pasqualini and Ruoslahti (1996) developed a methodology for the *in vivo* panning of phage displayed peptide libraries in order to select peptides that home to target organs. Phage were injected into the tail vein of mice, allowed to circulate for a few minutes, and were isolated and reamplified from brain and kidney tissue after the mice had been snap frozen in liquid nitrogen. After three rounds of *in vivo* panning, peptide sequences that selectively target brain and kidney were identified. In principle, *in vivo* panning should also be feasible by using phage displayed antibody libraries. Selective targeting by peptides or antibodies promises applications in gene and cancer therapy (Arap *et al*., 1998).

In conventional phage display approaches, molecular recognition and phage replication are linked by infecting the phage host *E. coli in vitro* with an affinity selected phage population. However, interesting strategies have been developed that enable direct coupling of antigen binding and phage replication, mimicking the antigen-driven selection and stimulation of B-cells (Duenas and Borrebaeck, 1994; Krebber, C. *et al*., 1995, 1997). The selectively infective phage (SIP) methodology (Krebber, C. *et al*., 1995, 1997) selects for protein-ligand interactions *in vivo* by making the infectivity of phage dependent on this binding. The three-domain structure of the phage pIII (1.4.1) is exploited by reassembling it from two parts when specific antibody-antigen interaction occurs. Firstly, a phage particle is made non-infective by replacing its N-terminal domains of pIII with an antibody fragment. Secondly, the antigen is linked to those N-terminal domains of pIII which are missing from the phage particle. Finally, infectivity is restored when the displayed antibody binds to the

antigen and thereby attaches the missing N-terminal domains of pIII to the phage particle. Krebber and co-workers used the SIP system for selecting cognate pairs of antibodies and antigens and found that even up to 10^6 antigen specific infection events occurred from 10^{10} input phages, compared to only one antigen-independent event (Krebber, C. *et al.*, 1997). Additionally, the system has a potential to examine library versus library interactions. This can be achieved by fusing the antigen to the N-terminal domains of pIII and encoding it in the same phage genome as the antibody fragment-pIII fusion. Antigen and antibody fusions will interact with each other in the periplasm of the phage-producing cell, restoring infectivity. This might in the future enable the cloning of a cDNA together with a specific antibody which binds it. In another recently reported approach antibody-antigen interaction dependent infection was achieved by expressing short peptide antigens (13 - 15 residues) on the surface of F pilus (Malmborg *et al.*, 1997). This fusion display blocked the natural infection process, avoiding the need for pIII deleted helper phage.

1.8 Labelling of antibodies for immunoassays

An analytical technique, in which the presence of an analyte is revealed by one or more antibodies and the reaction is monitored by a reporter group introduced to one of the reaction components, is the basis of current immunodiagnostics. The extreme variability, specificity and affinity of antibodies enable their use in analytical detection of a tremendous number of analytes of biological, biochemical and medical interest from complex biological samples. Immunoassays can be broadly classified into two categories. In competitive assays competition occurs between labelled and unlabelled analyte molecules for a limited number of antibody binding sites, whereas non-competitive assays are based on simultaneous binding of two antibodies independently to an analyte. Immobilised antibodies are used to catch all the analyte molecules from the sample and labelled antibodies to determine the amount of analyte.

Preparation of a conjugate of a label and an antibody or antigen is a key step in the development of an immunoassay. A great variety of different types of reporter molecules, such as radioisotopes, fluorescent and chemiluminescent molecules, and enzymes (with chromo-, fluoro- or luminogenic substrates) chemically linked to antibodies have been used to quantify antibody-antigen interactions (Hemmilä,

1994; Howanitz, 1992). The trend in label technology has been to make assay performance easier, e.g. by enabling visual detection or automation. Another aim is the development of labels that can improve assay sensitivity. Since the assay sensitivity especially in antibody-labelled two-site (sandwich) immunoassays is dependent on the detection limit for the label, multiple labelling of an antibody is often desirable in order to increase the detectability of the bound conjugate. Although a variety of labelling reactions are available, the methods of chemical modification often lead to partial loss of antibody binding activity, increased non-specific binding, and to batch-to-batch variation between different conjugate preparations. Such effects are further aggravated when much smaller recombinant antibody fragments are labelled to a high degree needed for sensitive detection. Nevertheless, the Fab or F(ab)$_2$ fragments lacking the Fc fragment often give lower unspecific signals than intact IgGs, which facilitates certain diagnostic assays.

Europium (Eu^{3+}) chelates are convenient, non-radioactive labels which can be quantified sensitively by time-resolved fluorescence. Therefore, Eu^{3+}-labelling has been widely utilised in clinical and research applications (Hemmilä, 1991; Hemmilä *et al*., 1984; Soini and Lövgren, 1987) including a commercial line of immunoassay kits based on the principle of dissociative fluorescence enhancement (DELFIA$^®$, Wallac). In this technology antibodies are usually labelled through the aliphatic ε-amine groups of lysine residues and the immunoreactivity is generally well preserved at labelling yields of 20 Eu^{3+}/IgG or more (Lövgren *et al*., 1990). In individual cases, as with the anti-hAFP antibody AF5, decreased affinity of certain monoclonal antibodies has been observed.

1.9 Steroid hormones

All steroid hormones have a common 4-ring cyclopentanoperhydro-phenanthrene as their chemical nucleus. Methyl side chains occur typically at positions 10 and 13, constituting C atoms 19 and 18, and a side chain at position 17 is usual. Although each of the 6-carbon rings of the steroid nucleus is capable of existing in the 3-dimensional conformation either of a 'chair' or 'boat', in naturally occurring steroids virtually all the rings are in the more stable 'chair' form. With respect to each other the rings can be either *cis* or *trans*. The B/C and C/D junctions are always *trans* but the junction between the

A and B ring is either *cis* or *trans* except in estrogens, which are not capable of this form of isomerism since their A ring is aromatic. Bonds to substituent groups above the plane of the rings are in β-configuration and are shown with a solid line, whereas those bonds attaching groups below the plane are indicated with broken lines (α-configuration). The methyl groups attached to C_{10} and C_{13} are invariably in the β-configuration, as are the chains attached to C_{17} and various substitutions at C_{11}.

Steroid hormones are produced in the adrenal cortex, testes and ovaries, and can be subdivided into 5 classes, each with characteristic functions: progestins, mineralocorticoids, glucocorticoids, estrogens, and androgens. The number of different steroids is significant, e.g. about 50 steroids have been isolated from the adrenal gland, although only a few of them are known to possess physiological activity. The large number and diversity of steroids necessitates excellent qualitative performance of any analytical technique in order to discriminate between derivatives with rather similar structures yet very different biological properties. Current immunoassays for small molecular analytes, such as steroids, often have a requirement for enhanced specificity. Cross-reactivity is measured by creating a standard curve of the potential cross-reacting compound and by comparing its ability to displace label to that of the standard. For convenience, cross-reactivity is usually reported as the mass required to displace 50% of the tracer in terms of percentage of the standard mass at the same point.

1.9.1 Testosterone

The testes and ovaries synthesise steroid hormones which control secondary sex characteristics, the reproductive cycle, and the growth and development of the accessory reproductive organs. These hormones also exert potent protein anabolic effects. Androgens are C-19 steroids that provide major regulatory influences on male reproductive function. Testosterone (TES), the principal androgenic steroid, is secreted by the Leydig cells of the testes. TES action in many organs, such as prostate and skin, is dependent on the conversion by 5α-reductase to the more potent 5α-dihydrotestosterone (5α-DHT). Oxido-reduction of androgens by 17-β-hydroxysteroid dehydrogenase and sulfurylation

by androgen sulfotransferase are two major pathways of androgen inactivation in target cells.

Determination of serum TES is important in the evaluation of hirsutism, virilism, infertility and other conditions associated with hyperandrogenism in women (Wheeler, 1994; Wilke and Utley, 1987). In men TES levels are used to diagnose reasons for delayed or premature puberty, hypogonadism, impotence and problems in spermatogenesis (Ismail *et al*., 1986a and b; Wang and Swerdloff 1992). Increasing interest in the use of hormone replacement therapy
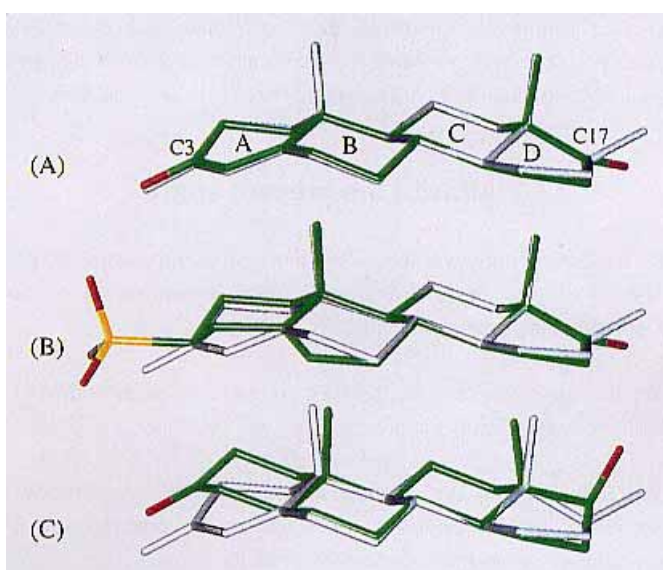
Figure 10. Structure of TES (4-androsten-17β-ol-3-one) and related steroids. The steroids were superimposed using the heavy atoms of the C-and D-rings. (A) TES (grey) and androstenedione (4-androsten-3,17-dione) (green) differ in the side group attached to C17 on the D-ring. (B) In DHEAS (5-androsten-3β-ol-17-one 3-sulfate) (green) both C3 and C17 have different side groups compared to TES (grey) and the position of the double bond in the ring system is different. (C) TES (grey) and 5α-DHT (5α-androstan-17β-ol-3-one) (green) differ in structure at the A-ring, TES having a double bond which is lacking in 5α-DHT.

in men to address problems associated with hypogonadism, in which natural production of TES starts to decrease in middle age and which may then result in decreased libido, impotence, fatigue, depression, loss of muscle and be linked to osteoporosis, is increasing the need to monitor TES levels (Ismail *et al.*, 1986a and b; Wang and Swerdloff 1997). TES levels are also used to monitor the treatment of patients with congenital adrenal hyperplasia (Korth-Schutz *et al.*, 1978). The normal value range for TES is in prepubertal children <1.0 nmol/l, in women 0.3 - 3.5 nmol/l and in men 10 - 35 nmol/l (Braunwald *et al.*, 1987).

Current diagnostic immunoassays for steroid hormones such as TES utilise polyclonal rabbit antiserum because the development of monoclonal antibodies with sufficient specificity and affinity has not been successful by hybridoma technology. The continuous supply of good polyclonal antibody reagents of uniform quality is a severe problem for the immunodiagnostic industry and requires continuous immunisation of large numbers of laboratory animals.

1.10 Aims of the present study

The general aim of this study was to produce immunoreagents for the purposes of the *in vitro* diagnostic industry by using novel antibody engineering technology, especially antibody phage display techniques.

One specific aim was to improve the labelling properties of an anti-human alpha-fetoprotein antibody for DELFIA applications.

Another specific aim was to develop high-affinity and -specificity anti-testosterone antibodies for clinical immunoassays by improvement of the binding properties of an existing monoclonal antibody.

The pre-set requirement for a clinically useful recombinant anti-testosterone antibody was a 10-fold improvement of affinity and a 60-fold reduction in dehydroepiandrosterone sulfate cross-reactivity of the original monoclonal antibody, without increased cross-reactivities to other related steroids.

2. Materials and methods

All the materials and methods used are described in detail in the publications I - IV and only a brief summary is provided here.

2.1 AF5 and 3-C$_4$F$_5$ antibodies

AFP is an important analyte in prenatal diagnosis and in tumor diagnosis and progress monitoring (Filly *et al.*, 1993). It is a protein normally produced by the liver and yolk sac of a fetus, where it has a function analogous to that of albumin. The adult plasma does not normally contain AFP, but it is often found in the blood of patients with hepatomas and teratomas. Abnormal AFP concentrations in pregnant woman are indicative of fetal disorders such as defects of neural tubes or abdominal wall. Too low concentrations could be caused by fetal chromosomal anomalies such as trisomy 21. A murine monoclonal anti-hAFP antibody AF5 (IgG$_1$, k) had been developed at Wallac. The binding characteristics of the AF5 antibody had been determined to be acceptable but the antibody suffered from a decrease in affinity upon labelling of its lysine ε-amino groups with an isothiocyanate derivative of an Eu^{3+}-chelate. The antibody was purified from the hybridoma cell culture supernatant, N-terminal amino acid sequences were determined and proteolytic F(ab)$_2$ and Fab fragments were made and purified (I).

The hybridoma cell line producing an anti-TES monoclonal antibody 3-C$_4$F$_5$ had been developed by hyperimmunising mice with a TES-3-carboxymethyl-oxime(CMO)-thyroglobulin conjugate at Orion Diagnostica. According to RIA, 3-C$_4$F$_5$ had a relatively high affinity (~0.3 x 10^9 M^{-1}) for TES and reasonably low cross-reactions (~11.6% and ~1%) with 5α-dihydrotestosterone (5α-DHT) and dehydroepiandrosterone sulfate (DHEAS). The 3-C$_4$F$_5$ antibody (IgG$_{2b}$,κ) was purified from the hybridoma cell culture supernatant, N-terminal amino acid sequences were determined and a proteolytic Fab fragment was made and purified (II).

2.2 Cloning of the AF5 and 3-C$_4$F$_5$ heavy and light chain cDNAs (I, II)

All basic recombinant DNA methods were carried out essentially as described by Sambrook *et al.* (1990). The cloning of Fd and L genes was performed from mRNA isolated from hybridoma cells. PCR was performed after the first strand cDNA synthesis (Orlandi *et al.*, 1989). The V$_H$-5' and V$_L$-5' PCR primers were synthesised according to the N-terminal amino acid sequences of the H and L chains, respectively, with the exception of the AF5 V$_L$ primer. The C$_H$1-3' and C$_L$-3' PCR primers were complementary to the conserved sequences in the 3' end of the C$_H$1 and C$_L$ regions of the heavy and light chains, respectively. The cloned cDNAs were sequenced and modified with PCR (Saiki *et al.*, 1985, 1988) to provide them with restriction sites allowing precise in-frame fusions in a Fab fragment expression unit in the pKK*tac* vector (Alfthan *et al.*, 1993; Takkinen *et al.*, 1991). The first constant domain of the 3-C$_4$F$_5$ heavy chain (IgG$_{2b}$) was replaced by a gene fragment encoding the first constant domain of the murine IgG$_1$ subclass heavy chain to promote secretion and folding of the Fab fragment in *E. coli* (Alfthan *et al.*, 1993; MacKenzie *et al.*, 1994).

2.3 Production and purification of recombinant Fab fragments

The *E. coli* strain RV308 was used as an expression host for all pKK*tac*-vector constructions. The expression in shake flasks and subsequent purification of Fab fragments from the culture supernatant in a single step using either a hAFP or a TES affinity column was performed as described in I and II, respectively.

Later on (II - IV), all purifications were performed using HiTrap protein G columns following the protocol recommended by the manufacturer. A high-cell-density *E. coli* bioreactor cultivation was set up (IV). The purified Fab fragments were analysed by SDS-PAGE and protein concentrations were determined from the absorbance at 280 nm.

After different panning approaches soluble Fab fragments were produced from isolated clones as described by Barbas *et al.* (1991). The XL1-Blue strain was used as an expression host for the pComb3 phagemid vector (II - IV).

2.4 Homology modelling (I, II)

A model was constructed for the AF5 Fab fragment in order to identify arginine residues exposed at the constant domain surfaces and available for mutation to lysine residues without affecting the antigen binding (I). The 3-C$_4$F$_5$ Fab model was used to identify amino acid residues interacting with the TES molecule (II).

Amino acid sequences of antibody crystal structures were aligned using MALIGN (Johnson *et al*., 1993; Johnson and Overington, 1993). The two 3-D structures with the highest sequence identities to either the H or the L chain were selected for model building. The structurally conserved regions (SCRs) of the antibodies were built by COMPOSER (Blundell *et al*., 1988, 1990; Sali *et al*., 1990; Sutcliffe *et al*., 1987a and b). Five out of the six CDRs in both antibodies were built in as canonical conformations. The CDR3 of the heavy chain does not have any canonical conformation available and therefore fragments from the database containing C$_\alpha$ x C$_\alpha$ distances generated from known crystal structures of better than 3.0 Å resolution were selected for these CDRs. The completed models were refined by energy minimisation using CHARMm (Brooks *et al*., 1988).

The TES structure was extracted from the Cambridge Structural Database (Allen *et al*., 1991) and energy minimised. TES was then docked into the antibody in four different conformations. Each complex was energy minimised and simulated for 100 picoseconds at room temperature by using a stochastic boundary molecular dynamics method, SBMD (Brooks and Karplus, 1989). The model of the complex in which TES had lowest interaction energy with the antibody was further simulated for 400ps by SBMD (Hoffrén *et al*., 1992). The molecular model was inspected by molecular graphics to identify the regions of the variable domains which are in close proximity to the antigen binding site.

2.5 Labelling of the AF5 antibody (I)

Three arginine residues selected from the C$_L$ of the AF5 Fab fragment were replaced by lysines by oligonucleotide-directed PCR mutagenesis. The affinity purified Fab fragments, AF5 antibody and its proteolytic fragments and hAFP were labelled through aliphatic ε-amine groups of lysines with 2,2',2",2"'-{[4-(4-isothio-cyanatophenylethynyl)pyridine-2,6-diyl]bis(methylennitrilo)tetrakis(acetic

acid) (Takalo *et al*., 1994). Thirty to 270-fold molar excess of the reagent and 0.30 - 0.50 mg/ml of the protein were used. The protein-chelate conjugates were purified by gel chromatography and the degree of labelling was calculated from the Eu^{3+} and protein concentrations of the gel chromatography fractions. Concentrations of Eu^{3+} were measured in a 1234 DELFIA fluorometer and the concentrations of proteins were calculated from the absorbance at 280 nm.

2.6 3-C$_4$F$_5$ CDR mutant libraries (II - IV)

The phagemid vector pComb3 (Barbas *et al*., 1991) used in this work was designed for the cloning of combinatorial Fab libraries providing sites for cloning of PCR-amplified heavy chain Fd and light chain sequences. In pComp3 the antibody Fd chain is fused with the C-terminal domain of pIII (P198 - S406) and a flexible five-amino acid linker has been placed between them. The Fd-pIII fusion and light chain genes are under the control of separate *lac* promoter/operator sequences and are directed to the periplasmic space for functional assembly on the membrane by *pelB* leader sequences. Since the pComb3 contains only the origin of replication of the filamentous bacteriophage *f1*, pComb3 needs a helper phage to provide the necessary phage proteins for replication and assembly of phage particles. In the presence of a helper phage on average one Fab/pIII fusion protein and 3 - 4 native pIII proteins will be exposed on the phage surface. A similar approach has been described for the production of hormone phage (Bass *et al*., 1990).

2.6.1 CDR3 mutant libraries (II)

The CDR3 loops of the hesavy and light chains were randomised by oligonucleotide-directed PCR mutagenesis (Hermes *et al*., 1989, 1990). To restrict the number of amino acid mutations in single clones, and thus to limit the number of clones not retaining original structural features of the CDRs, 'spiked', biased mixtures of nucleotides in the oligonucleotide synthesis for the CDR3 loops (80% wild-type and 20 % equal mixture of three other nucleotides) were used. The mutant libraries were cloned by the overlapping PCR method (Ho *et al*., 1989) into separate 3-C$_4$F$_5$ wild-type Fab fragment expression units cloned in the pComb3 phagemid vector, kindly provided by Dr. C. Barbas.

2.6.2 CDR1 and CDR2 mutant libraries (III)

The V_L and V_H CDR1 and CDR2 loops of the combined CDR3 mutant Fab fragment of the 3-C_4F_5 antibody were targeted to mutagenesis by using spiked PCR primers. The randomness of PCR primers were adjusted by nucleotide doping of the oligonucleotide synthesis (62.5% wild-type and 12.5% each of three other nucleotides). The V_H CDR2 mutant library was cloned by the overlapping PCR method while the three other libraries were constructed utilising unique restriction sites close to the CDRs.

2.6.3 Retargetted CDR3 mutant libraries (IV)

The CDR3 loops of the combined mutant clone (after CDR1, 2, and 3 mutagenesis) were re-randomised using spiked oligonucleotides. The bias of the oligonucleotides for the parental sequence was weaker than in the previous CDR3 mutant libraries (62.5%) and the libraries were cloned utilising unique restriction sites created by site-directed mutagenesis near to the CDRs.

2.7 Selection of the 3-C_4F_5 mutant libraries (II - IV)

2.7.1 Specificity panning (CDR3 libraries) (II)

The light and heavy chain CDR3 mutant libraries were selected by the phage display technique (McCafferty *et al*., 1990; Barbas *et al*., 1991) using a competitive specificity panning procedure. The mutant Fab fragment libraries displayed on the surface of the phage were first incubated with soluble DHEAS to inactivate those Fab phages having a high cross-reactivity to DHEAS. Thereafter, the phage pools were transferred to microtiter plate wells coated with the probe, TES-4-mercaptopropionic acid-BSA conjugate, to catch TES binders from the libraries. After washing (10 times during 1 h with Tris-buffered-saline-1% BSA-0.5% Tween 20) the binders were eluted with acidic buffer and immediately neutralised. For the next panning rounds the eluted phage pools were amplified by infecting *E. coli* XL1-Blue cells. The concentration of DHEAS was adjusted to achieve at least 50% inhibition on each round of panning. Seven rounds of panning were performed by increasing the concentration of DHEAS stepwise from

0.1 μM to 0.3 μM. The last panning round was performed without amplifying the previous phage eluate in *E. coli* and on this round the binders were eluted with soluble TES.

2.7.2 Affinity panning (CDR1 and CDR2 libraries) (III)

Mutant libraries were selected by the phage display technique. To improve the TES affinity, limiting, decreasing concentrations of soluble, biotinylated TES were used and binders were captured on immobilised streptavidin. The binders were eluted with a high concentration of soluble TES (100 μM) which was added together with *E. coli* host cells into the microtiter wells. To improve the specificity, the mutant libraries were selected by preincubating with a high concentration of the cross-reacting steroid, DHEAS, and then isolating TES binders using microtiter plate wells coated with a TES conjugate.

2.7.3 Combined panning (retargetted CDR3 libraries) (IV)

A combined selection procedure allowing simultaneous selection in respect of affinity and specificity was used. A high concentration of cross-reacting steroid as a protein conjugate (DHEA-3-SucH-BSA) was used to inhibit binding of cross-reacting phages to immobilised TES-3-CMO-BSA (first two rounds) and to limiting concentrations of biotinylated TES (next three rounds) The contact time with biotinylated TES (preimmobilised into the streptavidin wells) was decreased to 10 min. The elution of phages was performed using 50 mM NaOH, pH 12.6 for 15 min. The mutant libraries were also affinity selected without any competing steroid using limiting, gradually decreasing concentrations of biotinylated TES to catch the high-affinity binders. The concentration of TES used in this approach was clearly lower (1 nM - 10 pM) compared to approaches where the cross-reacting steroid was used.

2.8 Immunoassays

2.8.1 hAFP fluoroimmunoassays (I)

Affinities of Eu^{3+}-labelled antibodies were determined using the DELFIA immunoassay technique: 25 µl of DELFIA hAFP standard at 0 and 100 U/ml were reacted with an excess of the immobilised capture antibody. Simultaneously, six different dilutions of the labelled antibody (6.25 - 200 ng per 100 µl DELFIA assay buffer) recognising a different epitope on hAFP were added to react with the hAFP bound to the capture antibody. After incubation and washing, the fluorescence was developed and measured. After subtracting the nonspecific binding (0 U/ml) from the total binding (100 U/ml), the data was used to calculate the affinities according to the method of Scatchard (1949).

Affinities of unlabelled antibodies were determined accordingly: in a first incubation using non-coated microtiter strips, 5 ng of the anti-hAFP antibodies were incubated to equilibrium with increasing amounts of Eu^{3+}-labelled hAFP (0.625 - 20 ng of Eu^{3+}-hAFP per well). The incubation mixture was then transferred to prewashed DELFIA rabbit anti-mouse IgG strips and incubated to achieve complete immobilisation of the Eu^{3+}-hAFP-antibody complexes. After washing, the fluorescence was measured and the affinity constants were calculated as above.

2.8.2 Competitive TES fluoroimmunoassays (II - IV)

The relative affinities and cross-reactivities of the purified parental monoclonal antibody and different Fab fragments were determined by a competitive two-step time-resolved fluoroimmunoassay. First, goat anti-mouse IgG (Fab-specific) was coated on microtiter plate wells, the wells were blocked and samples were incubated in the wells. After a washing step, dilution series of the different steroids (in PBS) were added together with TES-3-CMO-polylysine labelled with an Eu^{3+}-chelate. After incubation, washing and fluorescence development, the amount of bound label was measured. Dilution series of TES and cross-reacting steroids were used to determine relative affinities (ED_{50} concentrations) and cross-reactivities for the corresponding steroids.

The improved sensitivity of the developed mutant Fab fragments allowed the use of serum-based standards and a competitive one-step immunoassay protocol to test the performance of the mutant Fab in a clinically relevant TES concentration range (III,IV). This method was compared to a currently used commercial reference immunoassay, in which rabbit polyclonal antibodies are used as TES specific reagents (DELFIA Testosterone kit, Wallac) and to gas chromatography-mass spectrometry analysis.

2.8.3 Competitive TES ELISA (II - IV)

After different panning approaches a rapid competitive ELISA assay was used to screen clones for further characterisation. Microtiter plate wells coated with TES-3-CMO-BSA-conjugate were used. Culture supernatant samples were added to the plate with dilution series of TES and DHEAS (in PBS) and after incubation and washing steps, labelled goat anti-mouse IgG (κ-specific) was used to determine the amount of bound Fab fragments. Clones having decreased DHEAS cross-reactivity but still retaining the TES binding activity at the wild-type level were selected for further characterisation.

2.9 Kinetic measurements by BIAcore^{TM} (I - IV)

The kinetics of binding of the purified mutant Fab fragments to hAFP, TES-3-CMO-BSA or DHEA-3-hemisuccinate(SucH)-BSA immobilised on a dextran-coated sensor chip were determined from the dependence of the surface plasmon resonance response on the concentration of the Fab fragments injected into the biosensor (BIAcore) (Karlsson *et al*., 1991; Jönsson *et al*., 1991). In the measurements six different concentrations of the Fab fragments were used.

Electrostatic adsorption of the affinity purified AF5 Fab fragments to a modified gold surface was analysed using a non-activated CM5 sensor chip in BIAcore. In the covalent immobilisation analysis the same AF5 Fab fragments were immobilised using a standard immobilisation cycle program.

3. Results

3.1 AF5 anti-hAFP Fab fragment (I)

In order to improve the labelling properties of the AF5 anti-hAFP antibody the genes encoding the L and the Fd chains of the Fab fragment were cloned and sequenced. High overall homology was found between the AF5 Fab and the known immunoglobulin structures (2HFL and 4FAB), used as templates for the model building. The local homology was even higher at the constant domains which were targets for the mutagenesis. Therefore, most parts of the 2HFL and 4FAB could be used as the conserved core and canonical structures were selected from a database for all other CDRs except for the V_H CDR3. The model was inspected by molecular graphics to identify arginine residues exposed at the constant domain surfaces, and available for mutation to lysine residues without affecting the antigen binding. Three arginine residues were selected from the C_L: R154, R187 and R210 (155, 188 and 211 according to Kabat *et al.* (1991)).

After site-directed mutagenesis the heavy and light chain gene fragments of the wild-type and lysine enriched Fab fragments were cloned into the *E. coli* expression vector (Alfthan *et al.*, 1993; Takkinen *et al.*, 1991) containing the *tac* promoter and *pelB* signal sequences. Purification of the recombinant and proteolytic Fab fragments was achieved in a single step procedure by affinity chromatography.

3.1.1 Labelling and affinity constants

Slight reductions in affinity constants were seen for the unlabelled proteolytic (1.3-fold) and recombinant Fab fragments (approx. 2-fold). The affinity constants of both the whole antibody as well as of its proteolytic or recombinant fragments decreased when the degree of labelling was increased, more dramatically so for the monovalent Fab fragments. Labelling of the recombinant Fab fragments under identical conditions resulted in an almost 40 percent higher degree of labelling for the lysine enriched Fab fragment. However, the decrease in the affinity constant caused by the labelling was essentially the same for the two Fab fragments.

3.1.2 BIAcore analysis

The kinetics of binding of Fab fragments to the antigen immobilised on dextran coated sensor chip were determined employing surface plasmon resonance by BIAcore. The calculated affinities of both the wild-type and lysine enriched Fab fragments decreased as the degree of labelling increased. This decrease was essentially due to lower association rates of the labelled fragments and to a lesser extent to higher dissociation rates. It was therefore likely that the lysine residues within the CDR loops were also labelled to some extent, increasing the proportion of inactive Fab fragments in the sample. However, the rate constants and affinity of the lysine enriched Fab, more efficiently labelled by Eu^{3+}, were comparable to those of the wild-type Fab. The Ka values determined by the BIAcore were systematically two to three times lower than those determined by fluoroimmunoassay, most probably due to the different conditions used in the binding assays; in the BIAcore the antigen was immobilised chemically to a solvated dextran hydrogel, whereas in the fluoroimmunoassay measurements the antibody and antigen were free in solution.

The electrostatic properties of the wild-type and lysine enriched Fab fragments were analysed by measuring the electrostatic interaction between negatively charged hydrogel matrix and the Fab fragments. The kinetics of adsorption of the wild-type and lysine enriched Fab fragments were almost equal at low pH but at higher pH the adsorption of the wild-type Fab was faster. The slower electrostatic interaction of the lysine enriched mutant compared to the wild-type Fab can be explained by the pKa difference between the ε-amino group of lysine residues and the guanidinium group of arginine residues. Despite the slower adsorption, the lysine enriched Fab was repeatedly better immobilised when the samples were passed over activated CM5 sensor chip surface. Accurate quantification of the amount of the immobilised Fab fragments was difficult due to variation of the individual sensor chip flow cell surfaces used for each immobilisation analysis. However, the amount of immobilised lysine enriched Fab fragment observed in six different assays was about 18 % higher than that of the wild-type.

3.2   3-C_4F_5 anti-testosterone Fab fragment (II - IV)

Production of monoclonal antibodies distinguishing selectively between TES and other steroids with very similar structures has usually been problematic. The binding properties of a monoclonal antibody to TES (3-C_4F_5, IgG_{2b},κ) were initially characterised by a radioimmunoassay: 3-C_4F_5 had a relatively high affinity (0.3×10^9 M^{-1}) for TES and reasonably low cross-reaction (~11.6%) with 5α-DHT. However, 3-C_4F_5 cross-reacted ~1% with DHEAS and due to the high DHEAS concentration in sera, prevented the use of 3-C_4F_5 in diagnostic applications. Thus a mutagenesis approach was taken to improve the binding specificity of 3-C_4F_5.

The genes of 3-C_4F_5 were cloned into a Fab fragment expression unit in the pKK*tac* vector (Takkinen *et al*., 1991) and at the same time the first constant domain of the heavy chain (IgG_{2b}) was replaced by the first constant domain of the murine IgG_1 subclass heavy chain to promote secretion and folding of the Fab fragment in *E. coli* (Alfthan *et al*., 1993; MacKenzie *et al*., 1994). The 3-C_4F_5 Fab fragment was well expressed in *E. coli*, was secreted as an active, soluble protein into the periplasmic space and was partially released to the culture medium during overnight inductions. The production levels obtained in the culture medium were 0.5 - 5 mg/l. The relative TES affinity and DHEAS cross-reactivity of the recombinant Fab fragment were highly comparable to those of the original 3-C_4F_5 antibody and its proteolytic Fab fragment as determined by a competitive two-step time-resolved fluoroimmunoassay. The heavy chain constant domain subclass replacement did not affect the binding properties.

3.2.1 CDR3 mutants (II)

According to the molecular model most of the contacts between TES and the 3-C_4F_5 antibody originated from the third CDR loops of the heavy and light chains. Thus, these CDRs were targeted for mutagenesis by using spiked PCR primers. The complete randomisation of 9 or 10 amino acid residue long CDRs (LCDR3 and HCDR3, respectively) would require the generation of a library far in excess of the number which is obtainable by transformation of *E. coli*. Therefore, the randomness of PCR primers was adjusted by using a defined, biased mixture of nucleotides during the oligonucleotide synthesis for the CDRs (80% wild-type and 20% equal mixture of three other nucleotides). The size of

both mutant libraries was ~ 10^5 clones. A number of individual clones were sequenced before selection and contained on average 3 - 5 nucleotide changes on the CDR loops.

The mutant libraries were expressed on the surface of filamentous phage and selected using a competitive panning procedure. A good binding inhibition was achieved by adding relatively high concentrations of soluble cross-reacting DHEAS (0.1 - 0.3 µM) into phage solutions. The TES-binders not blocked by soluble DHEAS were caught with an immobilised TES conjugate, TES-4-mercaptopropionic acid-BSA. A long combined washing/incubation step before acidic elution was used in order to mimic conditions routinely used in immunoassays. A total of seven rounds of panning were performed. The last panning cycle was done without amplifying the previous phage eluate in *E. coli* and the binders were eluted with soluble TES, resulting in a low number of output clones ($<10^3$) compared to previous outputs (10^5 - 10^7). Direct reselection without amplification may enable more efficient selection on the basis of binding characteristics (Hoogenboom *et al*., 1998).

After panning a rapid competitive ELISA assay was used to screen clones for further characterisation. As TES conjugated through the C4 on the A-ring was used in the panning as an immobilised catcher, the individual clones after the panning were screened and characterised utilising TES conjugated through the C3. The linker used for attachment with the carrier protein and its position in the molecule frequently affect the binding and maximal complementarity (affinity) that can be achieved for a particular hapten. The linker often becomes part of the unit which is recognised by the antibody (bridge effect) (Franek, 1987; Tiefenauer *et al*., 1989), leading to suboptimal binding of the unconjugated steroid hapten. The linker position is also associated with the antibody cross-reactivity since it has been found that antibodies are often unable to recognise structural differences in that part of the hapten (Arevalo *et al*., 1993a and b). By using these two different TES conjugates, isolation of binders recognising the linker of the labelled TES (used in subsequent fluoroimmunoassays) was avoided. After initial screening/ranking, clones showing decreased DHEAS cross-reactivity but still retaining the relative TES binding affinity at the wild-type level were characterised by a competitive fluoroimmunoassay. Only a few different clones were identified. In one light chain mutant (clone 28, Fig. 11) the DHEAS cross-reactivity was decreased to 0.52% and in one heavy chain mutant (clone 44, Fig.

11) to 0.09%. By combining the light and heavy chain CDR3 mutations the relative TES affinity was preserved at the wild-type level but the DHEAS cross-reactivity was further decreased to 0.03%. An important finding was that the overall binding specificity of the 3-C_4F_5 antibody was refined, since the cross-reactivities to 5α-DHT and androstenedione were also significantly decreased in the combined mutant.

The specificity improvement with respect to DHEA(S) was also determined directly by using BIAcore. In the BIAcore analysis TES-3-CMO-BSA and DHEA-3- SucH-BSA conjugates were used for determining the binding rate constants against the respective steroids, and affinities were calculated from these values. The observed 13-fold improvement in the specificity (Kd(TES)/Kd(DHEA)) was in good correlation with the relative specificity improvement determined using the competitive immunoassay method.

3.2.2 CDR1 and CDR2 mutants (III)

The combined CDR3 mutant showing the improved specificity profile was used as a new template for further mutagenesis and selection. The V_L and V_H CDR1 and CDR2 loops were targeted to mutagenesis by using spiked PCR primers. A higher frequency of mutations was allowed for these generally less important loops than for the CDR3 loops (62.5% wild-type and 12.5% each of three other nucleotides in oligonucleotide synthesis). The constructed HCDR1 library contained approximately 10^5, the HCDR2 library 5 x 10^5, the LCDR1 library 10^4 and the LCDR2 library 10^6 clones.

The mutant libraries were selected by the phage display technique. To improve the TES affinity the new libraries were selected by first incubating with limiting, decreasing concentrations of biotinylated TES (176 nM - 100 pM, four cycles) in solution and then capturing binders on immobilised streptavidin. The binders were eluted with a high concentration of soluble TES (100 µM) which was added together with *E. coli* host cells into the microtiter wells. In principle, those phages still bound to the immobilised antigen were also given a chance of infecting. To improve the specificity, mutant libraries were selected by using the same protocol as used earlier in the successful CDR3 mutant library selections. The libraries were preincubated with a high concentration of soluble cross-

reacting steroid (DHEAS) and TES binders were then caught using microtiter plate wells coated with a TES conjugate. The aim was to obtain 50% binding inhibition on each round of panning but this was not achieved with some of the libraries even with 10 mM DHEAS, which was the highest possible concentration due to the solubility limit of DHEAS.

The same screening and characterisation protocols as described for the CDR3 mutants were used after pannings. In two different light chain CDR1 mutant clones (A58 and A60, Fig. 11) isolated from the affinity panning the relative TES affinity was increased over tenfold, while the cross-reactivities to related steroids were preserved at the same level as in the parental combined CDR3 mutant clone. New heavy chain CDR1 (2B5, Fig. 11) and light chain CDR2 (1D7, Fig. 11) clones were isolated from the specificity selections showing decreased cross-reactivity to the related steroids. In the best combined mutant, clone A60/HCDR1/LCDR2, the TES ED_{50} value was over 12-fold lower than in the parental combined CDR3 mutant and the cross-reactivity to DHEAS was decreased to 0.024%.

The improved affinity of the mutant Fab fragments allowed the use of serum-based standards and a competitive one-step immunoassay protocol to test the performance of the mutant Fab fragments in a clinically relevant TES concentration range. The developed assay was compared to a currently used commercial reference immunoassay, in which rabbit polyclonal antibodies are used as TES specific reagents (DELFIA Testosterone kit, Wallac) and to gas chromatography-mass spectrometry analysis. The standard curve for the developed research assay method utilising the recombinant mutant Fab fragment (A60/HCDR1/LCDR2) was essentially identical to that obtained with the kit and covered the clinically relevant concentration range. The determined TES ED_{50} values were 5.6 nmol/l for the Fab and 4.3 nmol/l for the kit. The relative affinity of the parental wild-type 3-C_4F_5 monoclonal antibody could not be determined by this assay system, since no proper binding inhibition of the labelled TES was achieved with the clinically relevant standard concentrations. The correlation of the results obtained from clinical samples (28 samples, range 0.501 - 31.851 nmol/l, mean 9 nmol/l) using the recombinant Fab-based immunoassay to the values determined by gas chromatography-mass spectrometry analysis showed a good correlation (r = 0.983, slope = 1.11). However, the obtained intercept value (= 5) revealed that although the overall binding specificity of the

A60/HCDR1/LCDR2 Fab was significantly improved compared to the wild-type, it still had to be improved in order to achieve accurate quantitative measurement of very low serum TES concentrations in the presence of closely related cross-reacting steroids in clinical samples. The correlation to the DELFIA Testosterone kit results confirmed the same conclusion (r = 0.989, slope = 1.17, intercept = 3.95).

3.2.3 New CDR3 mutants (IV)

The combined mutant clone after CDR1, CDR2 and CDR3 engineering showing promising analytical performance with clinical samples was used as a template for further optimisation. The CDR3 loops were re-randomised by oligonucleotide-directed PCR mutagenesis. The bias of the oligonucleotides for the parental sequences was weaker than in the previous CDR3 mutant libraries (62.5%) and the libraries were cloned using unique restriction sites created by site-directed mutagenesis near to the CDRs, thus improving the size (both ~ 10^6 clones), diversity and quality of the libraries compared to the previous CDR3 libraries (II).

A combined selection procedure allowing simultaneous selection in respect of affinity and specificity was set up. To favour binders with a high dissociation rate from DHEA and rapid association with TES, a high concentration of DHEA-3-SucH-BSA was used in all phage solutions to inhibit the binding of cross-reacting phages to limiting concentrations of biotinylated TES (22 nM - 0.2 nM). The contact time of phages and biotinylated TES was decreased to 10 min and the subsequent elution was performed using 50 mM NaOH, pH 12.6. The previously used competitive and acidic elution conditions were found to be inefficient to regenerate the sensor chip surface in the BIAcore analysis of the improved Fab fragments. The mutant libraries were also affinity selected without any competing steroid by using limiting, gradually decreasing concentrations of biotinylated TES to catch the high-affinity binders. The concentration of TES used in this approach was clearly lower (1 nM - 10 pM) compared to the approach where cross-reacting steroid was used.

In the case of the best light chain CDR3 mutant clone, A4 (Fig. 11), isolated from the combined affinity/specificity panning approach, the DHEAS and 5α-

DHT cross-reactivities were clearly decreased (3- and 2-fold, respectively) whereas the relative TES affinity was preserved on the same level as in the parental mutant clone. In this assay system the relative affinity of the parental mutant Fab clone A60/HCDR1/HCDR2 was over 12-fold lower and the cross-reactivity with 5α-DHT over 3-fold higher than earlier determined by the two-step immunoassay protocol with buffer-based standards (III). In another light chain CDR3 mutant clone (A46, Fig. 11), selected by the affinity panning, the relative TES affinity was improved 3-fold but all cross-reactivities were increased.

LCDR1:

	24		26		27A		27C		27E		29		31		33	
wt	- R	S	S	Q	S	I	V	H	S	N	G	N	S	Y	L	E	-
A60				E	V			T	R				Y	T	P	I	
A58					R	M		Q	R				H	T	P		

LCDR2:

	50		52		54		56	
wt	- K	V	S	N	R	F	S	-
1D7		A	R	Y			P	

LCDR3:

	89		91		93		95		97	
wt	- F	Q	G	S	H	V	P	P	T	-
28					T					
A4		D						K		
A46		E			Q					

HCDR1:

	31		33		35	
wt	- T	Y	A	L	S	-
2B5	R					

HCDR3:

	95		97		99		100J		101	
wt	- E	Y	Y	G	Y	V	G	L	A	Y	-
44	A								V		

Figure 11. Deduced amino acid sequences of the CDR loops of the wild-type (wt) and selected mutant clones. Only amino acid changes are indicated for the mutants. Numbering is according to Kabat et al. (1991).

3.2.4 New mutation and CDR combinations (IV)

In order to further improve the overall binding properties of the TES-binding antibody, new light chain CDR3 sequences were created by combining

mutations from three different light chain CDR3 mutant clones (clones 28, A4 and A46). Finally, a number of new CDR combinations were created by recombining different CDR loop sequences selected during the stepwise optimisation of the CDRs of the 3-C_4F_5 antibody.

The optimisation of the light chain CDR3 sequence and the CDR combination resulted in four clones (S73, S77, S83 and S88) having excellent binding profiles. The one-step immunoassay ranked the clone S83 as the best one with respect to affinity and specificity. In S83 the affinity to TES was improved over 2-fold, and cross-reactivity to DHEAS was reduced 4-fold and to 5α-DHT 2-fold when compared to the parental mutant clone A60/HCDR1/HCDR2. No significant binding inhibition for the wild-type was achieved in this competitive one-step assay system with the relevant standard concentrations. The extrapolated TES ED_{50} value for the original antibody was 76.5 nM and the cross-reactivities were 0.3% (DHEAS), 13.7% (5α-DHT) and 16.6% (androstenedione). Thus, the TES-binding Fab fragment S83, having five optimised CDRs, showed 32-fold higher relative affinity to TES, 60-fold lower cross-reactivity to DHEAS, 2-fold lower cross-reactivity to 5α-DHT and over 9-fold lower cross-reactivity to androstenedione compared to the original monoclonal antibody.

The analytical performance and clinical usefulness of the developed antibody fragments were evaluated in the context of the competitive one-step immunoassay and compared to the commercial TES immunoassay. The standard curves for all the analysed Fab fragments were in the clinically relevant concentration range. The lowest standard (0.49 nmol/l) gave 86.5% - 93.1% (B/B_{max} %) binding for the Fab fragments and 85.9% for the kit. The highest standard (50.9 nmol/l) gave 8.6% - 15.6% and 10.4% binding for the Fab fragments and the kit, respectively. The determined TES ED_{50} values were 3.4 - 8.8 nmol/l for the Fab fragments and 4.3 nmol/l for the kit. Comparison of the one-step immunoassay using different Fab fragments with the reference immunoassay using 80 patient serum samples showed good correlations for all the Fab fragments (r = 0.943 - 0.971). However, the intercept value of the A4 Fab fragment (1.765) revealed that the final refinement by analysing the additivity of different mutations and different CDRs was a necessary and efficient step. Surprisingly, mutant S83 showing the best performance in the competitive one-step fluoroimmunoassay using serum-based steroids gave a relatively high intercept value (0.877). This bias is probably a consequence of a cross-reaction

with some other steroid(s) in clinical samples. However, the binding properties of some mutant Fab fragments, notably S77 (r = 0.943, slope = 1.105, intercept = 0.005) and S88 (r = 0.968, slope = 1.043, intercept = 0.059), in this modified research assay system were at least comparable to those of the rabbit polyclonal antiserum considered good enough for diagnostic immunoassay.

The binding kinetics of the purified Fab fragments (wt, S77 and S88) to immobilised TES-3-CMO-BSA were determined employing surface plasmon resonance by BIAcore. The most significant change in the rate constants was the about 15-fold slower dissociation rate of the mutants, which together with the association rate improvement resulted in about 40-fold higher affinity of the mutants to the TES conjugate compared to the wild-type Fab.
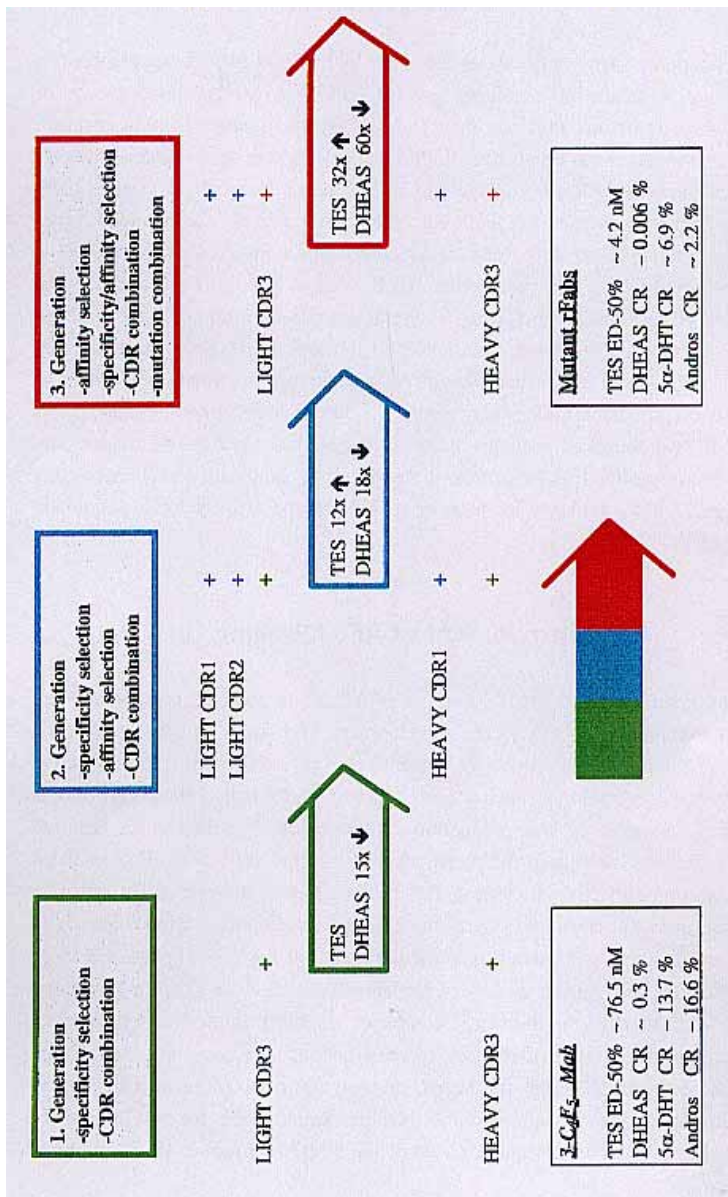
Figure 12. Stepwise in vitro affinity and specificity maturation of the anti-TES 3-C₄F₅ Fab fragm...

4 Discussion

The application of new antibody engineering technology to *in vitro* diagnostics can provide a means of obtaining greater control over the development of antibodies for immunoassays and may yield substantial savings in both cost and time. The absence of the Fc portion of an intact antibody in recombinant antibody fragments can provide lower non-specific binding and their small size makes them ideal for genetic engineering and e.g. for direct genetic fusion with other functional polypeptides. New fine-tuned recombinant antibodies, non-competitive assay techniques, and non-radioactive labels with a high specific activity can significantly improve the performance characteristics of immunoassays and are speeding up the demand for full automation (Self and Cook, 1996). In this study the application of novel antibody engineering techniques to the development of improved recombinant antibody reagents for *in vitro* immunodiagnostics was evaluated. Two common problems were studied; with the anti-hAFP monoclonal AF5 antibody partial loss of affinity upon labelling and with the TES-binding monoclonal $3\text{-}C_4F_5$ antibody inadequate specificity and affinity for a diagnostic immunoassay.

4.1 Improvement of AF5 labelling (I)

For many applications direct labelling of antibodies is required and one current trend in immunodiagnostics is the development of highly sensitive assays by combining the non-competitive labelled antibody approach with non-radioactive labels having a very high specific activity (Self and Cook, 1996). Hybridoma technology has enabled the production of monoclonal antibodies in required quantities and has simplified the preparation of labelled antibodies. Eu^{3+}-chelates provide a non-radioactive alternative for the labelling of antibodies for sensitive diagnostic immunoassays and e.g. lysine residues at antibody surfaces are ready targets for labelling by an isothiocyanate derivative of the Eu^{3+}-chelate. Up to 20 Eu^{3+}-molecules per IgG can usually be incorporated without significant loss of the antibody's affinity for the antigen (Lövgren *et al*., 1990). Unfortunately, but not unexpectedly, labelling usually has more pronounced effects on the affinity constants of the smaller Fab fragments, proteolytic or recombinant, than on the parental antibody. Much more potential residues are available for labelling in the additional heavy chain constant domains of the larger IgG molecule. The number

and location of these reactive groups on antibodies largely determine the efficiency of labelling and the final activity of the labelled product. However, in group-specific labelling it is not possible to direct the conjugation to amino acid residues at specific locations on the molecule. This leads to conjugates with a distribution of attachment sites, some of which may be located at or near the antigen binding site, decreasing the affinity of the antibody (Rodwell *et al*., 1986). However, in many cases the use of Fab fragments rather than complete immunoglobulins would offer a number of potential advantages in diagnostic immunoassays. Falsely elevated or decreased antigen concentrations are frequently seen as a consequence of interactions of e.g. human anti-mouse antibodies, rheumatic or complement factors, or heterophilic antibodies in the samples with whole IgG immunoreagents (Weber *et al*., 1990). Although a number of blocking methods have been used successfully to alleviate these interferences it would be of significant advantage if immunoreagents, such as Fab or $F(ab)_2$ fragments, devoid of effector functions but with full immunoreactivity could be employed.

Protein engineering provides the means for specific improvement of antibody properties, and has also been used to enhance covalent modification of antibody fragments. Site-specific dimerisation of antibody fragments to Fv (Glockshuber *et al*., 1990; Brinkmann *et al*., 1993), Fv-Fv (Cumber *et al*., 1992) as well as $F(ab)_2$ (Carter *et al*., 1992; Shalaby *et al*., 1992) has been achieved by introducing free cysteine residues at the carboxyl termini of the fragments. Lyons *et al*. (1990) reported site-specific labelling of an antibody by introducing cysteine residues to the surface of the C_H1 domain. In the present study protein engineering was used to increase the available lysine content of the anti-hAFP Fab fragment and to improve its labelling properties for sensitive immunoassay applications.

The monoclonal anti-hAFP antibody AF5 suffered from a decrease in affinity upon labelling of its ε-amino groups with an isothiocyanate derivative of an Eu^{3+}-chelate. Molecular modelling was used to identify three light chain constant domain surface arginine residues, R154, R187 and R210, which were mutated to lysine residues. The three conservative arginine to lysine mutations made did not alter the affinity constant of the Fab fragment but resulted in an approximately 40 percent increase in the labelling yield. However, decrease in the affinity constant as a function of the degree of labelling could not be avoided. The probable cause of the decrease in affinity is the labelling of the three lysine residues found in the

CDR loops and/or other lysines in the variable domains. It has been shown that in antibody-protein complexes the surface area buried on the antibody or antigen can be over 900 $Å^2$ (Tulip *et al*., 1992) compared with a modest 160 $Å^2$ for a typical antibody-hapten complex (Davies *et al*., 1990). In the anti-hAFP Fab fragment the contact surface to the antigen may thus involve a significant portion of the total surface. According to kinetic measurements most of the decrease in the affinity of labelled Fab fragments was due to the decreased k_{on} rate. It is therefore likely that the lysine residues within the CDR loops are also labelled to some extent, increasing the proportion of inactive Fab fragments in the sample. The decreased affinity caused by the labelling of lysine residues in the CDR loops could be avoided by site-directed randomisation of these residues and by subsequent selection of mutants with unaltered or even improved antigen binding activity by phage-display procedures. The labelling efficiency could be improved further by designing new constructions involving distinctive labelling tails fused to the Fab fragment. As many as 450 Eu^{3+}-chelates have been attached to a carrier molecule linked to streptavidin (Morton and Diamandis, 1990).

4.2 Improvement of 3-C$_4$F$_5$ binding (II - IV)

Steroid hormones are in many ways highly problematic analytes for immunodiagnostics. Firstly, they are small, rigid, and hydrophobic molecules containing only a few functional groups capable of direct interactions with antibodies. Secondly, the number of different closely related steroid structures in serum samples is high. Thirdly, their *in vivo* concentrations are low, down to picomolar level, and their relative concentrations can vary greatly even between normal healthy individuals. Furthermore, steroids are poorly immunogenic in mice and rats, two species from which monoclonal antibodies are usually generated, and it has been extremely difficult to produce monoclonal anti-steroid antibodies with sufficiently high specificity and affinity to achieve the high qualitative performance essential in diagnostic applications. A better immune response can normally be raised in rabbits, but the generation of monoclonal antibodies from rabbits has not been possible because a plasmacytoma fusion partner has not been available. Thus, the majority of commercially available immunodiagnostic test kits for steroid hormones still utilise polyclonal rabbit antibodies, not because of optimal performance and product quality, but because of the apparent lack of monoclonal antibodies fulfilling the clinical requirements.

The continuous supply of good polyclonal antibody reagents of adequate and uniform quality is a severe problem for the immunodiagnostic industry and requires continuous immunisation of large numbers of laboratory animals.

In the *in vivo* immune response, antibodies with moderate affinities are selected from primary repertoires and their affinities are improved stepwise by rounds of somatic mutation and clonal selection. Antibody phage display provides a means for the directed molecular evolution of antibody affinity and/or specificity *in vitro* and has successfully been applied for increasing affinities even to values rarely achievable using conventional hybridoma technology (Schier *et al*., 1996c). Generally, however, although low-affinity antibodies are readily improved, antibodies that already possess nanomolar binding of their antigen require more subtle refinements to improve their already high affinity (Barbas and Burton, 1996). In a typical antibody engineering approach the antibody sequence is first diversified and a phage display library is constructed, after which improved binders are selected by multiple rounds of affinity selection. In order to apply this approach successfully it is important first to consider where and how to introduce structural diversity (mutations) into the antibody structure and then how to efficiently select rare binders having the desired properties.

4.2.1 Generation of variation

The molecular basis of steroid-antibody interactions has been studied in detail by determining the 3D structures of a progesterone-binding antibody (DB3) in its free and complex form and with different high-affinity cross-reactive progesterone derivatives (Arevalo *et al*., 1993a and b, 1994). The binding site for progesterone is a hydrophobic pocket in which binding specificity is determined by the van der Waals interactions and a few hydrogen bonds formed with the ligand (Arevalo *et al*., 1993a). The high-affinity binding mode of a number of progesterone derivatives is due to two alternative docking orientations for the steroid skeleton and subtle conformational changes in the antibody structure (Arevalo *et al*., 1993b, 1994). The restricted number or complete lack of direct interactions between antibodies and small, hydrophobic and rigid ligand molecules is further demonstrated by the 3D structure of the anti-digoxin antibody (26-10) in the complex form (Jeffrey *et al*., 1993). The high-affinity binding mode to digoxin (1×10^{10} M^{-1}) is solely determined by

hydrophobic shape complementarity, and no direct interactions are formed between the antibody and the ligand (Jeffrey *et al.*, 1993; Schildbach *et al.*, 1994). Lamminmäki *et al.* (1997) modelled binding interactions between a monoclonal anti-estradiol antibody (57-2) and its ligands using extensive experimental data for the construction and evaluation of the model. Similarly to other antibody-steroid complexes, 57-2 appears to have a deep hydrophobic binding pocket with a large proportion of the hapten surface buried. One hydrogen bond between the antibody and the estradiol ligand was predicted at the bottom of the binding pocket.

In the absence of detailed structural data of the interactions involved in the binding of small haptens the *in vitro* maturation strategy, in which an initially selected antibody fragment is subjected to random or semirandom mutagenesis and improved variants are selected from large mutant libraries, has proved to be a feasible method. Short *et al.* (1995) used this approach to isolate antibodies having improved affinity towards digoxin from a library randomised in the HCDR1 region. Recently, Saviranta and co-workers engineered the steroid-specificity of an anti-estradiol Fab fragment by optimising the sequence of the V_H by error-prone PCR and phage display selection (Saviranta *et al.*, 1998). In the present study the binding properties of the anti-TES Fab fragment $3\text{-}C_4F_5$ were improved by a combination of the parallel and sequential CDR optimisation approaches.

In general, interactions of the CDR3 loops of the light and heavy chains with small antigens are known to dominate the overall binding interactions (Wilson and Stanfield, 1994). This was also predicted from the constructed molecular model for the $3\text{-}C_4F_5$ antibody (II). The constructed molecular model indicated that several amino acid residues of the CDR3 loops of the light and heavy chains were in close proximity to the TES ligand and could be in contact with the TES molecule. The selected CDRs are typically randomised by oligonucleotide-directed mutagenesis (e.g. Riechmann and Weill 1993, Barbas *et al.*, 1994) and the sequence complexity is limited by biasing the randomisation towards the wild-type sequence, i.e. oligonucleotides are synthesised by doping low concentrations of the three non-wild-type nucleotides at each step of the synthesis. This increases the proportion of clones preserving some of the original structural features of the CDR loops. In the case of $3\text{-}C_4F_5$ the 9 and 10 amino acid residues long CDR3s (LCDR3 and HCDR3, respectively) were mutated using 80% bias to the wild-type sequences (II). The CDR3s were optimised separately and then

recombined to yield clone 44+28, showing significantly reduced cross-reactivities. This clone was then used as a template for a subsequent round of maturation by targeting parallelly the remaining CDRs (III). A higher frequency of mutations was allowed for these generally less important loops (62.5% bias). Improvement of the affinity and specificity of the Fab fragment was possible by the additivity effect of the optimised CDRs isolated from the affinity and specificity selections. Next, the CDR3 loops were remutated at a higher frequency (IV). Final fine-tuning was performed by analysing new LCDR3 sequences by combining mutations from different light chain CDR3 mutant clones (IV). A number of new CDR combinations were also screened by recombining different CDR sequences isolated during the stepwise optimisation of the 3-C_4F_5 antibody (IV).

By directing mutagenesis to the CDRs it was possible to select new loop sequences with extensive amino acid changes even from modest sized libraries, although the synthesis of the randomised CDR oligonucleotides was biased to the wild-type sequence. For example, in the LCDR1 mutant A60 (II) 8 out of 14 and in the LCDR2 mutant 1D7 (II) 4 out of 7 amino acid residues were changed, demonstrating the power of the phage display selection to isolate even extremely rare phages with suitable binding properties. The best mutant Fab fragments (IV) (S73, S77, S83 and S88) contain 17-20 amino acid mutations compared to the original wild-type 3-C_4F_5 antibody. Only two of these residues were identified as contact residues (<3.5 Å distance from TES) by molecular modelling and e.g. the LCDR1, the optimisation of which resulted in over 10-fold improvement of the affinity, was located far from the binding site. This further supports the initial conclusion that rational design of even individual amino acid changes that would result in decreased cross-reactivities to the closely related steroids without affecting the TES affinity would require more detailed and refined structural data.

4.2.2 Selection of improved variants

Ideally, the selection would be determined strictly by the antigen-binding characteristics of the displayed antibodies. However, although the panning procedure itself tends to select for tight binders (Barbas *et al*., 1991), relative enrichment ratios of phage antibodies are not only dependent on affinity, but are

also affected by factors such as differences in expression and folding efficiency, toxicity to the host strain, and in the case of scFv their tendency to dimerisation (Deng *et al*., 1994; Malone and Sullivan, 1996; Schier and Marks, 1996). For example, in order to evaluate the ability of the panning process to yield antibodies with desired specificity and affinity, a family of anti-phenobarbital antibodies was cloned as scFvs and Fabs and displayed on phage (Malone and Sullivan, 1996). The range of panning efficiencies was observed to vary by two orders of magnitude for the expressed scFvs. Unexpectedly, the scFv with the highest panning efficiency had the lowest affinity and in competitive panning experiments this scFv was preferentially isolated over higher affinity antibodies. This clone expressed high levels of soluble protein compared to the higher affinity clones, demonstrating that the efficiency of functional expression can greatly influence the type of antibody selected by phage display.

The main problem with 3-C_4F_5 was its too high cross-reactivity with DHEAS. However, the panning technique provides tools to direct the selection towards specific epitopes; by preincubating libraries with related cross-reacting antigens (Parsons *et al*., 1996; Saviranta *et al*., 1998; Van Meijer *et al*., 1996) it has been possible to compete out cross-reactive clones during selection. In order to achieve inhibition of phages binding to DHEAS a competitive deselection method was established in which the mutant CDR3 libraries were panned by inactivating cross-reactive phages with a high concentration of soluble DHEAS and then selecting TES binders with an immobilised TES-protein conjugate (II). TES conjugated through the C4 on the A-ring was used in the selection and individual clones were subsequently screened and characterised by the competitive ELISA and fluoroimmunoassay using TES conjugated via the C3. By using these two different conjugates, isolation of binders recognising the linker of the labelled TES was avoided. With very low analyte concentrations, such as in many clinical samples, it is important to have an antibody with no affinity for the linker of the labelled analyte in order to keep the sensitivity of the assay as high as possible.

Selection of clones with decreased affinity to DHEAS and unchanged TES affinity was found to be problematic (II). Only after screening a relatively large number of clones were Fab fragments with improved specificity and only slightly decreased TES affinity isolated (LCDR3 mutant 28 and HCDR3 mutant 44). Nevertheless, the relative TES affinity of the combined CDR3 mutant (44+28) was maintained at the original level and the affinity to TES-3-CMO-BSA,

as determined by BIAcore analysis, was even increased. The improved selectivity of 44+28 to TES over DHEAS was achieved by a combination of the decreased affinity to DHEA(S) and the improved affinity to TES. An interesting finding was the overall specificity refinement; the cross-reactivity was not only decreased with DHEAS but also with 5α-DHT and androstenedione. However, the isolation of numerous clones having improved selectivities (decreased ED_{50} (TES)/ED_{50} (DHEAS) ratio) but unacceptably high TES ED_{50} values indicated that the panning conditions should further be optimised.

Optimal selection for monomeric high affinity antibody fragments has commonly been achieved by performing selections in solution, e.g. using biotinylated antigen, with subsequent capture on immobilised streptavidin (1.7.2). Modifications of the basic technique such as off-rate selection (Hawkins *et al*., 1992), or selections performed in the presence of the parental IgG as a competitor, can also been exploited. In the second stage of this work (III) the mutant libraries were selected using both immobilised antigen with a preincubation step with the cross-reacting antigen (specificity panning) and by limiting the concentration of the biotinylated antigen in solution and subsequently capturing the antigen-bound phages on immobilised streptavidin (affinity panning). Using these two different panning strategies the binding site of the Fab could be fine-tuned with respect to both relative affinity and specificity. Interestingly, each optimised CDR sequence was isolated only from one or the other panning approach, demonstrating the necessity to use different panning strategies in order to achieve an overall refinement of the binding properties.

Two light chain CDR1 mutant clones, A58 and A60, with clearly improved relative TES affinities (over 10-fold) but specificity profiles retained essentially at the parental mutant clone 44+28 level, were isolated from the affinity panning. In the affinity panning clones were selected for amplification by adding a high concentration of soluble TES (100 µM) together with *E. coli* host cells into the microtiter wells. This combined antigen elution-infection worked efficiently, although it has been reported that infecting without elution by adding magnetic beads with antigen-bound phage directly to *E. coli* cultures (Figini *et al*., 1994; Wind *et al*., 1997), and competitive elution with soluble antigen (Clackson *et al*., 1991; Hawkins *et al*., 1992; Riechmann and Weill, 1993) are the least effective means for selecting higher affinity antibodies for

the c-erbB-2 tumor antigen (Schier and Marks, 1996). When incubating antigen-bound phage with *E. coli*, the phage must probably in most cases dissociate from the antigen for infection to occur. Steric hindrance, due to the size of beads, may block the attachment of pIII on antigen-bound phage to the F-pilus on *E. coli* and result in preferential selection of clones with rapid k_{off} and lower affinity. Eluting with soluble antigen has a similar effect on the kinetics of selected clones, since the phage must first dissociate from immobilised antigen before the rebinding is blocked by binding of the phage to the soluble antigen. However, in some cases infection may also occur without dissociation from the antigen-coated surface of microtiter plate wells or magnetic beads, especially when a long linker between a small hapten and a carrier molecule is used, as in TES-3-biotin.

New light chain CDR2 and heavy chain CDR1 mutants showing moderately improved specificity were identified from the specificity panning. The specificity of the affinity-selected A60 mutant clone could be improved by combining it with the new mutant CDRs from the specificity panning. Additivity effects of independently selected mutations on a single binding characteristic (affinity) has been reported (Barbas *et al.,* 1994; Riechmann and Weill, 1993; Schier *et al.,* 1996c; Winter *et al.,* 1994; Yang *et al.*, 1995). Our results demonstrate that simultaneous improvement of the affinity and specificity of the anti-TES Fab fragment was possible by the additivity effect of the optimised CDRs isolated from the affinity and specificity selections. According to the BIAcore analysis the most significant change in the rate constants was the considerably slower dissociation rate compared to the wild-type. The affinity improvements in antibodies achieved by phage display selections are usually demonstrated to be due to slower off-rates (Foote and Milstein, 1991; Marks *et al.*, 1992b; Schier *et al.,* 1996c; Yang *et al.,* 1995). Evaluation of the mutants with clinical samples revealed that the specificity still had to be improved in order to achieve accurate quantitative measurement of very low serum TES concentrations. During the work, evaluation of the selected mutants in the real assay conditions with clinical samples was found to be highly important to choose the best clones for further optimisation and to exclude all unwanted cross-reactivities and other possible matrix interferences that might have been caused by the optimisation of the CDR sequences.

In the final stage of the study (IV) the CDR3 loops of the combined mutant clone were retargetted to mutagenesis. The previously established affinity panning technique was used for the selection in addition to a new combined selection procedure allowing simultaneous selection with respect to affinity and specificity. The combined selection approach was developed in order to avoid a frequently encountered problem in maintaining the TES affinity while decreasing the affinity to DHEAS. In both approaches the elution step was performed using 50 mM NaOH, pH 12.6. Previously used competitive (TES) and acidic elution conditions were found to be inefficient to regenerate sensor chip surface in the BIAcore characterisation of the improved Fab fragments obtained previously. Elution is one of the most critical steps in the whole panning procedure and eluent efficacy has been shown to be reflected in significant differences in the affinities of phage antibodies isolated from an antibody library (Schier and Marks, 1996). Schier and Marks showed that at least in the case of the c-*erb*B-2 tumor antigen, clones resulting from elutions with strong eluents (50 - 100 mM HCl, 100 mM triethylamine) had significantly lower Kd than clones resulting from elutions with 10 mM HCl or 1 µM soluble antigen, or from passive bacterial elution. Elution with 100 mM HCl resulted in the selection of the two highest affinity clones. Interestingly, the different eluents did yield clones with similar kinetic properties but different sequences. According to these results Schier and Marks (1996) suggested that one advisable selection approach would involve sequential elution, using a less stringent eluent to remove low affinity binders, followed by a more stringent eluent to remove high affinity binders. Alternatively, site-specific chemical (Griffiths *et al*., 1993; Thompson *et al*., 1996) and enzymatic (Orum *et al*., 1993; Ward *et al*., 1996) cleavage offer an opportunity for the retrieval of bound phage in a manner independent of the affinity of the antibody and enable effective but gentle selection of high-affinity clones.

Two different LCDR3 clones (A4 and A46) were isolated from independent selections, one with improved specificity and the other with improved affinity (IV). The LCDR3 sequence was optimised further by analysing all the mutant combinations from three different clones (28, A4 and A46). A number of new CDR combinations were also analysed by recombining different CDR loop sequences isolated during the study. After screening, four closely related clones were found with performances in the clinical TES immunoassay comparable to that of the commercial reference immunoassay in which rabbit polyclonal

antiserum was used as a TES-specific reagent. When compared to the original monoclonal the best mutant Fab fragments exhibited about 40-fold increase in the relative affinity ($K_d = 3 \times 10^{-10}$ M) and 50-fold lower cross-reactivity (0.006%) to DHEAS. The original hybridoma-based 3-C_4F_5 antibody was the result of prolonged efforts via hyperimmunisation. Therefore, in this study the efficacy and feasibility of antibody engineering technology were firmly demonstrated by achieving significant improvement of the binding properties of this originally promising antibody.

5 Conclusions and future perspectives

It was shown in this work that the Eu^{3+}-chelate labelling efficiency of the AF5 Fab fragment can be improved by increasing the number of reactive side chains at the surface of the constant domain. The labelling efficiency could be improved further by designing new constructions involving distinctive labelling tails fused to the Fab fragment. Furthermore, the decreased affinity caused by the labelling of lysine residues in the CDR loops could be avoided by site-directed randomisation of these residues and by subsequent selection of mutants with unchanged or even improved antigen binding activity by phage-display.

As described in this work for the case of the TES-binding $3\text{-}C_4F_5$ antibody, stepwise optimisation of the CDRs of reasonably good hybridoma cell-derived monoclonal antibodies using CDR mutagenesis and phage display selection techniques, and analysis of the additivities of different mutations and CDRs, are efficient and feasible approaches to the development of recombinant antibodies for sensitive diagnostic applications. Taking into account the simultaneous improvements in affinity and specificity, the overall functioning of the developed recombinant anti-TES antibodies was improved considerably and fulfilled the diagnostic demands for the affinity and cross-reactivities, comparable to those of a rabbit polyclonal anti-TES antiserum currently used in a diagnostic immunoassay. To the best knowledge of this author these developed Fab fragments are the first steroid hormone-binding recombinant antibodies shown to work accurately over the whole physiological concentration range of clinical samples. Additionally, the immunoassay system that was used to evaluate the performance of the mutants is being optimised for Fab fragments, which may further increase the sensitivity of the immunoassay. The established *E. coli* production system provides an economical, high level supply of stable Fab fragments with consistent quality.

It was firmly established during this study that antibody engineering technology provides excellent tools for the precise tailoring of antibodies for a given application, either by a process involving multiple, recursive cycles of mutagenesis, selection and characterisation ($3\text{-}C_4F_5$ Fab) or by exploiting detailed structural data and rational design of individual amino acid changes (AF5 Fab). In the future the generated know-how can be applied e.g. in the development of analytical tools and methods for analysis of drugs, metabolites and other small

analytes. The generation of new, fine-tuned antibodies using antibody engineering technology can significantly improve the performance characteristics of diagnostic and other immunomethods.

The rapid progress in antibody engineering technology is due to advances in several areas of research. Firstly, PCR has enabled the development of rapid and reliable methods for cloning and modifying antibody genes and antibody gene repertoires. Secondly, efficient and economical recombinant expression systems have been developed for antibodies and their derivatives, with the goal of maximising cell density and secretion productivity while minimising the number of downstream purification steps and other manipulations. Thirdly, selection approaches which permit the isolation of desired specificities from huge combinatorial libraries using phage display technology have been developed. Antibody engineering provides now rapid and reliable methods for generating desired fine-tuned antibody molecules for specific applications.

In the future, very large naive, semisynthetic and totally synthetic antibody gene libraries will probably totally supersede the hybridoma- and immunisation-based techniques for antibody development and significantly reduce the need for *in vitro* affinity maturation of antibodies derived from libraries. An antibody with superior expression and display properties can be selected as the framework for CDR randomisation and can be used as a source of structural diversity from which to select new antibodies for different applications. Furthermore, new selection strategies involving e.g. direct *in vivo* selection, ribosome display or direct selection for biological functions may provide interesting and efficient ways to tailor antibody properties and ensure fulfilment of the tremendous potential of genetically engineered antibodies for therapeutic, diagnostic and industrial applications.

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