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[14.00–14.30]
A binary code for antigen recognition
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Antibodies can evolve to recognize essentially any protein with high specificity and affinity. While natural antigen binding sites utilize all 20 natural amino acids to some extent, analysis of functional antibodies reveals clear biases for or against some amino acids. Most significantly, tyrosine and serine are highly abundant in antigen binding sites in general and at antigen contact sites in particular.

As it is now possible to construct antibody-phage libraries with synthetic CDR diversity, we decided to investigate whether a minimal genetic code may be sufficient for the recognition of protein antigens. Using a tetranomial genetic code that allows for only four amino acids (tyrosine, serine, alanine, and aspartate) we were able to generate antibodies against vascular endothelial growth factor (VEGF) that bound with high affinity ($K_d = 2$ nM) and specificity. In fact, we obtained two antibody families with distinct binding profiles; one bound with equal affinity to both human and murine VEGF while another bound only to the human protein. The crystal structures of the Fab-hVEGF complexes revealed that the binding epitopes of the two antibodies overlap with each other and with that of a natural VEGF receptor (Flt-1). Furthermore, most of the buried surface ($>$70%) was contributed by tyrosine side chains within the randomized CDRs.

The structural and mutational analyses indicated that tyrosine was the major mediator of binding energy at the antigen binding sites of the anti-VEGF Fabs, and the results suggested that it might be possible to further simplify the code for antigen recognition. To this end, we constructed Fab libraries in which CDRs were randomized with a binary genetic code that allowed for only tyrosine and serine. We envisioned that in these libraries, tyrosine might act as an effective "functional" amino acid with a large side chain that could provide significant binding contacts. In contrast, serine might act as an "auxiliary" amino acid with a small side chain that could provide space for the tyrosine side chains and also contribute to an overall hydrophilic surface.

Remarkably, naïve Fab libraries constructed with the binary code were extremely effective in generating highly specific antibodies against most targets tested (8 out of 9). Indeed, the libraries proved to be more efficient than the tetranomial library and the results rivaled those obtained with libraries that accessed the full diversity of natural amino acids. From a naïve library, without affinity maturation, we obtained Fabs that bound to VEGF with high affinity ($K_d = 50$ nM).

Our results suggest that certain amino acids possess features that increase the likelihood that they will be able to make productive contributions to binding affinity and specificity. Thus, biased libraries that favour such amino acids are likely to be much more effective in generating antibodies with high affinity and specificity. As a corollary, it seems that other amino acids are ill suited for productive contacts, and the absence or depletion of such amino acids will likely improve library performance. The results should aid the design and use of synthetic antibody libraries. In addition, and perhaps more importantly, the findings have significant implications for the fundamental principles and mechanisms that mediate molecular recognition at protein-protein interfaces.

[14.30–14.50]
Combinatorial yeast mating: Applications to affinity maturation of Fab antibody fragments
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Dyax has developed a yeast display technology platform which facilitates the rapid affinity maturation of Fab antibodies. We have applied this technology for the affinity maturation of antibodies specific to strep-

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tavidin (Van den Beucken et al., FEBS Letts, 2003: 288–294) and also to the orphan tyrosine kinase receptor Tie1 which is a novel tumor angiogenesis target. Further improvements have resulted in a novel technology whereby large combinatorial yeast displayed Fab libraries can be generated by yeast mating of separate light chain (LC) and heavy chain (HC) repertoires (PCT/US02/31113). For the proof of concept study two separate vectors were constructed that enable the inducible expression of an anchored HC and a soluble LC. The assembly and functional display of a Fab antibody on the diploid yeast cell surface was observed after combinatorial mating of yeast cells of opposite mating type carrying either LC or HC expression vectors. Large diverse Fab libraries in excess of 10^9 members have been routinely generated using this approach. In order to select these large yeast displayed libraries we have developed automated magnetic bead separation methods which are used to rapidly pre-select libraries prior to affinity selection using flow cytometric sorting.

Combinatorial yeast mating has broad applicability to antibody engineering and facilitates simple and rapid construction of large Fab antibody libraries. We have applied this technology to the affinity maturation of a target specific Fab antibody fragment using an iterative process of chain shuffling of distinct repertoires of LC and HC genes without the need for repeated cycles of *in vitro* library construction. To further extend the utility of this technology we have used combinatorial yeast mating to rapidly generate a large naïve yeast displayed human Fab antibody library of 1×10^9.

[14.50–15.10]
Selection of highly stable human antibody fragments for compartmental therapeutic approaches

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Single-chain antibodies (scFvs) show excellent tissue penetration features in comparison to full-length monoclonal antibodies due to their smaller size (Yokota et al. 1992; Larson et al. 1997; Thiel et al., 2002; Batra et al. 2002). Furthermore, since they lack the Fc part of immunoglobulins, these antibody fragments show reduced unspecific binding and associated toxicity towards non-target tissues, which is often conferred by binding to widespread Fc receptors (Patrick et al. 1998). However, their use as therapeutic tools is currently negatively affected by two disadvantageous properties: their rapid clearance from the body circulatory system and their general instability.

Due to their small molecular mass of 20–30 kDa, scFvs are rapidly excreted from the body by glomerular filtration, resulting in β-elimination half-life in the range of about 4–6 hours (Kang et al. 2000). Thus, their suitability for systemic applications is obviously limited, although their tissue penetration properties would in principle make them attractive therapeutic tools. On the other hand, if acting in a body compartment that is not directly exposed to the blood stream (e.g. the lung, the skin, the brain), the tissue penetration properties of scFvs might offer significant pharmacokinetic advantages in respect to full-length monoclonal antibodies. However, even in topical applications scFvs may not fully exploit their potential pharmacokinetic advantages, as they lack necessary properties such as sufficient thermodynamic stability and solubility (Willuda et al. 1999).

At ESBATech, we have overcome this obstacle by selecting highly stable and soluble natural human scFv frameworks that can be used to generate random-CDR scFv libraries for screening and identification of high-affinity binders or, alternatively, that can be used as scaffolds on which CDRs of existing antibodies can be grafted (Auf der Maur et al. 2001, Auf der Maur et al. 2002). Through the antigen-independent selection procedure developed at ESBATech, we have selected from the entire pool of more than 1.5 million possible human scFv VH and VL sequences, those that show high stability and low tendency to aggregate (high solubility). These properties of the selected antibody fragments enable them to be functional even in the reducing environment of the cell cytoplasm (Wörn et al. 2000, Barberis et al. 2004). Furthermore, these selected human VH and VL frameworks show increased stability and better expression properties compared to conventional scFvs also under natural, oxidizing conditions (Auf der Maur et al. 2004). Indeed, such selected scFvs can be produced in significantly higher amounts compared to benchmark scFvs, as production yields from 1-liter E.coli cultures (OD10) cultivated in conventional shaking flasks are in the range of 20–50 mg/l. High stability and solubility, taken together with the high degree of tissue penetration due to the small size, make these antibody fragments directed against specific antigens ideal for topical applications in particular therapeutic areas in body compartments. A detailed characterization of the different VH and VL domains of our selected human scFvs as well as their topical applications in defined body compartments will be presented.

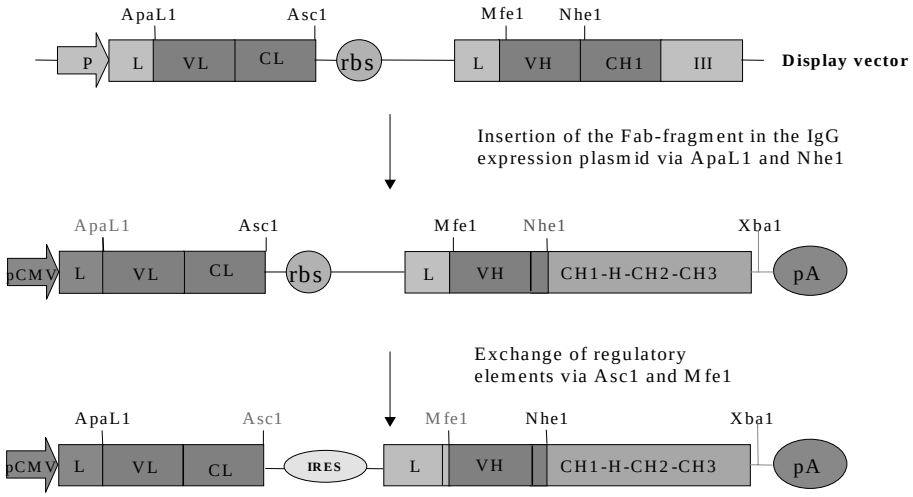

Fig. 1. Scheme of Reformatting Strategy The transfer of the LC and VH sequences from the phage display vector to the IgG expression vector is accomplished in two cloning steps. The restriction sites/enzymes utilized for each cloning step are highlighted in red. The same approach can be used for the conversion of Fabs to human IgG1, human IgG4, and mouse IgG2a.

[15.10-15.30]

Rapid generation of functional human antibodies derived from fab/phage display libraries

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Introduction: Soluble Fab fragments derived from phage display libraries can be screened in a range of *in vitro* assays that facilitate the ranking of binders and the identification of leads for reformatting in to IgG. However, as Fabs lack the effector functions of complete IgG antibodies, they cannot be studied in some functional assays. Furthermore, as soluble Fab fragments are often expressed in *E. coli*, they can be contaminated with endotoxin and are therefore unsuitable for many cell-based assays.

We have constructed a set of vectors, completely compatible with our phage display library, for expression of whole human IgG antibodies. This allows fast and "sequence-blind" reformatting of Fabs to IgGs, and this method permits reformatting of selected Fab-repertoires as well as individual Fab clones. Thus, we bypass the analysis of soluble Fab and proceed directly from Fab/phage to IgG constructs.

Here we show results of a reformatting campaign of Fabs directed against Tie-1, a receptor tyrosine kinase involved in angiogenesis [3] and a novel target for anti-tumor therapy. Furthermore, we describe high-level transient expression of the IgG molecules derived from our libraries in HEK293T cells, and purification of the expressed antibodies.

Antibody reformatting technology: We have developed mammalian expression vectors for the production of whole human IgG antibodies (subclasses IgG1 and IgG4) and mouse IgG2a antibodies [4]. The expression vectors are completely compatible with our Fab-on-phage display libraries and allow the transfer of Fab cassettes from the display vector to the IgG expression construct, either by "cut and paste" restriction fragment cloning, or with a strategy involving PCR-amplification of each individual Fab cassette.

The expression cassettes for the display and the IgG expression vectors share a similar modular bicistronic configuration. In both constructs, the expression of the two polypeptides is under control of one promoter, located $5'$ of the light chain (LC) cDNA, namely the prokaryotic LacZ promoter in the display vector and the strong immediate-early promoter of human cytomegalovirus in the IgG expression construct. Conversion of the displayed Fab from Fab-on-phage to a human IgG1 antibody, a process we call reformatting, is shown schematically in Fig. 1. First, the Fab cassette (minus C_H1), consisting of the antibody LC, a prokaryotic ribosome binding site (RBS), and the variable (V_H) region of the heavy chain (HC), is released from the display vector and inserted via the ApaL1 and Nhe1 restriction sites into the IgG expression construct, $3'$ of a eukaryotic antibody leader sequence and $5'$ of

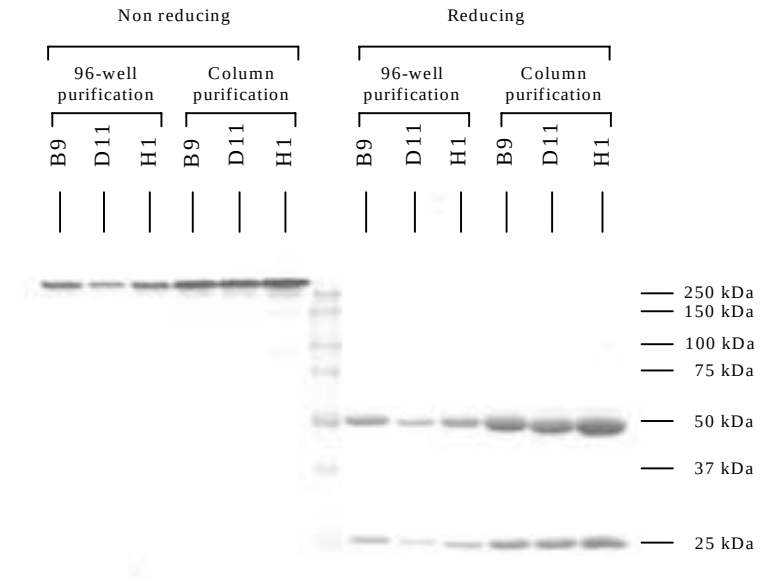

Fig. 2. Purification of IgG from Expression Culture Supernatants Three antibodies (S-B9, S-D11 & S-H1) purified either in a 96-well format or on a 1-ml HiTrap rProtein A column, were analyzed by reducing and non-reducing SDS-PAGE on a 12% polyacrylamide gel. Five μl (1 to 5 μg) of each antibody preparation was loaded. Following electrophoresis, the gel was stained with Coomassie blue. Note: No significant difference in the quality of the protein preps was observed when comparing larger scale purification with HT-96-well purification.

the HC constant region of a human IgG1 antibody. Subsequently, the prokaryotic RBS is exchanged with the encephalomyocarditis virus-derived IRES, a sequence motif that allows translation of two consecutive open reading frames from the same messenger RNA in mammalian cells [5]. Simultaneously, in this second cloning step, the prokaryotic leader sequence 5′ of the variable heavy chain region is replaced by a eukaryotic signal peptide.

This reformatting procedure maintains the linkage of LC/HC pairs selected from our display libraries. This approach is thus not limited to the transfer of individual Fabs to IgGs: the batch reformatting of repertoires of selected antigen-specific clones is also possible.

In addition, a reformatted Fab fragment can be easily transferred by a single restriction enzyme-based cloning step, from one IgG expression vector (e.g., human IgG1) to a different IgG expression construct (human IgG4 or mouse IgG2a). Via this "class-switching," a desired binding specificity can be combined with different effector functions.

Reformatting, expression and purification of human Tie-1 specific Fabs: In order to isolate Tie-1 specific Fabs, we selected our phagemid Fab-on-phage display library against recombinant Tie-1 protein. Next, 96 isolates obtained from the selection were screened as soluble Fab proteins against recombinant Tie-1 protein in an ELISA format, yielding 14 unique clones. These clones, along with an additional 13 unique Tie-1-binding isolates identified from Fab-on-phage screens, were chosen for batch reformatting to IgG.

The individual Fab cassette from each chosen Tie-1-binding isolate was PCR-amplified separately. The resultant 27 PCR products were pooled; the pool was reformatted in a single batch as outlined in Fig. 1. In order to "re-identify" the initial candidate Fabs, the expression cassettes of 288 IgG constructs (three 96-well plates) were amplified by colony PCR, and the variable heavy chain (VH) regions were sequenced and subsequently analyzed. DNA sequence data were obtained for 229 samples, and 24 of the initial 27 clones (89%) were re-identified.

The light chain and variable heavy chain regions of at least one isolate of each re-identified IgG were confirmed identical to the original Fab sequence by complete DNA sequence analysis before we continued with antibody production.

Our IgG expression vectors carry the SV40 origin of replication. These plasmids can be highly amplified in SV40 transformed cells, and are thus suitable for transient protein expression, e.g., in simian kidney COS cells or human embryonic kidney HEK293T cells. Each of the 24 Tie-1-IgG1 constructs was transiently expressed in HEK293T cells in one triple-layer flask.

Table 1
Amounts of Tie-1 specific IgG purified from 200ml conditioned culture medium*

Clone	Amount (mg)	Clone	Amount (mg)
P-A1	1.9	S-A2	1.5
P-A5	Not expressed	S-A10	1.1
P-A10	2.0	S-B2	1.4
P-B1	2.2	S-B9	2.2
P-B3	3.3	S-C2	3.6
P-C6	2.8	S-C7	2.3
P-D6	Not expressed	S-C10	1.7
P-D10	Not expressed	S-D11	3.0
P-D12	2.1	S-E11	1.3
P-F3	1.8	S-G10	2.9
P-F4	2.5	S-H1	1.9
P-G3	3.7	S-H4	3.4

* Antibodies were transiently expressed in one T-175 triple-layer flask and purified from culture supernatants on a 1-ml HiTrap rProtein A column. Following purification, protein concentrations were measured, and the amounts recovered calculated.

Culture supernatants were harvested 72 hours and 144 hours after transfection, and both harvests were combined. High antibody expression levels were detected for 21 clones.

The cell culture supernatants were purified on a 1-ml Protein A column, as summarized in Table 1. The

purification yield ranged between 1.1 and 3.7 mg, with an average of 2.2 mg per clone.

The quality of the antibody preparations (shown for S-B9, S-D11, S-H1) was analyzed by SDS-PAGE, both under reducing and non-reducing conditions (Fig. 2). Under reducing conditions, the antibody light and heavy chains appeared as approximately 25 kDa and 50 kDa bands, respectively. Under non-reducing conditions, the antibodies migrated as one major "high molecular weight" band, indicating that heavy and light chains were correctly disulfide bonded. On the basis of Coomassie staining, the sample purity was estimated to exceed 95%.

Conclusion: The method described allows isolation and expression of antigen-specific fully human IgG molecules within less than 3 months, as fast or even faster than animal immunization and subsequent hybridoma generation.

Antibodies are the most important and fastest growing group of protein therapeutics. Rapid reformatting of phage-selected antibody fragments to mammalian-expressed whole IgG molecules allows screening and characterization of high numbers of candidate clones in the final format of the therapeutic agent.

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