The SH3 domain of fyn kinase as a scaffold for the generation of new binding proteins

Author(s):
Grabulovski, Dragan

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The SH3 Domain of Fyn Kinase as a Scaffold for the Generation of New Binding Proteins

A dissertation thesis submitted to the ETH Zürich to obtain the degree of
DOKTOR DER WISSENSCHAFTEN

presented by
Dragan Grabulovski
Eidg. Dipl. Apotheker, ETH Zürich
born 9.3.1978
citizen of Winterthur (ZH)

accepted on the recommendation of
Prof. Dr. Dario Neri, examiner
Prof. Dr. Gerd Folkers, co-examiner

2007
To my parents
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Summary

Therapeutic proteins have become an increasingly important class of medicines during the last two decades. After the first successes with recombinant proteins in replacement therapies, monoclonal antibodies are currently representing the second wave of innovation created by the biotechnological industry. Antibodies have proved useful as human therapeutics because they bind to drug targets with high affinity and specificity and exhibit a favorable pharmacokinetic profile.

However, even blockbuster biologics suffer from certain drawbacks. Nature did not evolve them for manufacture \textit{ex vivo} and for this reason, difficulties in their production often arise. Poor expression yields, low solubility or immunogenicity are sometimes problematic aspects of protein-based therapeutics. Additionally, thermal instability and aggregate formation are known to increase the immunogenic potential of therapeutic proteins. Therefore, there is a growing interest in the development of stable and highly soluble proteins from human origin, which may be less immunogenic in therapeutic applications and easier to produce in microbial expression systems.

According to this rationale, an emerging field in pharmaceutical biotechnology is represented by the generation of novel binding molecules based on small globular domains serving as protein frameworks ("scaffolds"). In this approach, certain amino acid residues of the surface of a single domain are combinatorially mutated to produce a protein library, which can then be screened for binding specificities of interest. Thus, the concept of a universal binding site from the antibody structure is transferred to alternative protein frameworks with suitable biophysical properties. On one hand, these new proteins will provide further insights into the processes of molecular recognition, on other hand, they could also have commercial applications, as therapeutic agents, diagnostic reagents or affinity ligands.

In this thesis, we describe the design, construction and characterization of a human Fyn SH3 phage library. After test selections and successful isolation of Fyn SH3 derived binders to mouse serum albumin (MSA), a novel, optimized phage library was created, comprising $1.2 \times 10^9$ library members. Using this second library, we present the isolation and \textit{in vitro}
characterization of Fyn SH3 derived proteins binding to the extra-domain B of fibronectin (EDB), a marker of angiogenesis. One specific binding clone, named D3, was further evaluated and showed a remarkable ability to stain vascular structures in tumor sections. Furthermore, quantitative biodistribution studies in tumor bearing mice revealed the ability of D3 to selectively accumulate in the tumor. In contrast to human scFv antibody fragments administered to mice, neither Fyn SH3wt nor the D3 mutant were immunogenic in mice, after four i.v. injections.

The EDB-binding D3 protein opens new biomedical opportunities for the in vivo imaging of solid tumors and for the delivery of toxic agents to the tumoral vasculature. In addition, the single-pot library of Fyn SH3 mutants described in this thesis may represent a rich source of useful reagents for many biochemical and biomedical applications as an alternative to more conventional IgG-based immunochemical technologies.
Zusammenfassung


Ein aufstrebendes Gebiet in der pharmazeutischen Biotechnologie stellt daher die Entwicklung von neuen bindenden Molekülen dar, basierend auf sogenannten kleinen, globulären Domänen, die als Protein-Gerüste dienen. In diesem Ansatz, werden bestimmte Aminosäuren auf der Oberfläche der kleinen Domäne randomisiert, um eine Bibliothek an Varianten zu generieren, die dann auf gewünschte Bindungsspezifitäten geprüft werden können. Somit wird das erfolgreiche Konzept der Antikörper (eine variable Bindungs Oberfläche und konstante Regionen) auf diese alternativen Gerüste übertragen, die im Gegensatz zu den Antikörpern vorteilhaftere biophysikalische Eigenschaften aufweisen. Einerseits können diese alternativen, bindenden Proteine neue Einsichten gewähren in die molekularen Erkennungsprozessen von Protein-Protein Interaktionen, andererseits könnten...
sie auch kommerziell genutzt werden, zum Beispiel als Therapeutika, Diagnostika oder als Bindungsreagenzien in Laborexperimenten.


Das EDB-bindende Protein D3 öffnet neue biomedizinische Möglichkeiten für die in vivo Darstellung von soliden Tumoren und für die zielgerichtete Anreicherung von toxischen Wirkstoffen im Tumorgewebe. Zusätzlich bietet die präsentierte Fyn SH3 Phagenbibliothek womöglich eine reiche Quelle an nützlichen Molekülen für viele biochemische und biomedizinische Anwendungen, und stellt somit eine Alternative dar zu den herkömmlichen immunochemischen Technologien, die auf Antikörper als Bindungspartner basieren.
1 Introduction

1.1 Recombinant proteins as biopharmaceuticals

The development of genetic engineering and progresses in molecular biology have allowed the cloning of genes in living organisms such as bacteria and yeast for their efficient expression. Large-scale production of many proteins has become possible, thus opening several industrial applications, including the production of enzymes for food processing or the synthesis of chemicals and medicinal products. Therapeutic proteins produced via such methods have been coined biopharmaceuticals (1).

The first biopharmaceutical to gain marketing approval was that of „humulin“ (recombinant human insulin), initially approved in the United States in 1982 (2). In the intervening years, the biopharmaceutical industry has grown rapidly: by 2006, 165 biopharmaceutical products have gained approval, with a market size estimated at some $33 billion in 2004 (3) and projected to reach $70 billion by the end of the decade (4,5). The majority of initially approved biopharmaceuticals may be classified as „simple replacement proteins“, i.e. proteins displaying an identical amino acid sequence to a native human protein and administered in order to replace or augment levels of that protein (e.g., insulin, growth hormone or blood factors). More modern biopharmaceuticals have been engineered to tailor their therapeutic properties. Such second-generation products display either an altered amino acid sequence (achieved via protein engineering or site directed mutagenesis), an altered glycomponent (in the case of some glycosylated biopharmaceuticals) or a covalently attached chemical moiety such as polyethylene glycol or toxins.

1.1.1 Monoclonal antibody based drugs

30 years after the advent of hybridoma technology (6), antibodies represent nowadays the best established class of binding molecules that can be rapidly isolated with high affinity and specificity to virtually any target. It is therefore not surprising that antibodies are routinely
used in biomedical research for analytical and separation purposes, are essential ingredients in many diagnostic procedures, and represent the fastest growing sector of pharmaceutical biotechnology. By 2008, engineered antibodies and fragments thereof are predicted to account for >30% of all revenues in the biotechnology market (7). Not only the high-affinity and specificity, but also the variety of mechanism of actions (e.g., neutralization, interfering with cell-signalling, antibody-dependent cellular toxicity and/or complement activation) and favorable pharmacokinetic profiles contribute to the great success of this class of drug. Table 1.1 gives an overview of antibodies approved by FDA for therapy and diagnosis.

<table>
<thead>
<tr>
<th>Product name</th>
<th>Generic name</th>
<th>Target</th>
<th>Type</th>
<th>Indication</th>
<th>Year</th>
</tr>
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<tr>
<td>Orthoclone OKT3</td>
<td>Muromonab</td>
<td>CD3</td>
<td>murine IgG2a</td>
<td>Transplant rejection</td>
<td>1986</td>
</tr>
<tr>
<td>OncoScint</td>
<td>Satumomab pendetide</td>
<td>TAG-72</td>
<td>murine IgG 111In</td>
<td>Colorectal and ovarian cancer</td>
<td>1991</td>
</tr>
<tr>
<td>NeoPro</td>
<td>Aboximab</td>
<td>GP IIb/IIIa</td>
<td>chimeric Fab</td>
<td>Cardiovascular</td>
<td>1994</td>
</tr>
<tr>
<td>CEA-Scan</td>
<td>Arcitumomab</td>
<td>CEA</td>
<td>murine Fab 99mTc</td>
<td>Colorectal cancer</td>
<td>1996</td>
</tr>
<tr>
<td>Prostascint</td>
<td>Capromab pendetide</td>
<td>PSA</td>
<td>murine IgG1 111In</td>
<td>Prostate carcinoma</td>
<td>1996</td>
</tr>
<tr>
<td>Verluma</td>
<td>Nofetumomab</td>
<td>CD20</td>
<td>murine Fab 99mTc</td>
<td>Small cell lung cancer</td>
<td>1996</td>
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<tr>
<td>Zenapax</td>
<td>Dacizumab</td>
<td>CD25</td>
<td>humanized IgG1</td>
<td>Transplant rejection</td>
<td>1997</td>
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<td>Rituxan</td>
<td>Rituximab</td>
<td>CD20</td>
<td>chimeric IgG1</td>
<td>B-cell lymphoma (Non-Hodgkin)</td>
<td>1997</td>
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<tr>
<td>Simulect</td>
<td>Basiliximab</td>
<td>CD25</td>
<td>chimeric IgG1</td>
<td>Transplant rejection</td>
<td>1998</td>
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<tr>
<td>Synagis</td>
<td>Palivizumab</td>
<td>RSV</td>
<td>humanized IgG1</td>
<td>RSV bronchiolitis</td>
<td>1998</td>
</tr>
<tr>
<td>Remicade</td>
<td>Infliximab</td>
<td>TNF</td>
<td>chimeric IgG1</td>
<td>Rheumatoid arthritis; Crohns disease</td>
<td>1998</td>
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<tr>
<td>Herceptin</td>
<td>Trastuzumab</td>
<td>HER2/neu</td>
<td>humanized IgG1</td>
<td>Breast cancer</td>
<td>1998</td>
</tr>
<tr>
<td>Mylotarg</td>
<td>Gemtuzumab</td>
<td>CD33</td>
<td>humanized IgG4 toxin conjug. (Calicheamycin)</td>
<td>Acute myeloid leukaemia</td>
<td>2000</td>
</tr>
<tr>
<td>MabCampath</td>
<td>Alemtuzumab</td>
<td>CD52</td>
<td>humanized IgG1</td>
<td>Chronic lymphatic leukaemia</td>
<td>2001</td>
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<tr>
<td>Zevalin</td>
<td>Ibritumomab</td>
<td>CD20</td>
<td>murine IgG1 90Y</td>
<td>B-cell lymphoma</td>
<td>2002</td>
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<td>Humira</td>
<td>Adalimumab</td>
<td>TNF</td>
<td>human IgG1</td>
<td>Rheumatoid arthritis</td>
<td>2002</td>
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<td>Xolair</td>
<td>Omalizumab</td>
<td>IgE</td>
<td>humanized IgG1</td>
<td>Asthma</td>
<td>2003</td>
</tr>
<tr>
<td>Bexxar</td>
<td>Tositumomab</td>
<td>CD20</td>
<td>murine IgG1 131I</td>
<td>B-cell lymphoma</td>
<td>2003</td>
</tr>
<tr>
<td>Raptiva</td>
<td>Efalizumab</td>
<td>CD11a</td>
<td>human IgG1</td>
<td>Psoriasis</td>
<td>2003</td>
</tr>
<tr>
<td>Avastin</td>
<td>Bevacizumab</td>
<td>VEGF</td>
<td>humanized IgG1</td>
<td>Colorectal cancer</td>
<td>2004</td>
</tr>
<tr>
<td>Erbitux</td>
<td>Cetuximab</td>
<td>EGFR</td>
<td>chimeric IgG1</td>
<td>Colorectal cancer</td>
<td>2004</td>
</tr>
<tr>
<td>Tysebri</td>
<td>Natalizumab</td>
<td>alpha4 integrin</td>
<td>humanized IgG4</td>
<td>Multiple sclerosis</td>
<td>2004</td>
</tr>
<tr>
<td>Lucentis</td>
<td>Ranibizumab</td>
<td>VEGF</td>
<td>humanized Fab fragment</td>
<td>age related macular degeneration</td>
<td>2006</td>
</tr>
<tr>
<td>Vectibix</td>
<td>Panitumumab</td>
<td>EGFR</td>
<td>human IgG2</td>
<td>Metastatic colorectal cancer</td>
<td>2006</td>
</tr>
</tbody>
</table>

Table 1.1 List of antibodies approved by FDA for therapy or diagnosis.

1.2 Small globular proteins as antibody substitutes

Even antibody blockbusters suffer from certain drawbacks, such as the requirement for mammalian cell production systems and dependence on disulfide bonds for stability. In addition, some antibody fragments tend to aggregate and display limited solubility. Finally, the success, and consequently the extensive use, of antibodies has led to a complicated patent situation of antibody technologies and applications. Therefore, several research groups and
small and medium-sized companies have recently focused on the development of small globular proteins as scaffolds for the generation of a novel class of versatile binding proteins.

As therapeutics, globular proteins with engineered binding properties might be particularly interesting, (i) if the neutralization of a target protein is the desired pharmacological effect (in contrast to a full length antibody, where the Fc portion may stimulate immune processes), (ii) as fusion proteins, for the targeted delivery of bioactive molecules to sites of disease, (iii) as receptor-binding drugs, thus interfering with the cell-signalling and (iv) as enzyme inhibitors.

Common to all approaches of finding a suitable scaffold are the following steps (8-11):

- choosing a small protein (domain) that is well expressed in bacteria or yeast and has good biophysical properties (stability, solubility)

- creating a library of protein mutants by introducing diversity at a contiguous patch of the surface of the protein (e.g., loops)

- using a genotype-phenotype display system (such as phage or ribosome display, see chapter 1.4) to select a range of binders to a therapeutic target of interest

- screening the binders obtained by the selection procedure for the desired biological activity, e.g., by ELISA

Mutations introduced in the protein scaffold to produce diversity may compromise the three-dimensional structure, stability and solubility of the protein scaffold, thus making the isolation of protein binders based on other folding frameworks than the immunoglobulin fold difficult. Nevertheless, more than 40 scaffolds have been described for the generation of new protein binders (reviewed in (12)). Being mainly a topic of academic interest at first, nowadays the development and use of non-antibody classes of proteins is being pursued by small- and medium-sized biotechnology companies (13). The common basis for the successful development of well designed protein drugs lies in the availability of both a well behaving protein scaffold and an efficient selection or screening technology in order to find suitable candidate proteins in the large repertoire created by mutagenesis. The next sections
will introduce selected classes of protein scaffolds that have been successfully used and commercialized by small- and medium sized companies.

1.2.1 Single domain antibodies

Although not belonging to the strict definition of „antibody alternative protein frameworks“, single domain antibody fragments are included as well in this chapter since they share a lot of common properties (e.g., small domains with high expression yields in bacteria). Moreover, they represent a valuable source for the generation of new binding proteins.

In a seminal early publication (14), mouse single variable domains (VH) were shown to be functional, and it was proposed that they could potentially target cryptic epitopes normally hidden for whole antibodies and even for smaller fragments thereof. However, they were described to be very sticky, poorly soluble and often prone to aggregation, because of their exposed hydrophobic surface normally „capped“ by the VL domain. Interest was recently revived when it was discovered that at least two types of organisms, the camelids and cartilaginous fish have evolved high-affinity V-like domains, mounted on an Fc-equivalent constant domain framework as an integral and crucial component of their immune system (15,16) (Fig. 1.1).

Figure 1.1 Schematic representation of different antibody formats. Intact „classic“ IgG molecule, the corresponding intact camel Ig and shark IgNAR with their single domain fragments are shown.

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Unlike mouse V_H domains (14), camelid VhH (termed nanobodies) and shark V-NAR domains are in general soluble and can be produced as stable \textit{in vitro} reagents. However, for \textit{in vivo} administration, humanization (or deimmunization) may be crucial to reduce immunogenicity. Human single domain antibodies (dAbs) would be even more preferable, and very recently the problems of poor stability and solubility have been solved for some human V domains by the identification and design of mutations that minimize the hydrophobic interface (17,18). Moreover, in a recent publication of Jespers and co-workers (19), aggregation-resistant domain antibodies could be directly selected on phage by heat denaturation. Starting from a DP47d domain antibody (a typical human V_H dAb), which unfolds irreversibly and forms aggregates if heated above 55°C, a repertoire containing approximately 1 billion different mutants was cloned by diversification of the CDR loops. The library was multivalently displayed on phage and after three rounds of heat denaturation followed by selection on protein A (a ligand common to folded dAbs), 179 out of 200 colonies secreted dAb phage that retained more than 80% of protein A-binding activity after heating (in contrast to the starting protein DP47d, which loses binding by a factor of 560 after heating). Twenty clones were sequenced and revealed many unique dAb sequences with a large variability in the CDR length and sequences, which shows that mutations located only in the CDR loops of the dAbs are sufficient to confer resistance to aggregation. Interestingly, the T_M of the mutants was not higher than the one of the parental clone DP47d, which shows that the selected dAbs are not heat stable, but can fold reversibly.

In summary, progress has been made towards the design of human dAbs which are soluble and can be expressed in \textit{E.coli} in acceptable yields. However, the disulfide bond in the immunoglobulin fold of the dAb can lead (when fused to other proteins containing an additional disulfide bonds or free cysteine residues) to the formation of misfolded and inactive proteins. To circumvent this problem, in 2004 Wittrup and colleagues have engineered a V_L domain devoid of disulfide bonds that is stable and efficiently expressed in the cytoplasm of yeast (20). Intracellular antibodies, also called intrabodies, are attracting interest as tools to manipulate and study biological systems intracellularly.

The company having commercialized the use of single domain antibodies, Domantis Ltd., was recently acquired by GlaxoSmithKline for £230 million. Together with the acquisition of
Avidia by Amgen (see chapter 1.2.3), this trade-sales certainly reflects the growing interest of big pharmaceutical companies in single domain protein scaffolds.

1.2.2 Tenth type III domain of fibronectin

The tenth type III domain of human fibronectin ($^{10}$Fn3) is a 94 aminoacid residue structural protein with an immunoglobulin-like fold that is used as an antibody-mimic scaffold. High thermal stability ($T_M = 90^\circ C$) and solubility (> 15mg/ml), high expression yields in *E.coli* and the lack of cysteines in its structure are attractive properties that make the $^{10}$Fn3 a good scaffold candidate (21). Three of the solvent exposed loops in $^{10}$Fn3, named BC, DE and FG are structurally analogous to the $V_H$ complementary-determining regions (CDR) (Fig. 1.2).

![Figure 1.2](image)

**Figure 1.2 Structural comparison of a llama $V_{HH}$ domain and the wild-type human $^{10}$Fn3 domain** (21). Despite the lack of significant sequence identity, both domains fold into similar beta sheet sandwiches. The CDRs of the $V_{HH}$ domain and the residues randomized in the $^{10}$Fn3 domain are shown in color.

The first report on the use of the $^{10}$Fn3 domain as a protein with artificial binding sites was published in 1998 (22). In that study, a library of $10^8$ distinct $^{10}$Fn3 mutants was made by deleting three residues in the FG loop and by randomizing five residues in the BC loop and four residues in the FG loop. The library, displayed on phage, was used in affinity selection experiments with immobilised ubiquitin as model target protein. After five rounds of panning
clones were randomly picked for sequencing. A clone designated as Ubi4-Fn3 dominated the population of selected protein mutants and was subjected to further analysis. Ubi-Fn3 was demonstrated to bind in phage enzyme-linked immunosorbent assay (ELISA) experiments. However, when expressed as a single domain protein in E.coli, Ubi4-Fn3 exhibited low solubility at neutral pH, unspecific binding to the dextran matrices of the size-exclusion chromatography column and the dextran coated biosensor chips used for the subsequent characterization of Ubi-Fn3. More importantly, the affinity was relatively low, the IC_{50} determined by competition ELISA was 5μM.

In order to select 10Fn3 domains binding to a target of interest with improved properties than Ubi4-Fn3, an alternative library design in combination with a fully in vitro selection system (mRNA display (23)) was used for the isolation of 10Fn3 variants binding to tumor necrosis factor-α (TNF-α) (21). Clones from the ninth and tenth round of selection were cloned in E.coli, sequenced and expressed. Affinity constants were determined by incubating the in vitro translated 35S-methionine labeled proteins with biotinylated TNF-α at different concentrations. The solution containing the 10Fn3 mutants bound to TNF-α were aspirated by vacuum onto a membrane coated with streptavidin, therefore capturing protein complexes on the membrane. Binding was analysed by measuring the radioactivity on the membrane, the K_D for selected mutants were found in the range of 1-24 nM. Further affinity maturation procedures revealed K_D values around 100 pM, the best being 20 pM, and analytical gel filtration showed that the apparent molecular weight of purified, soluble wild-type (wt) and mutant domains was consistent with the variants being monomeric. The reported affinities, especially for TNF-α, are very high. However, these values should be evaluated critically because first, the capture step of the biotinylated TNF-α-Fn3 protein complexes was performed in a solid phase format, and second, TNF-α is homotrimeric protein, so avidity effects may contribute to the high apparent affinity observed.

Other 10Fn3 variants were shown to recognize αvβ3-integrin expressed on cell surface and inhibiting αvβ- dependent cell adhesion (24) and to bind to vascular endothelial growth factor receptor 2 (VEGF-R2) (25,26). For all 10Fn3 variants reported so far, their thermodynamic stability was worse than that of the wt protein. In addition, some VEGFR-R2 binding mutants tend to dimerize and/or to aggregate (25).
As a conclusion, it seems difficult to obtain high-affinity and stable proteins with this scaffold. In addition, the use of engineered $^\text{10} \text{Fn3}$ in therapeutic applications may turn out to be problematic: an immune response of the patient to the injected protein might generate cross-reactive antibodies binding to the wild-type $^\text{10} \text{Fn3}$, which is ubiquitous in the human body, thus leading to an immune attack in the patient and/or formation of circulating complexes, with a potential kidney toxicity. However, in the near future we will learn more about this scaffold, since a VEGF-R2 antagonist, termed „Angiocept CT-322“, has just entered into phase I clinical trials, in patients with advanced solid tumors and non-hodgkin’s lymphoma (www.clinicaltrials.gov).

1.2.3 A-domains

A family of A-domains (27,28) has been described to be suitable as a scaffold for the generation of new binding proteins (29). A-domains occur as strings of multiple domains in several cell-surface receptors (Fig. 1.3a). Domains of this family bind over 100 different known targets, including small molecules, proteins and viruses (30,31). Such a target is typically contacted by multiple A-domains with each domain binding independently to a unique epitope, thereby generating avidity. Each of the 217 human A-domains comprises approximately 35 amino acids and domains are separated by linkers that average five amino acids in length. Native A-domains fold quickly and efficiently to a uniform, stable structure mediated by calcium binding and disulfide formation. A conserved scaffold motif of only 12 amino acids is required for this common structure (32). Stemmer and co-workers have designed a phage display library of A-domains, consisting of a conserved consensus sequence in the scaffold motif and variable amino acids in different positions (Fig. 1.3b) (29). Like other directed evolution technologies, the process the authors developed included multiple recursive cycles, each consisting of library generation and screening in functional assays. However, rather than mutagenizing the protein between cycles (e.g., for affinity maturation purposes), they added a new pool of domains adjacent to the domain(s) selected in previous rounds (Fig. 1.3c).
**Figure 1.3 Overview of avimer technology** (29). (a) Domain organization of several human receptor containing A-domains. Multivalent binding of several natural ligands to known combinations of A-domains is also depicted. α-2M, α-2 macroglobulin; LRP, low density lipoprotein-related protein; RAP, receptor-associated protein; VLDLR, very low density lipoprotein receptor; VLDL, very low density lipoprotein; ApoE, apolipoprotein E; ApoB100, apolipoprotein B100; LDL, low density lipoprotein; PAI-1, plasminogen activator inhibitor 1; uPA, urokinase-type plasminogen activator. (b) Fixed and variable positions of the A-domain library, as well as the disulfide topology, are indicated. Each circle represents an amino acid position. Calcium-coordinating scaffold residues are yellow, structural scaffold residues are blue, cysteine residues are red and variable positions are green. A ribbon diagram of a prototypical A-domain structure is included (PDB ID 1AJJ). (c) Flow chart depicting the sequential panning and screening referred to as „domain walking“. The end result is a single protein chain containing multiple domains, each of which represents a separate function. The A-domains are therefore called „avimers“, from avidity multimers. A heterotetramer consisting of three IL-6 binding A-domains and an IgG binding domain (named C326) showed a remarkable affinity (picomolar range) to its target and exhibited sub-picomolar IC₅₀ in cell-proliferation assays (29). Moreover, C326 completely abrogated acute-phase protein induction by human IL-6 in mice, in a dose dependent manner, suggesting that C326 inhibited IL-6 functions in vivo. A placebo-controlled phase I study of C326 was enrolled in the United States in September 2006, with patients suffering from Crohn’s disease (www.clinicaltrials.gov). The completion of this study is expected in September 2007, giving new insights about safety and pharmacokinetics of C326.
The technology of using the modular platform of A-domains was commercialized by Avidia Inc., that was acquired by Amgen for 290$ million in October 2006, proving the interest and significant potential of antibody alternatives in the field of pharmaceutical biotechnology.

1.2.4 Z-domain of staphylococcal protein A

The immunoglobulin binding domain of staphylococcal protein A (SPA) is widely used as an immunochemical ligand for the purification or detection of antibodies and serves as affinity tag in fusion proteins (33). The IgG binding domain of SPA consists of five highly homologous three-helix bundle domains of approximately 58 amino acid residues each. Because SPA is known as a non-cysteine containing, highly soluble, proteolytically and thermally stable protein, an engineered version of one of the SPA domains, the so-called Z-domain (34) was chosen as a scaffold for the development of novel affinity proteins designated as "affibodies" (Fig. 1.4). Utilizing structural data available for the complex between a native SPA domain and the Fc fragment of human IgG1, 13 positions distributed across two helices, located at the surface of the domain and involved in the Fc interaction, were chosen for random mutagenesis, and subsequently, for the creation of phage libraries (35). In 1997, from two medium sized libraries comprising about $4 \times 10^7$ individual clones, binding proteins against three model target proteins (Taq DNA polymerase, human insulin, and a human apolipoprotein A-1 variant) were isolated, with binding affinities in the micromolar range (36). In the meantime, affibodies have been isolated against a variety of protein targets (e.g., human factor VIII (37), human immunoglobulin A (38) and CD28 (39)). In a more recent paper (40), an affibody ligand binding to human epidermal growth factor receptor 2 (Her2) was isolated with a dissociation constant ($K_D$) of 50 nmol/L. Dimerization of this affibody molecule resulted in improved target binding affinity ($K_D = 3$ nmol/L), and radiiodination of the dimer allowed selective targeting and imaging of Her2-expressing xenografts in vivo (41). Further affinity maturation strategies led to an affibody molecule with a dissociation constant in the range of 20 pmol/L and to better tumor targeting and imaging results in the same tumor mouse model (42). The most recent paper describes fully synthetic affibody molecules, site-specifically and homogenously conjugated with a DOTA
(1,4,7,10-tetraazacyclododecane-N,N',N''',N'''''-tetraacetic acid) chelator, produced in a single chemical process by peptide synthesis (43).

Figure 1.4 Structure of an in vitro evolved affibody in complex with protein Z. (44) (a) Structure of the complex, the ZSPA-1 affibody in blue and protein Z in green. The ordered sulfate ions with partial occupancy and putative magnesium ion from the mother liquor are also shown; however, they do not seem to influence the interaction surface. (b) Superposition of the two molecules. Notice the shift of helix 1 in the affibody (blue) compared with protein Z (green). (c) Electron density for all 13 mutated residues in the affibody; for clarity, only the electron density around the side chains is displayed.

Importantly, first clinical data of molecular imaging of breast cancer metastases using $^{111}$In radiolabeled anti-Her2 affibody molecules were presented (Next-Generation Protein Therapeutics Conference, Basel, November 2006), showing high-resolution images of metastases within hours. Although the data obtained from patients have not been published in peer reviewed journals yet, it seems that the affibody molecule works well for imaging applications. However, future clinical data will show whether affibody molecules are good enough for therapeutic applications, especially concerns about the immunogenic potential of this class of proteins should be seriously considered, owing to its bacterial origin.
1.2.5 Kunitz type domains

Proteases have important biological functions, they have been identified as key regulators of cellular processes such as ovulation, fertilization, wound healing, angiogenesis, apoptosis, peptide hormone release, coagulation, and complement activation (45). Because unregulated proteolysis in any of these processes will have undesirable effects, effective control of protease activity is critical. Indeed, unregulated activity has been implicated in the pathogenesis of many diseases (e.g., cancer, inflammatory diseases, chronic obstructive pulmonary disease (COPD), chronic pancreatitis, muscular dystrophy and Alzheimer’s disease (46-51)), making proteases important target proteins. It is notable, that there are only few marketed drugs (such as HIV protease inhibitors, angiotensin-converting enzyme inhibitors, the proteasome inhibitor bortezomib and the very recently approved renin-inhibitor aliskiren), since a key challenge in the discovery of new inhibitors is the ability to identify drugs which are both potent and specific. Structural similarities within the active site of most proteolytic enzyme families often result in a simultaneous inhibition of several family members, which can lead to an unacceptable toxicity profile. One solution to this problem might represent the use of endogenous, engineered protease inhibitors that make more contacts with the target protease and therefore allow for tighter control over specificity. One type of endogenous inhibitors include the kunitz domain inhibitors, e.g., bovine pancreatic trypsin inhibitor (BPTI) (52).

In 1992, Ladner and co-workers produced a small library of mutants of kunitz domain BPTI, displayed on phage and screened for the ability to bind to elastase (53), a serine protease that is believed to play a causative role in a variety of lung diseases, including COPD and cystic fibrosis (54). Isolates that were able to bind to elastase were expressed, purified and tested for inhibition of elastase. The P-loop (see Fig 1.5), the region that was varied in the library, of the best inhibitor (K_i ~ 1 pM) was transferred to a human kunitz domain without subsequent loss of potency (55). The resulting engineered molecule, depelestat (Dyax/Debiopharm SA), inhibited human neutrophile elastase (HNE) activity in the sputum of cystic fibrosis patients, was demonstrated to be safe in an aerosolized format in monkeys and no adverse effects were reported in phase I clinical trials (45,56,57). Pharmacodynamic reports of a phase IIa clinical trial have been shown to inhibit completely sputum HNE in 52.6 % of the patients and to
decrease interleukin-8 levels, a biomarker of inflammation, in the sputum of treated patients (58).

Figure 1.5 Schematic representation of a representative kunitz type domain (LAC-D1). (59) Varied positions are depicted in black, the P1 and second loop positions are enclosed.

In 1996, another human kunitz domain, the lipoprotein-associated coagulation inhibitor (LACI-D1), was used as a scaffold for the successful isolation of a very specific and potent inhibitor of human kallikrein (60) (Fig. 1.5). Kallikrein is believed to be an important mediator of hereditary angioedema (HAE), a rare disorder with attacks of edema in the hands, face, feet, abdomen, and/or throat. This condition is caused by a genetic deficiency of C1 esterase inhibitor (C1-Inh). In Europe, HAE is treated with plasma derived C1-Inh which attenuates attacks and may prove life saving, but C1-Inh is expensive and requires the use of pooled blood product (45). After an iterative selection and screening approach chosen by the authors (60), the plasma kallikrein inhibitor DX-88 (Dyax Corp / Genzyme Corp) was isolated and proved to be a potent inhibitor ($K_i = 40$ pM) with high selectivity (Table 1 in (61)). The use of DX-88 in a C1-Inh deficient mouse model demonstrated that DX-88 is effective in preventing changes in vascular permeability (62). DX-88 is currently being used to treat acute attacks of HAE in clinical studies, positive phase II trial results have been reported (61) and double-blind, placebo-controlled phase III clinical trials have been enrolled to assess the efficacy and safety of DX-88 for the treatment of acute attacks of HAE.

With respect to clinical development, kunitz type domains represent the most advanced
scaffold in this field. The strategy of taking a human protease inhibitor as a scaffold and improving it by protein engineering works well for certain therapeutic applications. At the same time, kunitz type domains have the limitation of binding only to proteases, and thus they do not represent a universal source for the generation of binding proteins to a variety of targets.

1.2.6 Ankyrin repeat proteins

Ankyrin repeat (AR) proteins, first isolated in mammalian erythrocytes, are involved in the targeting, mechanical stabilization and orientation of membrane proteins to specialized compartments within the plasma membrane and endoplasmic reticulum. Natural ankyrin repeat proteins consist of many 33-amino-acid modules, each comprising a β-turn and two anti-parallel α-helices (63). In most known complexes, the β-turn and the first α-helix mediate the interactions with the target, and different numbers of adjacent repeats are involved in binding. The reported target binding affinities of natural AR proteins are in the low nanomolar range (64). Ankyrin repeat proteins were built and diversified to create a library from which ankyrin variants were selected binding to maltose-binding-protein and two eukaryotic kinases (65-67). In the approach chosen by the authors, a consensus ankyrin repeat module consisting of six diversified potential interaction residues and 27 framework residues was designed based on sequence alignments and structural analyses (Fig. 1.6). Varying numbers of this repeat module were cloned between capping repeats, which are special terminal repeats of ankyrin domains shielding the hydrophobic core. Two libraries were created with two and three, respectively, randomized ankyrin repeats in between an N-terminal and a C-terminal cap.
Figure 1.6 Schematic representation of the library generation of designed ankyrin repeat proteins (65). (upper part) Combinatorial libraries of ankyrin repeat proteins were designed by assembling an N-terminal capping ankyrin (green), varying numbers of the designed ankyrin repeat module (blue) and a C-terminal capping ankyrin (cyan); side chains of the randomized residues are shown in red. (lower part) Ribbon representation of the selected MBP binding ankyrin repeat protein (65) (colors as above). This binder was isolated from a library of N-terminal capping ankyrin repeat, three designed ankyrin repeat modules and a C-terminal capping ankyrin repeat.

Using a library with more than $10^{10}$ individual members in combination with the ribosome display selection methodology (see chapter 1.4.5), binding variants (termed DARPins) were selected by performing four or five rounds of selection. Dissociation constants were determined by surface plasmon resonance and found to be in the range of 2-20 nM against these model target proteins. More recently, more ankyrin repeat variants have been isolated against target proteins such as intracellular kinases, phosphotransferases and human epidermal growth factor receptor 2 (Her2), the latter being an important target for cancer therapy and diagnosis (66-68). However, in vivo data are not available yet, and, because the DARPins were newly designed and are not found in nature in this format, their immunogenic potential should be investigated in detail.
1.2.7 Lipocalins

The lipocalins represent a family of functionally diverse, small proteins that comprise 160-180 amino acid residues and have weak sequence homology but high similarity at the tertiary structural level (69). Members of this family have important biological functions in a variety of organisms, from bacteria to humans. The majority of lipocalins are responsible for the storage and transport of compounds that have low solubility or are chemically sensitive, such as vitamins, steroids and metabolic products (70). An example of human lipocalin is the retinol binding protein (RBP), of which the 3D-structure has been elucidated by X-ray crystallography (71). RBP transports the poorly soluble and oxidation-prone vitamin A from the liver, where it is stored as a fatty acid ester, to several target tissues. Despite their extremely poor sequence homology, the lipocalins share a structurally conserved β-barrel as their central folding motif, which consists of eight antiparallel β-strands that are arranged in a cylindrical manner (Fig 1.7). The binding specificity for low-molecular weight compounds is well-characterized for many lipocalins, some having high ligand specificity whereas others form complexes with a considerable range of lipophilic molecules (72). Typically, the ligand affinities of lipocalins are moderate with dissociation constants of approximately 1 μM, which is consistent with their presumed function as a physiological buffer for the bound substance; however, there are notable exceptions, e.g., the tick histamine-binding protein (HBP)-2 with a K_D of 1.7 nM (73). The insect bilin binding protein (BBP) from Pieris brassicae served as a model lipocalin in initial studies to create artificial binding sites for several ligands. Sixteen amino acid positions located within the four loops at the open end of the β-barrel and adjoining regions of the β-strands were subjected to targeted random mutagenesis (74). The resulting molecular random library with about 4 x 10^8 members was subjected to selection towards several low-molecular weight compounds using phage display. BBP variants, so-called „anticalins“, that specifically recognize fluorescein, digoxigenin, phthalic acid esters and doxorubicin were obtained, with affinities in the nM range (74-76). In order to extend the concept of anticalins, several human lipocalins were subjected to random mutagenesis generating libraries that enable recognition of macromolecular protein targets. Because proteins have larger molecular dimensions than small compounds, they cannot penetrate into the ligand-binding pocket of lipocalins. Consequently, side chains at more exposed positions, close to the tips of the four loops at the open end of the β-barrel,
were subjected to random mutagenesis. Following this strategy, a panel of anticalins based on human lipocalins with specificities for several therapeutic targets such as cytotoxic T-lymphocyte-associated antigen (CTLA-4), VEGF and other undisclosed targets (www.pieris.biz) were isolated. However, detailed data about this work has not yet been published and it has to be seen how these engineered proteins will behave in experiments with animal models and, subsequently, in clinical trials.

Figure 1.7 General structure of human retinol-binding protein, a prototypic lipocalin. Ribbon diagram of the crystal structure of RBP (adapted from (72)) with the bound ligand retinol (magenta, ball and stick representation). The eight antiparallel strands of the conserved $\beta$-barrel structure are shown in blue with labels A to H, and the four loops, which are highly variable among the lipocalin family, are colored red and numbered. The typical $\alpha$-helix that is attached to the central $\beta$-barrel in all lipocalins, the loops at the closed end the N- and C-terminal peptide segments are shown in grey. The three disulfide bonds of RBP are depicted in yellow.

1.2.8 Other domains

The preceeding sections introduced important domain scaffolds that have been commercialized and for which published data are available. In a recent review of Binz et al. (12) more than 40 scaffolds have been described for the generation of new binding proteins. The majority of them have been used for research purposes only (e.g., probing the specificity determinants of WW-domains to their ligands (77), elucidating target recognition rules of SH3 domains (78,79) or identifying signal transduction pathways with the staphylococcal nuclease as scaffold (80)). Other domains have been commercialized by companies for in
vitro applications (e.g., PDZ domains for the generation of high-affinity detection reagents (Biotech Studio (81)) or for intracellular signaling interference applications (thioredoxin, Aptanomics (82)). A few other scaffolds have also been commercialized by small- and medium sized companies, however, no refereed publications are available (human γ-crystallin and human ubiquitin by Scil Proteins, C-type lectin domain by Borcan Pharma, monoclonal soluble T cell receptors by Avidex and a not disclosed scaffold by Amunix; informations obtained from the corresponding websites.)

1.2.9 Concluding remarks

After reviewing the most important scaffolds described in the literature, it can be summarized that an ideal scaffold should comprise the following features:

- fully human protein scaffold (reduced immunogenic potential)

- conserved amino acid sequence in different species from mouse to man (immunogenic potential can be tested in preclinical experiments)

- high-expression yields in bacteria (cheap production)

- scaffold devoid of cysteine residues (site-directed introduction of cysteine residues for chemical modification at a later stage possible; less folding problems if scaffold is expressed as fusion protein with cysteine residues, e.g., cytokines)

- thermally stable and soluble (no formation of aggregates -> less immunogenic)

- wild-type domain involved in protein-protein interaction (starting point for a first library design)

- structure solved (further rational randomization possible)
Although some rational justification may be given for the choice of almost any scaffold described so far, in our opinion the protein scaffolds used for the development of therapeutic proteins introduced in chapter 1.2 are not perfectly suited for the generation of binding proteins. None of the protein scaffolds discussed before meets all the requirements for an optimal scaffold candidate, maybe with the exception of ubiquitin. All other scaffolds have at least one deviation from the set of desired properties. For example, camelid single domain antibody fragments (nanobodies), protein A derived binders (affibodies), A-domains and ankyrin repeat proteins are from non-human origin or are synthetically designed scaffolds; A-domains, T cell receptor domains, human C-type lectin domains, human single domain antibody fragments, kunitz type domains, lipocalins and γ-cristallins contain cysteine residues; 10Fn3 variants may lead to devastating effects in human beings, if such a therapeutic variant elicits an immune response and if the antibodies cross-react with the wild-type domain, which is ubiquitously expressed in the body.

It is difficult to predict how these intrinsic disadvantages of the scaffolds translate into complications and increased costs in clinical development, but starting with a scaffold that meets all requirements described may be more advantageous.
1.3 The SH3 domain of Fyn kinase as a scaffold for the generation of new binding proteins

1.3.1 Src homology-3 (SH3) domains

SH3 domains are globular protein modules typically comprising 50-60 amino acid residues found in many different proteins, particularly those involved in cellular signal transduction (83) and in those involved in the regulation of intracellular dynamic processes occurring at the plasma membrane, such as the organization of the cytoskeleton and the internalization of membrane receptors (84). Two hundred seventy human SH3-containing proteins have been identified, with a total of ~300 different human SH3 domains (85). Despite the variability in their primary structures, crystal and solutions structures from several SH3 domains revealed a completely conserved fold: a compact β-barrel, formed by two perpendicular, two or three stranded β-sheets, and a single turn of $3_{10}$ helix (Fig. 1.8) (86-90).

![Figure 1.8 Crystal structure of the Src-SH3 domain](image)

Figure 1.8 Crystal structure of the Src-SH3 domain (87). The structure shown is PDB code 1SHG.
SH3 domains mediate inter- and intramolecular interactions by binding to ligands that contain a region with a secondary structure known as the polyproline type II (PPII) helix. These ligands can bind to SH3 domains in two opposite orientations and show typically a PXXP core-binding motif, flanked by a basic residue (RXXPXXP or PXXPXAR) (91-93). However, examples of unconventional SH3 binding sites are also known (94,95). The regions involved in the interaction with the proline-rich core of target sequences is a surface patch rich in aromatic residues (forming the "XP grooves") and by various polar residues located in the RT- and n-src-loops (Fig 1.9) (96,97). SH3 domains bind their targets with low-affinities ($K_D = 1$ to 200μM), a property that is probably necessary for finely regulated, transient signaling interactions (98). However, most of the affinity measurements reported so far used the SH3 domain and a minimal binding motif (such as XPXXPXAR), so the "real" affinity between a SH3 domain and its interacting protein carrying a PPII helix is considered to be higher (85).

Figure 1.9 Structure of the Crk SH3-N domain in complex with a proline rich peptide (97). The structure shown is PDB accession code 1CKB. The SH3 domain is depicted in ribbons with secondary-structural elements shown in different colors and labeled. The bound peptide, PPPALPPKKR, is shown in blue with side chains. Interface residues on the SH3 domain are shown in pink, for aromatic residues, and in light blue, for non-aromatic residues. The locations of the XP grooves and specificity pocket on the SH3 domain arc identified by broken arrows.
1.3.2 SH3 domains as scaffolds for the generation of engineered binding proteins

Since it has become clear that also molecular contacts outside the PPII helix interface can provide specificity and strength to SH3 binding (99), e.g., by contacting loop regions of SH3 domains, efforts have been made by using engineered SH3 variants to elucidate further the specificity and affinity determinants of the interactions between SH3 domains and their ligands.

Saksela and colleagues constructed a phage display library of ~10^8 Hck SH3 variants, where six amino acid residues in the RT loop were randomized (79). Wild-type Hck is known to bind to the HIV-1 Nef protein and to a Nef mutant (F90R) with an affinity of 250 nM or 2 μM, respectively (99). Performing phage display selections against HIV-1 Nef, the authors could isolate individual Hck SH3 variants which bound to HIV-1 Nef with up to 40-fold higher affinities than the parental Hck SH3 domain, indicating that the RT loop contributes to the binding strength in this particular interaction. Interestingly, some of the selected clones bound also well to the F90R Nef mutant (NefR90), while others were dependent on the presence of the Nef Phe90 residue even more critically than wild-type Hck SH3, and showed over a 100-fold decreased affinity towards NefR90. Conversely, Hck SH3 derived binders to NefR90 could be isolated using NefR90 as target in phage display selections, whose affinity depended on structural determinants involving the side chain of Arg90 residue. However, no binding proteins could be isolated to other epitopes than the polyproline motif of Nef.

In conclusion, the group of Saksela succeeded in isolating affinity matured Hck SH3 binding proteins to the known Hck SH3 ligand HIV-1 Nef. The authors concluded that artificial SH3 domains in general, like the ones isolated against HIV-1 Nef, could be potent competitors for natural SH3 interactions, an important feature in basic research of intracellular physiological pathways.

Cesareni and co-workers designed a repertoire of ~10^7 SH3 domains by grafting 12 residues that are represented in the binding surfaces of natural SH3 domains onto the scaffold of human Abi SH3 domain (78). This phage-displayed library was screened by affinity selection for SH3 domains that bind to the synthetic peptides, APTYPPLPP and LSSRPLPTLPSP, which are peptide ligands for the human Abl or Src SH3 domains, respectively. By analyzing
the isolates, the authors identified three amino acid positions of the Abl SH3 scaffold that are crucial for recognition specificities, favoring either the "Abl-peptide" or the "Src-peptide": one amino acid in the RT loop, one in the n-Src-loop and a tryptophan residue in the β4 strand. By further site-directed mutagenesis experiments with these 3 residues in the wild-type Abl SH3 scaffold, the authors evolved evolution pathways of protein interactions (e.g., an Abl SH3 variant carrying the residues TNW in the 3 positions and specific for the Abl peptide can be mutated to TCW without substantially changing its recognition properties whereas a second substitution, TCL, causes a sudden shift in ligand preference to the Src peptide). The authors created then a position-specific scoring matrix, based on the amino acid frequencies observed in the domains selected from the artificial repertoire, in order to rank natural SH3 domains according to their probability of binding to the 2 peptides. Twenty-nine SH3 domains in the yeast proteome were predicted to bind to either of the two poly-proline peptides; the predictions were challenged by ELISA binding assays, which revealed false negative and false positive results, as the underlying scaffold has a stronger influence on binding specificity than assumed by the authors. The authors concluded nonetheless that their library can be used to select Abl SH3 variants capable of binding to intracellular targets with a proline-rich motif, in order to interfere with signaling cascades.

In summary, SH3-derived proteins have been used so far only for the generation of binders against known SH3 ligands, such as HIV-1 Nef protein or synthetic proline-rich peptides (78,79).

1.3.3 SH3 domain of Fyn kinase

Fyn kinase was identified in 1986 as a new member of the Src family of tyrosine kinases (100,101). Originally denoted Syn or Slk, this related novel protein has been renamed Fyn and encodes a 59 kDa kinase. As a result of alternative splicing, the Fyn protein is expressed as two isoforms, differing in approximately 50 amino acids in a region between their SH2 and kinase domain. One form is found in thymocytes, splenocytes and some hematolymphoid cell lines (FynT), while the second form accumulates principally in the brain (102). Its biological functions are diverse and related to Fyn’s ability to associate and to phosphorylate
a variety of intracellular signaling molecules. One of the best known functions of Fyn kinase is the phosphorylation of SLAM (signaling lymphocyte activation molecule) in T cells, inducing a signaling complex modulating interferon-γ expression (103) (Fig. 1.10). The interaction between Fyn and SLAM occurs via the SH3 domain of Fyn and the adaptor protein SAP (SLAM associated protein), forming a ternary complex. Interestingly, Fyn SH3 associates with SAP through a surface-surface interaction that does not involve the canonical PXXP recognition motif of SH3 domains (94). Interacting regions of the Fyn SH3 domain include the RT-loop, n-src loop and single residues of the β3- and β4 strands (94).

Like other SH3 domains, Fyn SH3 is composed of ~60 amino acids [amino acids 83–145 of the sequence reported by Refs. (100,101)] and shows the typical SH3 topology of two perpendicular β-sheets and a single turn of 310 helix.

With its biophysical properties and its primary structure, Fyn SH3 perfectly matches our criteria for a scaffold to be used as alternative to antibodies: (i) it is expressed in bacteria at high level in soluble, monomeric form (104), (ii) it is stable (Tm: 70.5° C) (105), (iii) it does not contain any cysteine residues, (iv) it is from human origin and (v) its amino acid sequence is conserved in mouse, rat, monkey (gibbon) and man. Moreover, its binding mode to SAP has been resolved and indicates that this particular domain does not necessarily need a PXXP core binding motif, which is an important prerequisite to be well suited for the isolation of binders against a variety of different target epitopes. Moreover, since the groups of Saksela and Cesareni showed that phage display technology is suitable for the isolation of artificial Hck - and Abl - SH3 domains, the probability that this established methodology might also work for the Fyn SH3 domain was very high.
Figure 1.10 The role of Fyn kinase in T cells (103). (a) Signal transduction through the SLAM receptor is regulated by SAP. Fyn is kept in an auto-inhibited state in the absence of SAP. Recruitment of Fyn to the SLAM receptor by SAP leads to the activation of the kinase and subsequent phosphorylation of SLAM on multiple tyrosine residues. Phosphorylated tyrosine residues serve as docking sites for the SH2 domain of SHIP (SH2-containing inositol phosphatase). Binding of SHIP to SLAM initiates a signaling cascade that ultimately leads to modulation of IFN-γ production in T cells. (b) Structure of a ternary complex of Fyn SH3-SAP-SLAM-peptide. The Fyn SH3 domain, in green, binds to SAP, in cyan, via surface-surface association. SAP engages the SLAM peptide, in magenta, on the opposite surface using a groove conserved for SH2 domains. The structure shown is PDB code 1M27.
1.4 Isolation of binding domains

1.4.1 General aspects

The identification and isolation of proteins exhibiting desired binding properties from large repertoires of mutant domains is a daunting task. Due to the large number of different clones, individual members of the repertoire cannot be assayed one by one due to time limitations and practical reasons. Therefore, methodologies have been developed which allow the simultaneous evaluation of many different protein mutants in a short period of time. Screening methodologies represent procedures where all members of the repertoire are individually assessed in a biological assay in a highly parallel fashion, thus opening the opportunity to screen large numbers of protein candidates for desired activities. The researcher then chooses those proteins, which were positive in the screening assay.

In addition to the screening techniques, selection technologies are often used, which allow to mimic the natural process of evolution in a test tube in the laboratory. Common to all selection technologies is the linkage of the genetic information (genotype) with the encoded polypeptide (phenotype). This allows the amplification of the genetic information of the isolated protein mutants, leading to the survival of genotypes which code for favorable polypeptides. By repeating the two steps of generating genetic diversity followed by selection for a desired protein activity, proteins can be evolved in vitro.

1.4.2 Iterative colony filter screening

In colony filter screening, clones (typically $<10^6$) are simultaneously assayed for their ability to generate the binding specificities of interest. A filter screening assay was described (106) for the identification of the few clones secreting an antibody of given antigen specificity, out of several thousand hybridoma clones. A modified version of this methodology was used for the isolation of antibodies from a large library with millions of different clones (107) (Fig.
The authors used the naïve ETH-2 antibody library (108), in the form of a pool of antibody secreting bacteria, to isolate monoclonal antibodies specific for the extra domain-B (EDB) of fibronectin (see chapter 1.6).

Figure 1.11 Schematic representation of the iterative colony filter screening method (107). Bacteria expressing potentially each a different antibody fragment were spread on a Durapore filter membrane (A). On the filter, placed on a solid medium that represses expression of the antibody fragments, colonies were visible after 8 h incubation at 37°C (here three different colonies are depicted schematically). A second filter membrane (B) was coated with the antigen of interest (represented by pyramids), laid on a solid medium capable of inducing the expression of scFvs, and in contact with membrane A. Antibody fragments that diffused from membrane A and that bound to the antigen (in red) were captured on membrane B, and could be detected by an enzymatic colorimetric reaction. The corresponding colonies could be identified on membrane A, regrown and the procedure could be repeated until single positive colonies producing monoclonal antibodies were identified.

One hundred million bacteria from the ETH-2 library, potentially expressing a different scFv antibody fragment, were grown on a porous master filter until a lawn of small confluent colonies was visible. The master filter was then laid on a capture filter embedded with the antigen (an EDB containing fibronectin fragment), and bacteria on the master filter were induced to express the recombinant antibodies by placing the filter membranes on solid medium containing IPTG. Recombinant antibodies can diffuse from the master to the capture
membrane, where the ones specifically binding the antigen are trapped onto the capture filter. A Flag-tag at the C-terminal extremity of the scFv antibody fragments allowed the detection of their binding to the antigen coated-capture membrane by an enzymatic colorimetric reaction. Due to the huge number of bacteria on the master filter, the first round of screening did not allow the direct identification of single positive bacterial colonies, but only of positive areas of confluent colonies that, by superposition of the two filters, could be rescued from the master membrane and grown to perform a further round of screening. Three rounds of screening were performed, plating $10^6$ bacteria on a master filter for the second round and $1000$ for the third round. At this stage, colonies on the master filter were no longer confluent and approximately 30% of the plated colonies produced scFvs that bound to filter B, therefore representing potential anti-EDB monoclonal antibody fragments (107). The positive clones at the third round of filter screening were regrown and the binding specificity of the corresponding antibodies was analysed by ELISA. 16% produced antibodies that bound specifically to the ED-B domain of fibronectin and the best clone, named scFv ME4C, was further characterized. The affinity of scFv(ME4C) towards the ED-B domain of fibronectin was measured by real-time interaction analysis using surface plasmon resonance detection, yielding a $K_D = 1 \times 10^{-7} \text{ M}$ (107).

Two general methodologies based on filter-sandwich assays were described by Heinis et al. (109) for isolating enzymatic activities from a large repertoire of protein variants expressed in the cytoplasm of E. coli cells. The enzymes were released by the freezing and thawing of bacterial colonies grown on a porous master filter and diffused to a second "reaction" filter that closely contacted the master filter. Reaction substrates were immobilized either on the filter or on the enzyme itself (which was, in the latter case, captured on the reaction filter). The resulting products were detected with suitable affinity reagents. Biotin ligase was used as a model enzyme to assess the performance of the two methodologies. Active enzymes were released by the bacteria, locally biotinylated the immobilized target substrate peptide, and allowed the sensitive and specific detection of individual catalytically active colonies.
1.4.3 Phage display

Phage display is a powerful methodology that allows the selection of a particular phenotype (e.g. a ligand specific to a desired antigen) from repertoires of polypeptides displayed on phage. It was originally described in 1985 by Smith (110), who reported the use of the non-lytic filamentous bacteriophage fd for the display of specific binding peptides on the phage coat. The power of the methodology was further enhanced by the groups of Winter (111) and Wells (112) who demonstrated the display of functional folded proteins on the phage surface (an antibody fragment and a hormone, respectively). The technology is based on the fact that a polypeptide (capable of performing a function, typically the specific binding to an antigen of interest) can be displayed on the phage surface by inserting the gene coding for the polypeptide into the phage genome. Thus, the phage particle links phenotype and genotype (Fig. 1.12).

![Phage displaying a binding protein as fusion protein of a minor coat protein pIII.](image)

Figure 1.12 Phage displaying a binding protein as fusion protein of a minor coat protein pIII.

It is possible to create repertoires of phage (phage display libraries) in which the proteins displayed on each phage represent a population of different molecules with different properties. If a phage particle is isolated by virtue of its phenotype displayed on the surface (e.g. the binding specificity), the genetic information coding for the protein is co-isolated. As an example, one can consider the selection of a binding specificity from a displayed protein library. The library on phage is panned against an antigen of interest; unbound phage are
discarded whereas specifically binding phage are enriched and amplified in bacteria. Several rounds of selection can be performed (typically 2-4 rounds). As a consequence, even very rare phenotypes present in large repertoires can be selected and amplified from a background of phage carrying undesired phenotypes (Figure 1.13).

**Figure 1.13 Selection of binders from a phage display library.** A library of proteins displayed on the phage surface is used as input for the selection. Phage which display a binding protein (red and light blue) are captured on immobilized target molecules, and after unbound phage are washed off, bound phage can be eluted. The eluted phage population is then used to infect E.coli cells and can be propagated in bacterial cultures. The resulting phage population is enriched with target-specific binders and can be used for subsequent rounds of panning.

Phage particles are very stable, they remain infective when treated with acids, bases, denaturants and even proteases. These properties allow a variety of selective elution protocols and have been used for applications other than selection for binding, such as the selection of proteins with altered thermal stability (113,114), the selection of catalytically active enzymes (115-117), or aggregation resistant domain antibodies selected on phage by heat denaturation (19).
Filamentous phage particles, approximately 6 nm in diameter and 900 nm in length, are covered by several thousand copies of a small major coat protein (pVIII). Few copies of the minor coat proteins pIII and pVI are displayed at one extremity of the phage particle, while pVII and pIX are present at the other extremity. The minor coat protein pIII, the product of gene III, is displayed in 3-5 copies and mediates the adsorption of the phage to the bacterial pilus. Peptides and/or proteins have been displayed on phage as fusions with the coat proteins pIII (110,118), pVIII (119), pVII and pIX (120,121). Display of proteins encoded by a cDNA library as carboxy-terminal fusion with the minor coat protein pVI has also been reported (122). The first peptides and proteins were displayed on phage using phage vectors (essentially the phage genome with suitable cloning sites for pVIII or pIII fusions and an antibiotic resistance gene). Phage vectors carry all the genetic information necessary for the phage life cycle. Using phage vectors, most peptides and folded proteins can be displayed as pIII fusions, while only short peptides of 6-7 residues without cysteine give rise to functional phage when displayed as pVIII fusions (123).

Phagemids, a more popular vector for display, are plasmid vectors that carry only the gene III with appropriate cloning sites and a packaging signal as phage derived sequences. For the production of functional phage particles, phagemid containing bacteria have to be superinfected with helper phage particles, which contain the complete phage genome. Phagemid vectors encoding the polypeptide-pIII fusion are preferentially packaged into the phage particles, because helper phage used for superinfection (such as M13K07 or VCS-M13) have a defective origin of replication, which also serves as packaging signal. The resulting phage particles may incorporate either pIII derived from the helper phage or the polypeptide-pIII fusion, encoded by the phagemid. Depending on the type of phagemid, growth conditions used and the nature of the polypeptide fused to pIII, ratios of (polypeptide-pIII): pIII ranging between 1:5 and 1:10000 have been reported (114,118). Furthermore, the proteolytic cleavage of protein-pIII fusions has been described, contributing to further decreased levels of polypeptide-pIII fusions on the phage (111). Therefore, it can be concluded that phage particles, obtained by using a phage vector, are polyvalent (i.e. 3 to 5 identical polypeptides displayed on one phage particle), whereas the use of phagemids often delivers monovalent phage, which is instrumental for the isolation of high-affinity binders. If desired, performing superinfections with hyperphage, a phage which lacks most of the gene III in the genome, polyvalent phage can still be generated with phagemids (124).
Not only antibody fragments, but also other proteins and enzymes have been successfully displayed and selected on phage, e.g., domain antibodies (dAbs) (19), lipocalins (69), A-domains (29), fibronectin type III domains (22), Kunitz type domains (59), SH3 domains (78, 79) biotin ligase and trypsin (117). However, not every protein can be displayed on phage as fusion to pIII. Even proteins that fold well in bacteria are frequently displayed poorly on filamentous phage. The main reasons for inefficient display on phage are proteolytic cleavage of the pIII fusions and poor incorporation of the pIII fusion protein into the phage coat, because of a competition with the pIII protein of the helper phage. Low protein presentation on phage might be also caused by premature cytoplasmic folding, leading to inefficient translocation into the periplasm (125). In order to obtain a sufficient number of phage particles displaying the fusion protein, high titers of phage are needed, thus limiting the diversity of protein mutants that can be accessed for selection. A strategy to improve the display of proteins on phage was published by Jestin et al. (126). In this work, optimized leader peptides were selected, which are cloned upstream of pIII fusion proteins and serve as a signal for the export of pIII fusions to the bacterial periplasm. More recently, the group of Plückthun showed that redirecting the pIII fusion proteins to the cotranslational SRP pathway (instead of the post-translational Sec pathway) using an appropriate signal sequence results in an efficient display of otherwise display-refractory proteins (125).

An additional drawback of phage display is the need for transformation of bacterial cells by electroporation when cloning a library because the limiting factor in making large primary libraries is the efficiency of introduction of plasmid or phage DNA into bacteria. This limits the size of phage display libraries to maximum $10^{10} - 10^{11}$ individual library members (127).

1.4.4 Yeast display

Yeast display of antibody fragments has proven to be an efficient methodology for the directed evolution of single chain Fv (scFv) antibodies for increased affinity and thermal stability and, more recently, for the display and screening of a non-immune scFv and immune Fab libraries. Yeast display is compatible with fluorescent-activated cell sorting (FACS) and enables the screening of mid-sized libraries replacing the needs for selections (128). However, when working with large libraries, the huge number of cells cannot be handled
anymore by a flow cytometer. If this is the case, magnetic beads coated with the antigen are used for the first or two rounds of selection followed by screenings with FACS (129). A big advantage of yeast display is that affinities and further important parameters like expression or stability can be determined without the need for subcloning, expression and purification (130). In yeast display, recombinant proteins are fused to the Aga2 protein, which engages in a disulfide bonded complex with the Aga1 protein on the surface of *Saccharomyces cerevisiae* (128). By labeling surface displayed antibodies with fluorescent antigen, yeast cells can be screened by FACS for desired binding properties (Fig. 1.14).

![Image of the scFv Aga2 fusion protein surface expression system](image)

**Figure 1.14 The scFv Aga2 fusion protein surface expression system.** Aga1 is bound to a cell wall glucan and connected by a disulfide bond to Aga2. The protein to be displayed is cloned in frame with Aga2 protein. Using suitable antibodies, N-terminal hemagglutinin (HA) tag and C-terminal c-myc tag allow the monitoring of fusion protein expression. By addition of labeled antigen, yeast cells displaying antibody fragments binding the antigen can be isolated by affinity purification (e.g. biotinylated antigen) or FACS (e.g. fluorescently labeled antigen).

By means of a peptide tag which is appended to the scFv (hemagglutinin and myc tag in Fig. 1.14), a second fluorescent probe can be used to detect the amount of displayed antibodies. This enables the discrimination between good expression and bad expression clones, and eventually the affinity ranking of clones during screening (see also Fig. 1.15).
Enrichment of antigen-binding clones can be achieved by multiple (typically four in the case of antibody libraries) subsequent rounds of enrichment on a cell sorter. Sort gates are drawn such that the best 0.05-1% clones are collected, depending on the stage of enrichment. This includes that a threshold is set for scFv surface display (1st fluorophore) and for antigen binding (2nd fluorophore), and the best clones, i.e. clones with a high ‘antigen binding to surface display’-ratio above these two thresholds, are collected.

Yeast surface display combined with FACS allows to perform selections in solution and does not rely on immobilisation on a solid support which often hamper efficient selections due to avidity effects. Moreover, doing selections in solution allows the investigator to precisely control the antigen concentration and consequently establish conditions which allow to optimally distinguish between high-affinity and low-affinity antibody clones (131) and also to identify subtle affinity improvements (132).

![Flow cytometric analysis of yeast-displayed antibody fragments](image)

**Figure 1.15** Flow cytometric analysis of yeast-displayed antibody fragments in a population greatly enriched for antigen binding (129). Yeast cells were labeled with biotinylated antigen/streptavidin-phycoerythrin (detection of bound antigen), and anti-c myc/ anti-mouse-FITC
(monitoring of scFv display level). The subpopulation of yeast cells in the blue circle does not display scFv fragments. Despite good display of scFv, the subpopulation in the yellow circle does not bind to the antigen. The subpopulations in the red and in the green circles represent clones with high levels of scFv display (meaning good expression) and antigen binding. However, the subpopulation in the green circle represents binders with higher affinities than the red population.

Characterization of clones, such as determination of the dissociation constant ($K_D$), the kinetic dissociation rate ($k_{off}$) and stability analysis, which is usually a laborious and slow process, can be done with monoclonal antibody populations displayed on the yeast surface with high reliability, without the need for subcloning or purification of antibodies. Affinities are determined using equilibrium-based kinetic analysis by measuring the degree of binding over a wide range of antigen concentrations by FACS. The kinetic dissociation rate $k_{off}$ is determined by competition assays using an excess of unlabelled antigen as competitor (133).

There are several examples of antibodies with extremely high affinities, which have been affinity matured by yeast display. Boder et al. (130) used a scFv fragment called 4-4-20 recognizing the hapten fluorescein for affinity maturation. Mutagenesis and screening was repeated three times, resulting in affinity matured scFv antibodies with dissociation rates over four orders of magnitude slower than that of the parental scFv 4-4-20, and slower than the dissociation rate of the streptavidin-biotin complex.

However, yeast display has its limitations. First, FACS throughput is limited and therefore, big libraries (above $10^7$) are not suited for FACS screening. Large naïve yeast surface display antibody libraries have been used for subsequent rounds of panning using first magnetic bead capturing in order to reduce the complexity of clones, followed by FACS screening (129). Nevertheless, the strength of the methodology clearly resides in FACS. In cases where the antigen is not monovalent, strong avidity effects may come into play due to dense scFv display on the yeast cell wall, making yeast display inappropriate for the isolation of high-affinity antibodies. Moreover, yeast cells are less resistant to harsh experimental conditions if compared with filamentous phage.
1.4.5 Ribosome display

The screening and display methodologies mentioned so far all have in common that library size is limited to $\sim 10^{10}$ individual variants due to the initial transformation of living cells with library DNA. In addition, the cloning of libraries in the size of $10^9$ can consume a considerable amount of time and work. In order to circumvent this problem, selection techniques have been proposed which can take place fully in vitro. This not only opened the possibility to work with very large repertoires ($>10^{12}$ members), but also shortened significantly the time needed to generate sequence diversity, thereby allowing to repeatedly introduce new mutations after each round of selection.

Ribosome display was first described by Mattheakis and colleagues (134), who displayed short peptides on polysomes. Considerable improvements were needed to allow the display and selection of folded proteins (135), such as scFv antibody fragments with dissociation constants as low as $10^{-11}$ M (136). Proper folding in vitro is supported by introducing an unstructured spacer region to the C-terminal end of proteins. This peptide spacer fills the ribosomal tunnel and provides some extra flexibility, thus allowing the protein of interest to fold as an independent unit and bind to the target. Additionally, chaperones and protein disulfide isomerases (for proteins depending on disulfide bonds) are added to the translation reaction (137). In ribosome display, a DNA library encoding a repertoire of proteins is transcribed in vitro, the mRNA is purified and finally used for in vitro translation. As the mRNA lacks a stop codon, the ribosome stalls at the end of the mRNA, giving rise to a ternary complex of mRNA, ribosome and functional protein. Thus, the ribosome links the phenotype and genotype. The ribosomal complexes, which are stabilized by high concentrations of magnesium ions and low temperature (138), are directly used for selections on a ligand either immobilized on a surface or in solution.

The mRNA incorporated in the bound ribosomal complexes is eluted by addition of EDTA, purified, reverse-transcribed and amplified by PCR. During the PCR step, the T7 promoter and the Shine–Dalgarno sequence are reintroduced by appropriate primers. Therefore, the PCR product can be directly used for further selection cycles. Ribosome display is schematically depicted in Figure 1.16.
Figure 1.16 Schematic representation of a selection cycle of ribosome display. Linear DNA fragments coding for a protein library (here scFv variants) are transcribed \textit{in vitro} and purified before subsequent translation \textit{in vitro}. After having reached the end of the mRNA during translation, the ribosome is unable to dissociate from the mRNA because the stop codon is missing. The resulting ternary complex comprising the ribosome, mRNA and the nascent polypeptide can be stabilised by high concentrations of magnesium ions and low temperature, thereby creating a stable linkage between the mRNA (genotype) and the encoded polypeptide (phenotype). Ribosomes displaying a binding protein can be isolated by affinity selection on immobilised antigen, the selected mRNA molecules can be eluted, and the genetic information is amplified by reverse transcription and PCR, which allows to introduce further variability by error prone PCR.

Hancs and colleagues have used a large synthetic antibody library, HUCAL-1 (139), for the selection of antibodies specific to bovine insulin (140). Six rounds of selection were performed, and after each round, further diversity was introduced into the selected genes by error prone PCR. This procedure mimics to a certain degree the process of somatic hypermutation of antibodies \textit{in vivo}.

Ribosome display has been shown to work especially well for affinity maturation of scFv
fragments. So far, two studies have been reported in which a given antibody was evolved to higher affinity. In both cases, stringent off-rate selections combined with error-prone PCR were used. An antibody fragment specific to fluorescein with a dissociation constants of about 100 pM was generated by Jermutus and colleagues (138). Interestingly, the majority of the 4 to 11 mutated residues which were present in selected antibodies were found to be unlikely to contact the antigen. In a second study, the dissociation constant of a scFv fragment specific to a peptide deriving from the transcription factor GCN4 was improved from 40 to 5 pM (141). Libraries of antibody mutants were generated with error-prone PCR and DNA shuffling, and selected for decreased off-rates. Crystallographic analysis of the scFv in its antigen bound and free states showed that only a few mutations, which do not make direct contact to the antigen, lead to the affinity improvement. These results might suggest that the affinity optimization of very high affinity binders is achieved by modulating existing interactions via subtle changes in the framework rather than introducing new contacts.

In a recent publication, ribosome display was successfully used for the isolation of binders based on the ankyrin repeat scaffold (see chapter 1.2.6 and Ref (65)) which bound their target with nanomolar affinities.

Throughout the selection process, high Mg$^{2+}$ concentrations and low temperature are needed in order to maintain the integrity of the ternary ribosome-mRNA-polypeptide complexes, and therefore, selection conditions can only be varied to a certain extent, which might hamper the isolation of proteins that are intended to be stable under harsh conditions, e.g. high temperature or extremes of pH. Since mRNA is used as carrier of genetic information, care should be taken to avoid contamination with RNase molecules.

1.4.6 Covalent DNA display

Bertschinger and Neri recently proposed a technology called covalent DNA display (142) in which a library of linear DNA molecules is co-packaged with an *in vitro* transcription/translation mix in the compartments of a water-in-oil emulsion (143) (Figures
1.17 and 1.18). Experimental conditions are chosen so that, in most cases, one compartment contains one DNA molecule. A covalent linkage between DNA and the encoded polypeptide is achieved by using fusion proteins which contain the Hae III DNA-methyltransferase domain of Haemophilus aegypticus, an enzyme which is able to form a covalent bond with DNA fragments containing the sequence 5'-GGFC-3' (F = 5-fluoro-2'-deoxycytidine) (144). M.Hae III fusion proteins expressed in each compartment of the water-in-oil emulsion are extracted from the emulsion, and DNA molecules displaying a protein with the desired binding specificity are selected from the pool of DNA–protein fusions by affinity selection. The genetic information of the selected DNA–protein fusions is amplified by polymerase chain reaction (PCR) and can be used either for a further round of selection or for cloning and characterisation of the selected mutants.

Figure 1.17 Concept of the selection method. A library of linear DNA molecules is co-packaged with an in vitro transcription/translation mix into a water-in-oil emulsion (1). Ideally, one compartment contains one DNA molecule. The DNA molecules each code for a Hae III DNA-methyltransferase fusion protein and contain a mechanism-based inhibitor for the covalent cross-linking of the DNA-methyltransferase fusion proteins (see also Fig. 1.18). After in vitro expression
and formation of the DNA–protein complexes, the water phase is extracted from the emulsion and DNA molecules displaying a protein with desired binding properties are selected from the pool of DNA–protein fusions by affinity panning. The genetic information of selected DNA–protein fusions is amplified by PCR and can either be used for a further round of selection or for cloning and characterisation of the selected mutants.

Figure 1.18 Enlarged view of a water compartment of the water-in-oil emulsion. The DNA molecule is transcribed into mRNA, which is translated into a fusion protein consisting of two domains: the N-terminal Hae III DNA-methyltransferase (yellow) and a C-terminal potential binding domain (red). Owing to the catalytic activity of the Hae III DNA-methyltransferase, the fusion proteins form a covalent bond via the modified methylation target sequence 5'-GGFC-3' (F = FdC = 5-fluorodeoxycytidine) with their encoding DNA molecule, which is present in the same compartment of the water-in-oil emulsion.

Bertschinger et al. demonstrated successful enrichment of DNA molecules on the basis of the binding specificity of the protein they encode from an excess of DNA molecules coding for a protein with irrelevant binding specificity: mixtures of DNA templates coding either for M.Hae III-calcium (CaM) or M.Hae III-EDB were prepared in such a way that the ratio of M.Hae III-EDB / CaM molecules was ~1000. CaM-binding peptide was used to enrich DNA coding for M.Hae III–CaM from the DNA mixtures, resulting in enrichment factors in the range of $10^2$. 

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The technology was further optimized by the authors, showing that the covalent cross-linkage of the M.Hae III fusion proteins with their encoding DNA takes place within the water compartment and that the covalent bond is indeed necessary for the efficient recovery of DNA molecules coding for binding proteins. In addition, several steps of the selection protocol were optimized, such as emulsion preparation, elution of the selected DNA molecules and their subsequent PCR amplification. With the optimized protocols, we recently successfully isolated affinity matured Fyn SH3 derivatives binding to mouse serum albumin (MSA) (146).

Overall, covalent DNA display represents a useful addition to the protein engineer’s toolbox with the potential to deliver good-quality binding proteins for biomedical applications. However, so far only model selection and affinity maturation experiments have been performed. It remains to be seen, whether the technology is feasible for selections with naïve libraries of small globular proteins.
1.5 Serum albumin as a target protein

In this thesis, two different target proteins have been chosen, mouse serum albumin (MSA) and the extra-domain B (EDB) of fibronectin, a marker of angiogenesis. The latter one is described in the next chapter within the context of vascular tumor targeting.

Albumin (molecular mass ~ 67 kDa) is the most abundant protein in plasma, present at 50 mg/ml (600 µM), and has a half-life of 19 days in humans (147). It maintains plasma pH, contributes to colloidal blood pressure, functions as a carrier of many metabolites and fatty acids, and serves as a major drug transport protein in plasma. There are several major small molecule binding sites in albumin that have been described. Warfarin is known to bind at site I, benzodiazepines at site II and cardenolides and biliary acids at site III. In addition, there is an important metal ion binding site.

Because the kidney generally filters out molecules below 60 kDa, efforts to reduce clearance have focused on either increasing molecular size through protein fusions (e.g., fusion to albumin or to the Fc portion of an IgG), glycosylation, the addition of polyethylene glycol (PEG) or on albumin binding polypeptides (148).

The noncovalent association with albumin has been shown to extend the half-life of short-lived proteins. A recombinant fusion of the albumin binding domain from streptococcal protein G to human complement receptor type I increased its half-life 3-fold to 5 h in rats (149). In another example, when insulin was acylated with fatty acids to promote association with albumin, a protracted effect was observed when injected subcutaneously in pigs (150). Genentech recently engineered fusion proteins consisting of a Fab fragment of Herceptin® and different albumin binding peptides, each of them differing in their affinity to albumin, ranging from 0.04 to 2.5 µM (151). Reduced affinity for albumin correlated with a reduced-half-life and higher clearance rates. Since it has been observed that size and half-life of tumor targeting agents can have a dramatic effect on their ability to accumulate in the tumor (152), the fine-tuning of albumin binding affinities may improve tumor to organ ratios for these classes of ligands.
Beside pharmacokinetic aspects, albumin binding molecules can be used for bioseparation purposes, since the identification of biomarkers from serum or plasma is often hindered by a few proteins present at high concentrations, which may obscure less abundant proteins. Moreover, the identification of these biomarkers is hindered not only by their low absolute abundance (ng/mL serum or less) but also by the complex and heterogeneous nature of serum and plasma, where a modest number of proteins (such as albumin, IgG, IgA, haptoglobin, transferrin, a1-antitrypsin and a2-macroglobulin) are present at high concentrations (mg/mL), accounting for more than 85% of the total serum proteins (153). By probing a plasma sample over an affinity resin conjugated to an albumin binding domain, efficient removal of albumin can be achieved.

In this thesis, albumin served as target protein for model selections with libraries of the Fyn SH3 domain.
1.6 Vascular tumor targeting

1.6.1 Concept and definitions

The treatment of solid tumors with most chemotherapeutic drugs relies on the expectation that the drugs will preferentially kill rapidly dividing tumor cells, rather than normal cells. However, the lack of selectivity towards tumor cells leads to toxicities in normal tissues with enhanced proliferation rates, such as the bone marrow, gastrointestinal tract and hair follicles. As the high interstitial pressure and the irregular vasculature of the tumor impairs the accumulation of the active agents at the tumor site, the efficiency of conventional therapy is further decreased (154). Moreover, the activity of multidrug resistance proteins minimizes drug uptake and leads often to failure of the therapy (155). A promising approach to circumvent the hurdles of tumor therapy is the emerging field of vascular targeting.

Vascular tumor targeting aims at the rapid and selective shutdown or damage of the established tumor vasculature. Consequently, this strategy will lead to tumor cell death as the blood supply to these cells has been cut off. Potential advantages of this strategy over attacking tumor cells are:

- Occluding one blood vessel will trigger cell death of a large number of tumor cells which depend on it for supply of nutrients and oxygen

- One single vascular targeting agent could in principle be used to treat a wide range of solid tumors

- Endothelial cells and cells of the surrounding stroma are genetically more stable than tumor cells, so therapy resistance is less likely to occur. Moreover, these cells are usually easily accessible for any compound
A broader definition of vascular targeting, which is not limited to the thrombosis of neo-vasculature, may be “the targeted delivery of bioactive agents to new blood vessels”. The schematic shown in Fig. 1.19 illustrates the overall concept.

![Figure 1.19 Illustration of the concept of vascular targeting](image)

**Figure 1.19 Illustration of the concept of vascular targeting.** The targeted drug is delivered intravenously and homes to the tumor-induced antigen that might be either on the endothelial cell or in the perivascular space.

Vascular tumor targeting is only possible because the endothelium and the surrounding stroma in tumors differ from that in normal tissue; this fact has long been known but only recently have these differences begun to be characterized at the molecular level. Compared with the vasculature in normal tissue, the tumor vasculature is strikingly disorganized and tortuous (156-158). The flow of blood through the tumor capillaries is frequently sluggish, and at times might be stationary or even experience a reversal in the direction of flow (159,160). The microenvironment – including blood and the endothelium lining the vessels – is profoundly hypoxic (161). In this environment, the endothelial cell proliferates rapidly and contributes to active angiogenesis (162). The tumor is also nutrient starved, acidic and under oxidative stress (163,164); it has been shown that the endothelial cell responds transcriptionally to these stimuli and remodeling activities, giving rise to the production of new proteins either on their surface or in the surrounding extracellular matrix. Some of this specific markers can be used as targets for vascular targeting approaches.
1.6.2 Classes of vascular targets

*Extra-Domain B (EDB) of fibronectin*

Fibronectin is a large glycoprotein that is present in large amounts in the plasma and tissues. EDB is a 91-amino-acid type III homology domain that becomes inserted into the fibronectin molecule under tissue-remodelling conditions by a mechanism of alternative splicing at the level of the primary transcript (145). EDB is essentially undetectable in healthy adult individuals whereas EDB containing fibronectin is abundant in many aggressive solid tumors and displays either predominantly vascular or diffuse stromal patterns of expression, depending on the tumor type (165). Despite its very restricted expression, the function of EDB does not seem to be indispensable, as mice lacking the EDB exon develop normally (166). The EDB sequence is identical in mouse, rat, rabbit, dog, monkey and man. This feature facilitates animal experiments in immunocompetent syngeneic settings, but has, so far, prevented the isolation of anti-EDB antibodies using hybridoma technology, probably due to tolerance.

A few years ago, using phage display technology (167) and other technologies (107) our group in collaboration with the group of L. Zardi (Genova, Italy) succeeded to isolate a number of human monoclonal antibodies to EDB (168-170). These include the high-affinity human antibody L19, which has been shown to efficiently localize to tumor blood vessels in animal models (171-173) and in patients with cancer (174) following intravenous injection. A large number of therapeutic derivatives of the L19 antibody have been produced and tested in animals, including conjugates to fluorophores and photosensitizers (175,176), therapeutic radionuclides (177,178), liposomes (179), procoagulant agents (180), cytokines (181-185), enzymes (186) and other proteins (187,188). Importantly, the anti-EDB antibody L19, in homodimeric single-chain Fv format and labeled with iodine-123, has been studied in over 40 patients with cancer. The results obtained in the first 20 patients have recently been described (174) and confirm the ability of the antibody to localize to tumor masses exhibiting rapid growth. L19-interleukin 2 (L19-IL2), L19-tumor necrosis factor (L19-TNF) and Small-
Immuno-Protein [SIP]-L19-I\textsuperscript{131} arc three therapeutic derivatives of the L19 antibody which are currently in clinical development (189).

*Large Tenascin-C isoforms*

Tenascin-C, a polymorphic high molecular mass extracellular matrix glycoprotein exists in several isoforms which are generated as a result of different patterns of alternative splicing in the region between domains A1 and D (190). These large isoforms of tenascin-C, containing extra domains, have long been known to be tumor associated antigens. Though not completely absent in healthy tissues, they show a more restricted pattern of expression compared to the isoforms without extra domains (191). Especially the C domain of tenascin-C shows the most restricted expression pattern: while being undetectable in normal human tissues and only barely detectable in most carcinomas, it is extremely abundant in high grade astrocytoma (grade III and glioblastoma) and in lung cancer, particularly around vascular structures and proliferating cells (192,193).

A critical immunohistochemical analysis of the expression pattern of the different isoforms in various cancer types is needed to evaluate their potential as targets for biomolecular intervention. Radiolabeled derivatives of monoclonal antibodies to domains A1 and D of tenascin-C have been used for imaging and radioimmunotherapy in patients with cancer for over a decade (194-198). The pattern of staining of these antibodies varies between different tumors, the two extremes being a predominantly vascular and a diffuse stromal staining. Recently, Silacci et al. (193) and Brack et al. (199) reported about the isolation of antibody fragments specific for the domain C and the domain A1 of tenascin-C, with excellent quantitative biodistribution results in mouse xenograft U87 glioma models.

*Phosphatidyl Serine*

Phosphatidyl serine (PS) phospholipids are major components of the cell membrane which are preferentially found in the inner leaflet of the lipid bilayer. However, under conditions of cellular stress, apoptosis, platelet activation and endothelial-cell proliferation in tumors, PS becomes exposed on the outer leaflet of the cell membrane (200,201). Annexin V and
monoclonal antibodies have been used to confirm the surface accessibility of the phosphatidyl serine moiety on endothelial cells in vitro (for example, after treatment with hydrogen peroxide) and in vivo (200,202). The impressive microscopic analysis of tumor targeting performance by monoclonal antibodies to PS have not yet been complemented by a quantitative biodistribution analysis, but the 9D2 and 3G4 antibodies displayed potent antitumor activities even when used as naked antibody in rodent models of cancer. Recently it could be shown that the vascular targeting antibody, 3G4, significantly enhances the therapeutic efficacy of docetaxel against the growth and dissemination to the lungs of MDA-MB-435 human breast tumors in mice without concomitant increase in host toxicity (203). A chimeric version of 3G4 has been developed and is scheduled to enter clinical trials now.

Annexin A1

Annexins are cytosolic proteins that can associate with cell membranes in a calcium dependent manner. Some annexins may translocate the lipid bilayer to the external cell surface. Annexin A1 was recognized as a tumor endothelial target by Schnitzer and co-workers (204). A monoclonal antibody to this antigen has been used for the radioimmunoscintigraphic detection of solid tumor lesions in a rat model. Furthermore, relatively low radioactive doses of the same antibody labeled with iodine-125 have shown a therapeutic benefit in rats.

Integrins

During vascular remodeling and angiogenesis, endothelial cells show increased expression of several cell-surface molecules that potentiate cell invasion and proliferation. One such molecule is the integrin-αvβ3, which has a key role in endothelial cell survival during angiogenesis in vivo and which might serve as a target for therapeutic molecules, particularly those that require internalization in endothelial cells. Monoclonal antibodies to the integrin-αvβ3 have been shown to display anti-angiogenic activities and to preferentially stain tumor

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blood vessels. However, expression of integrin-α,β3 and other integrins has been reported in several normal tissues.

Efforts to influence the biology of blood vessels by gene delivery have been pursued using cationic nanoparticles coupled to an integrin-α,β3 targeting ligand for the selective gene delivery to angiogenic blood vessels, with a substantial therapeutic benefit in tumor bearing mice (205).

A high-affinity humanized anti-α,β3 antibody is in clinical development as an anti-angiogenic therapeutic (206), however, so far its tumor-targeting performance in patients with cancer has been unsatisfactory (207).

Anti-α,β3 antibodies have been shown to preferentially localize to tumor blood vessels using ex vivo fluorescence microscopy detection (208). Furthermore, a paramagnetic contrast agent targeted to the LM609 monoclonal antibody, which is specific to α,β3, has been described for the in vivo imaging of angiogenesis using magnetic resonance (209).

Vascular endothelial growth factors (VEGFs) and their receptors

VEGFs represent a class of proteins that mediate angiogenesis. The overexpression of VEGFs and their receptors in tumors (204,210) makes them attractive targets. Especially, the recent approval of the humanized anti-VEGF monoclonal antibody bevacizumab for first-line cancer treatment (211,212) has highlighted the contribution of VEGF-A to cancer progression. In addition, the selective localization of monoclonal antibodies to VEGF-A, VEGF receptor 2 and the VEGF-A/VEGF receptor 2 complex has been studied (213-217). The targeting efficiencies reported so far were modest, which possibly reflects kinetic limitations in the targeting of low or medium abundance antigens, even when they are readily accessible to binding agents injected into the bloodstream (183).

Prostate-specific membrane antigen (PSMA)

PSMA is a membrane glycoprotein with proteolytic activity. It is predominantly expressed in the prostate and serum concentrations are often elevated in patients with prostate cancer...
The interest for vascular targeting applications of PSMA has been stimulated by the observation that PSMA is over-expressed in the neo-vasculature of several solid tumors, whereas expression around blood vessels in normal tissues is limited to breast, kidney, duodenum and prostate. Specific antibody-derivatives to PSMA with alpha-emitting radionuclides in rodent cancer models have been reported. More recently, the radiolabeled antibody J591 has been used for tumor imaging and is currently being evaluated for therapeutic applications.

**Endoglin**

Endoglin (CD105) is a transforming growth factor-β (TGF-β) co-receptor that is overexpressed in tumor neovasculature. Even though immunohistochemical analysis has shown endoglin expression in normal adult tissues, monoclonal antibodies to endoglin have been used in biodistribution studies and for imaging in rodents and dogs.

**Nucleolin**

Ruoslahti and colleagues reported about a 31-amino acid synthetic peptide (F3) that accumulates in the nuclei of tumor endothelial cells and tumor cells. The cell surface protein that is recognized by F3 was then identified as nucleolin. The internalization of F3 takes place in a nucleolin-dependent manner, as antinucleolin antibodies that were previously injected inhibited F3 cellular uptake. The restricted expression pattern of nucleolin and its ability to internalize binding agents make it an attractive target for directed tumor therapy.

**Other possible targets**

Over the past few years, a range of antigens have been proposed as vascular targets for imaging and for the selective delivery of drugs to the tumor.
Peptides to the endothelial antigen CD13 have been isolated (233,234), and peptide fusions with tumor necrosis factor (TNF) have shown to markedly increase the therapeutic ratio of this biopharmaceutical (235), even at low doses (236).

Radiolabeled monoclonal antibodies against a CD44 isoform, which is a cell-surface receptor, have shown impressive performance in tumor targeting experiments in animal models (237).

Magic roundabout or ROBO4, a member of the roundabout receptor family, is a promising target as it is absent in adult tissues except at sites of angiogenesis (238). This gene product is believed to be intimately involved in the development of the vasculature. Biodistribution studies, e.g. with radiolabeled antibodies, will give more information about the suitability of ROBO-4 as a vascular tumor target.

Developmental endothelial locus-1 (Del-1) is a unique integrin ligand produced by endothelial cells, thus mediating autocrine signals. It has been reported to mediate angiogenesis in the adult (239). Biodistribution studies are needed for the evaluation of the suitability of Del-1 as a target.

Endothelial-specific protein disulfide isomerase (EndoPDI) is a hypoxia induced gene that is predominantly expressed by the endothelium (240). Functionally, EndoPDI has been shown to protect the endothelium from apoptosis during hypoxia-induced stress. Use of RNA interference has shown to that EndoPDI is required for the folding of endothelial-protective molecules, including endothelin-1 (EDN1), adrenomedullin (ADM) and CD105, which are produced when endothelial cells are exposed to hypoxic stress. Inhibition or ablation of EndoPDI should, therefore, make the hypoxic tumor endothelium more sensitive to apoptosis, particularly if it is concurrently stressed by a cytotoxic drug or radiotherapy.

1.6.3 Imaging applications of vascular tumor targeting

One of the most straightforward biomedical applications of ligands capable of selective localization around tumor vascular structures may reside in their use for the macroscopic in vivo imaging of sites of disease. The visualization of the homing of ligands to vascular structures imposes two main requirements: (i) the use of suitable chemical modification which makes the ligand visible, and, (ii) the choice of macromolecular targets which are
abundant enough to counterbalance the small contribution of vascular structures to the overall solid tumor mass.

In principle, four main chemical modification strategies can be envisaged for the molecular imaging of angiogenesis-related diseases:

- the radioactive labeling of ligands, for SPECT or PET imaging modalities (241-243)

- the use of near-infrared (NIR) fluorophores, which can be detected using epi-illumination (170,175), optical coherence tomography (244) or diffuse optical tomography methodologies (245,246)

- the use of ligands for the targeted delivery of microbubbles, to be used as contrast agents for sonographic imaging applications (247,248)

- the use of magnetic nanoparticles (249)

The ability of radioactively or fluorescently labeled ligands to image tumors in vivo is by now well established. For macroscopic imaging applications, NIR fluorescence imaging may be limited by the reduced light penetration of tissues (176,250). However, fluorescently labeled antibodies may facilitate the microscopic imaging of superficial lesions (e.g., using endoscopic methods (251)) and of transparent structures within the body (e.g., angiogenesis-related ocular disorders (176,252)). Furthermore, the use of NIR dyes may open novel angiographic imaging opportunities for solid tumors and other conditions, such as atherosclerosis (246,252).

It is much more debatable whether ligand-modified microbubbles and nanoparticles can efficiently extravasate and reach abluminal antigens in tumors and other diseases. Promising tumor imaging results with antibody-quantum dot conjugates suggest that even large particles can selectively accumulate at the tumor site. This contrasts with experience of our group, suggesting that highly charged antibody derivatives (183,187,188) and large antibody derivatives (253) may completely abrogate the tumor targeting ability of the parental antibody in vivo, while retaining unperturbed antigen binding properties.
1.6.4 Therapy applications of vascular tumor targeting

The following discussion is limited to derivatives of tumor targeting antibodies, a class of therapeutic agents for which a substantial amount of preclinical experimental data is now available. But, some of the considerations made in this section may be applicable for other classes of ligands, in particular for small globular proteins.

Figure 1.20 illustrates some of the main classes of antibody-derivatives which have been considered for tumor targeting applications. Some of these strategies (e.g., antibody-photosensitizer and antibody-pro-coagulant conjugates) appear to be ideally suited for vascular targeting applications, since they may lead to intravascular blood coagulation, causing an avalanche of tumor cell death (254,255).

Figure 1.20 Antibody derivatives which could be considered either for tumor imaging or therapy.

Many of the antibody functionalization strategies depicted in Figure 1.20 have been applied both to full immunoglobulin and to antibody fragments. In most cases, smaller antibody fragments are preferred because of easier expression, rapid blood clearance and lack of Fc
(which avoids the undesired targeting of bioactive moieties to cells bearing Fc receptors). However, some therapeutic strategies such as antibody-drug conjugates, may benefit from the long blood circulation times of antibodies in the IgG format.

Antibody fragments have successfully been coupled to cytokines (181-185), chemokines (256), fluorophores and photosensitizers (175,176,255), drugs (257), pro-coagulant factors (254), enzymes for pro-drug activation (258) radionuclides (259) and liposomes (260), but also to more exotic functional moieties such as uranium-loaded ferritin for neutron capture therapy (261).

Figure 1.21a shows the selective accumulation at the tumor site of the SIP-L19 antibody labeled with the infrared fluorophore Cy7. Figure 1.21b illustrates a striking therapeutic effect, observed in orthotopic animal models of hepatocellular carcinoma, using the fusion protein L19-IL2. As mentioned above, the vascular targeting properties of the L19 antibody, specific to the EDB domain of fibronectin, can be used to dramatically improve the tumor accumulation of the pro-inflammatory cytokine IL2, thus enhancing the therapeutic index of this anti-cancer biopharmaceutical.

Most of the studies performed with antibody derivatives suggest that the tumor targeting properties of judiciously chosen antibodies can substantially potentiate the therapeutic action of the bioactive moiety chosen for antibody coupling.
Figure 1.21 (a) Selective accumulation of L19 in the tumor. Twenty four hours near-infrared image of 129SvEv mouse subcutaneously grafted with F9 teratocarcinoma, i.v. injected with SIP(L19), labeled with the infrared fluorophore Cy7. The tumor is indicated by an arrow. (b) The therapeutic efficacy of L19-IL2 in the orthotopic HuH7 mouse model for hepatocellular carcinoma. HuH7 tumors (tumor volume: 70-100 mm³ pretreatment) were treated with two treatment cycles consisting of 5 consecutive daily i.v. bolus injections of L19-IL2 or PROLEUKIN (free IL2; two dose levels for each drug) followed by two drug-free days. After the second treatment cycle, the animals were sacrificed and tumor volumes were measured. Compared with the non-targeted IL2 (proleukin), L19-IL2 showed a superior efficacy.
1.7 Aim of this thesis

The aim of my PhD thesis was the development of technologies for the isolation of single domain binders based on the Fyn SH3 scaffold, with a special interest in the isolation of binders to mouse serum albumin (MSA) and the extra-domain B of fibronectin (EDB).

The creation of proteins that have new binding properties is an important goal in protein engineering. Beside giving new insights into processes of molecular recognition, such binders also have potential commercial applications, as therapeutic agents, diagnostic reagents or affinity ligands (10).

In biotechnology and biomedical research, antibodies and fragments thereof are currently the most widely used specific, high-affinity binding molecules; they exhibit a favorable pharmacokinetic profile and can be generated against essentially any target either by immunization or by using natural or rationally designed antibody libraries in vitro (36). However, antibodies suffer from certain drawbacks, as for example relatively low expression yields, requirement for an expensive mammalian cell production system, dependence on disulfide bonds for stability and, in addition, some antibody fragments tend to aggregate (12).

There is a clear need for the creation of an alternative protein scaffold, that may lead to the same affinity and specificity as antibodies without any of the above mentioned drawbacks. An appropriate scaffold would be human, devoid of cysteine residues, well expressed in bacteria or yeast and would have good biophysical properties (solubility, stability). As previously discussed in more detail, the Fyn SH3 domain scaffold fulfills important criteria for being an optimal scaffold candidate.

I have constructed libraries of mutants of the Fyn SH3 domain and performed selection experiments with mouse serum albumin (MSA) and EDB, a marker of angiogenesis (145). The characterization of the EDB-binding Fyn SH3 derivatives in vitro and in vivo has been an important goal of the thesis. Moreover, the complete identity of Fyn SH3 in mouse, rat, gibbon monkey and man has facilitated the demonstration of a low immunogenic potential of Fyn SH3 mutants in immunocompetent mice.
Fyn SH3 derived EDB-binders may be considered as useful molecules for the targeted delivery of bioactive agents to the tumor neo-vasculature in full analogy to derivatives of the L19 antibody, which are currently being investigated in clinical trials (189). In addition, the single-pot library of Fyn SH3 mutants described in this thesis may represent a rich source of target protein binding specificities, thus, it may provide useful reagents for many biochemical and biomedical applications as an alternative to more conventional IgG-based immunochemical technologies.
2 Results

2.1 Expression of Fyn SH3 wild-type and Fyn SH3 mutants

2.1.1 Cloning and expression of Fyn SH3 wild-type

The gene encoding the Fyn SH3 wild-type domain [amino acid residues 83-145, numbering according to the Fyn kinase sequence reported by Refs (100,101)] was amplified from a human cDNA library and cloned into the bacterial expression vector pQE-12, appending a C-terminal His tag. The 8.6 kDa recombinant protein was expressed in *E. coli* and purified from cleared lysate by immobilised metal affinity chromatography (IMAC) using Ni-NTA resin. The quality of the protein was assessed by SDS-PAGE. Expression yields were ~ 60 mg protein per liter of bacterial culture in shake flasks (Fig. 2.1).

![Diagram of vector pQE-12 and SDS PAGE](image)

**Figure 2.1 Cloning of Fyn SH3 wild-type.** (a) Schematic map of vector pQE-12, appending a hexahistidine tag to the C-terminus of the inserted protein. (b) SDS PAGE of the purified Fyn SH3 domain. The protein runs at the expected size of 8.6 kDa.
2.1.2 Test library designs and characterization

In order to introduce novel binding specificities into the Fyn SH3 domain, amino acid residues in the RT- and n-Src-loop were randomized (Fig. 2.2).

**Figure 2.2 Fyn SH3 wt structure, amino acid sequence and library cloning strategy.** (a), left structure of the Fyn SH3 domain (PDB code 1M27) prepared with the software Pymol, www.pymol.org. The RT loop is in green, and the n-Src loop in red. right DNA and amino acid sequence of the Fyn SH3 domain. The loop regions are also indicated by the corresponding colors. (b) Library cloning strategy: Mutations were introduced either in the RT (lib 1) or in the n-Src (lib 2) or in both loop regions (lib 3) by PCR using partially degenerate primers. PCR fragments were then assembled by PCR and cloned into the pQE-12 vector.
Since it was not clear \textit{a priori}, whether mutations in these regions of the wild type domain would have an influence on expression, three small test libraries of Fyn SH3 mutants were constructed by combinatorial mutagenesis, using the Fyn SH3 wt gene as template. In the first library, 6 amino acids of the RT loop were randomized (residues 94-99), in the second library the n-Src loop was extended from 4 to 6 randomized residues (residues 113-116, plus 2 residues of extension), and in the third library both loops were randomized simultaneously. Figure 2.2 shows the structure and sequence of Fyn SH3 wt, the corresponding loops and the library construction strategies.

Individual clone members of the libraries were randomly picked and grown in a 96-well plate. After induction of protein expression, a dot blot experiment with the lysates revealed that about 60\% of the selected clones of library 1, ~ 90\% of library 2 and 60\% of library 3 expressed a detectable amount of soluble Fyn SH3 mutants (Fig. 2.3). These levels of functional expression were considered as good enough for phage library constructions.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{dot_blot_experiments.png}
\caption{Dot blot experiments of the test libraries. Dot blot analysis of induced lysates of individual clone members. Fyn SH3 mutants were detected with anti-His-HRP antibody conjugate. (a) About 60\% of the clones of library 1 (RT loop randomized), (b) 90\% of the clones of library 2 (n-Src loop randomized), and (c) ~ 65\% of the clones of library 3 (simultaneous randomization of RT and n-Src loops) expressed a detectable amount of soluble protein. The positive control represents Fyn SH3 wt, the negative control bacterial growth medium (2xYT).}
\end{figure}
2.2 Phage library design, cloning and characterization

The previous section showed that at least ~ 60% of mutants with altered RT and \( n \)-Src loops expressed a detectable amount of soluble protein. In a first attempt, we were interested in creating a phage display library of Fyn SH3 variants with 6 randomized residues in the RT loop (postions 94-99, Fig 2.4a), since Saksela and colleagues reported that the RT loop of the Hck SH3 domain contributes to target binding (79). After PCR amplification of the randomized gene fragment of library 1 (see previous section) and thereby introducing appropriate restriction sites (Fig 2.4b), a total of 3.3 \( \mu \)g of insert was ligated into 17 \( \mu \)g of double-digested phagemid vector pHEN1 and electroporated into freshly prepared electrocompetent \( E.coli \) TG1 cells, resulting in a library containing \( 1.2 \times 10^7 \) individual clones (theoretical library size of \( 6.4 \times 10^7 \)).

![Figure 2.4 Visualization of the Fyn SH3 wt RT loop and library cloning strategy. (a) Structure of Fyn SH3 wt domain (1M27); the 6 randomized amino acid residues of the RT-loop-library are shown in green (positions 94-99). (b) Library cloning strategy. The randomized fragments of the test library 1 were amplified by PCR and cloned into the pHEN1 vector (262).](image)

The quality and functionality of the library were assessed by PCR colony screening, dot blot, Western blot and DNA sequencing. PCR colony screening showed that 77% of randomly picked clones contained an insert of the correct size. As expected from the previous test-libraries, the dot blot analysis of this newly created library revealed that ~ 62% of the analyzed library members expressed soluble Fyn SH3 mutants (Fyn SH3m). The efficient
The display of Fyn SH3m-pIII fusion proteins on the surface of filamentous phage is an absolute prerequisite for performing phage display selections. The performance of the display of Fyn SH3m-pIII fusion proteins was evaluated by Western blot, using an anti-pIII antibody as primary detecting reagent. From the ratio of intensities of the bands corresponding to pIII or Fyn SH3m-pIII fusions (Fig 2.5), and considering that 3-5 copies of pIII are present at the tip of the phage, we estimated that approximately 1:4 to 1:7 phage particles display the Fyn SH3m fusion protein on its surface. This level of display is very high and comparable to recently described antibody scFv libraries (118).

Figure 2.5 Western blot analysis to evaluate the efficiency of the display of the Fyn SH3 mutants (Fyn SH3m)-pIII fusion protein on the surface of the phage particle. Different amounts of purified phage were analyzed: lane 1, 4\( \times 10^9 \) transforming units (t.u.); lane 2, 1\( \times 10^9 \) t.u.; lane 3, 2\( \times 10^9 \) t.u.; lane 4 1\( \times 10^9 \) t.u. The protein pIII and the Fyn SH3m-pIII fusion protein were detected with a monoclonal anti pIII antibody.
Fifteen randomly picked clones were sequenced, revealing that all amino acid sequences in the RT loop region were diverse; only one clone was not in frame due to a single nucleotide insertion (Table 2.1).

Table 2.1 Amino acid sequences of the loop regions of randomly selected FynSH3 mutants (RT-loop library). Single amino acid codes are used according to standard IUPAC nomenclature. Numbering is according to the Fyn kinase sequence reported by Semba et al. (101) and Kawakami et al. (100). For the sake of completeness, the sequence of the n-Src loop is also shown.

<table>
<thead>
<tr>
<th>Clone</th>
<th>sequence RT-loop 94-99</th>
<th>sequence n-Src-loop 113-116</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fyn SH3 wt</td>
<td>E A R T E D</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT 1</td>
<td>S P M H I L</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT 2</td>
<td>G Q L M L T</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT 3</td>
<td>A L T S G P</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT 4</td>
<td>C R P A K P</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT 5</td>
<td>N N A A N K</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT 6</td>
<td>S Q R R G A</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT 7</td>
<td>K Q L P E V</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT 8</td>
<td>L V E L H S</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT 9</td>
<td>Q P P Q S A</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT10</td>
<td>H L M D S P</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT11</td>
<td>M S F L P P</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT12</td>
<td>R H L K V S</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT13</td>
<td>K S W V P T</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT14*</td>
<td>N H K M Q A</td>
<td>N S S E</td>
</tr>
<tr>
<td>RRT15</td>
<td>V R Y C V L</td>
<td>N S S E</td>
</tr>
</tbody>
</table>

*single nucleotide insertion (frame shift).
2.3 Generation of Fyn SH3 derived proteins binding to mouse serum albumin (MSA)

2.3.1 Phage display selections of Fyn SH3 mutants binding to MSA

In order to evaluate the above described phage library (RT-loop randomized), commercially available mouse serum albumin (MSA) coated on immunotubes was used as a model target protein for phage display selections. After three rounds of panning, 13 clones out of 59 (22%) gave a positive signal in phage ELISA (Fig. 2.6). Sequence analysis of all 13 clones revealed that 2 unique sequences were enriched (termed G4 and C4) (Table 2.2). In both sequences, 4 amino acid residues in the artificial RT-loop were conserved, suggesting that they are mainly involved in the binding. Interestingly, the two glycine residues were encoded by different codons (data not shown), indicating a selection pressure for the observed sequence.

![Absorbance vs Clones](image)

*Figure 2.6 Phage ELISA after the third round of panning.* Phage ELISA signals of 59 clones are shown, 13 clones gave a positive signal.
Table 2.2 Isolated unique binding sequences after 3 rounds of panning against MSA. The amino acid sequences of the loop regions are shown (for the sake of completeness, the sequence of the n-Src loop is also shown). Interestingly, 4 amino acids are conserved in both sequences.

<table>
<thead>
<tr>
<th>Clone</th>
<th>sequence RT-loop 94-99</th>
<th>sequence n-Src-loop 113-116</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fyn SH3 wt</td>
<td>E A R T E D</td>
<td>N S S E</td>
</tr>
<tr>
<td>G4</td>
<td>Y S A G F G</td>
<td>N S S E</td>
</tr>
<tr>
<td>C4</td>
<td>R T A G F G</td>
<td>N S S E</td>
</tr>
</tbody>
</table>

2.3.2 Affinity maturation of G4 using phage display

As shown in Figure 2.6, the binding of Fyn SH3 variants to MSA could be detected by phage ELISA, but ELISA experiments performed with the soluble protein G4 (after subcloning the sequence into the cytosolic expression vector pQE-12) failed to yield signals even at concentrations > 150 μM (shown below). For this reason, we aimed at affinity maturing the clone G4, by randomizing amino acid residues in the vicinity of the RT-loop. Starting from the sequence coding for G4, a sublibrary was created (Sublib1) in which the n-Src loop was extended from 4 to 6 randomized amino acid residues (see Fig. 2.7a, (positions 113-116, plus 2 residues of extension)), since the expression tests (chapter 2.1.2) showed that the n-Src loop allows for randomizations. In addition, a second sublibrary was created (Sublib2) based on the G4 sequence, in which the n-Src loop was randomized in 4 positions (113-116), and 2 other residues of the Fyn SH3 binding surface (Trp119 and Tyr132) (Fig. 2.7b). These two residues were shown in literature to be also involved in protein-protein interactions (see Ref (94)). As before, the randomizations were introduced by PCR using partially degenerate primers. The resulting fragments were assembled by PCR, amplified, and cloned into the phagemid vector pHEN1. The cloning strategy for the construction of the affinity maturation libraries is outlined in Figure 2.7c. Two and a half μg insert and 13.5μg vector per sublibrary were ligated and electroporated into freshly prepared electrocompetent TG1 bacteria. The obtained library sizes for Sublib1 and Sublib2 were 2.0 x 10^7 and 4.0 x 10^7, respectively (theoretical size: 6.4 x 10^7). The quality and functionality of the libraries were assessed by
PCR colony screening and dot blot. PCR colony screening showed that all of 10 randomly picked clones (for each sublibrary) contained an insert of the correct size and a dot blot analysis of both libraries revealed that ~62% of the analyzed library members expressed soluble Fyn SH3 mutants (Fyn SH3m). Both affinity maturation libraries were subjected separately to one round of phage panning, using MSA as target protein, that was previously coated on immunotubes. Additionally, both sublibraries were mixed and submitted for panning in a third immunotube. Elution conditions of phage in this immunotube were chosen to favor binders with a long $k_{off}$ value, by adding a solution containing soluble MSA as a competitor after the washing step. Subsequently, the solution containing MSA and binders with a fast $k_{off}$ value was discarded and bound phage were eluted. After one round of affinity selection, clones from all 3 affinity selection tubes were screened for binding by phage ELISA. Several clones gave at least a twofold higher signal than the signal of the parental clone G4 (summarized in Figure 2.8), suggesting that the affinity maturation strategy was successful. In addition, it was observed that Sublib1 worked better than Sublib2, since only one clone isolated from Sublib2 gave a twofold higher signal than G4. Moreover, the ELISA signals of the clones isolated from the affinity selection round with soluble competing MSA were much higher as compared to the other two standard elution conditions, with single colonies having a nine-fold higher value of the signal.
Figure 2.7 Affinity maturation libraries of G4. (a) The structure of Fyn SH3 is shown with its randomized and extended region of the n-Src loop in red, giving raise to Sublib 1. The G4-coding sequence in the RT-loop is kept constant. (b) The same as (a), except that in Sublib 2 the randomized positions comprise the n-Src loop (not extended) and, in addition, the positions Trp119 and Tyr132 are randomized (all in red) (c) Library cloning strategy: (left) Mutations were introduced either in the n-Src loop (Sublib1) or (right) in the n-Src and at the positions Trp119 and Tyr132 (Sublib2) by PCR using partially degenerate primers. PCR fragments were then assembled by PCR and cloned into the pHEN1 vector.
Figure 2.8 Phage ELISA screening after one round of panning with affinity maturation libraries Sublib1 and Sublib2. (a) Phage ELISA signals of different clones from the affinity maturation Sublib1 are shown. Nine clones gave at least a twofold higher signal than the parental clone G4. (b) The same is shown for Sublib2, revealing that only one clone was significantly better binding to MSA. The signal produced by G4 is also indicated. (c) Phage ELISA signals from the panning method favoring longer $k_{off}$ values are shown. In all three figures "-" means negative control (PBS was added to the wells instead of phage).

2.3.3 Characterization of affinity matured MSA binders isolated by phage display

Six affinity matured Fyn SH3 variants (B1, E11, C6, C3, H9 and D12), which gave at least a twofold higher signal than G4 in phage ELISA, were sequenced (Table 2.3) and subcloned into the cytosolic expression vector pQE-12. Five out of 6 Fyn SH3 variants had a histidine residue in position 3, other conserved residues were a proline in position 4 and a threonine in position 6. The clones were expressed and purified by affinity chromatography. The purity of the proteins was assessed by SDS-PAGE (Fig. 2.9). Expression yields varied between 0.4 and 42.2 mg/L of bacterial culture in shake flasks (Table 2.3). Using the purified proteins, the binding specificities of the clones was examined by ELISA. Mouse serum albumin (MSA), human serum albumin (HSA), rat serum albumin (RSA), bovine serum albumin (BSA), ovalbumin (OVA) were coated on plastic, and after addition of the purified Fyn SH3 mutants,
bound proteins were detected with anti-His tag HRP antibody conjugate (Fig. 2.10). All affinity matured clones clearly bound to MSA. Two of them (B1 and E11) were very specific and did not cross-react with any albumin of other species, whereas C3 bound to all tested albumins. Binding to MSA of the parental clone G4 used for affinity maturation was not detected, however, a weak signal was observed towards OVA. Fyn SH3 wt did not bind to any of the tested target proteins.

Figure 2.9 SDS-PAGE of Fyn SH3 variants binding to MSA.

Figure 2.10 Specificity ELISA of Fyn SH3 variants isolated after affinity selections.
Table 2.3 Sequences of MSA binding clones after affinity maturation selections. The amino acid sequences of the loop regions are shown, including Fyn SH3 wt and G4. Expression yields are also indicated (expression under non-optimized conditions in shake flasks).

<table>
<thead>
<tr>
<th>Clone</th>
<th>RT-loop 94-99</th>
<th>n-Src-loop 113-116</th>
<th>Expression yield (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fyn SH3 wt</td>
<td>E A R T E D</td>
<td>N S S E - -</td>
<td>60.0</td>
</tr>
<tr>
<td>G4 (parental)</td>
<td>Y S A G F G</td>
<td>N S S E - -</td>
<td>58.0</td>
</tr>
<tr>
<td>B1</td>
<td>Y S A G F G</td>
<td>R P H P F T</td>
<td>0.4</td>
</tr>
<tr>
<td>E11</td>
<td>Y S A G F G</td>
<td>S I H P N T</td>
<td>12.3</td>
</tr>
<tr>
<td>C6</td>
<td>Y S A G F G</td>
<td>R P H R L A</td>
<td>5.3</td>
</tr>
<tr>
<td>C3</td>
<td>Y S A G F G</td>
<td>C K H P M S</td>
<td>18.4</td>
</tr>
<tr>
<td>H9</td>
<td>Y S A G F G</td>
<td>N V H Y K T</td>
<td>6.8</td>
</tr>
<tr>
<td>D12</td>
<td>Y S A G F G</td>
<td>G K S P L T</td>
<td>42.2</td>
</tr>
</tbody>
</table>

The binding properties of the only specific binder with reasonable expression yields (E11) was further characterized by real-time interaction analysis on a MSA coated BIAcore chip revealing an apparent dissociation constant ($K_d$) of 87 nM. The sensogram is shown in Fig. 2.11.

![Figure 2.11 BIAcore sensogram of the MSA binding clone E11. E11 was injected at two different concentrations on a MSA coated chip.](image-url)
2.4 Construction of a novel Fyn SH3 phage library

2.4.1 Phage library design, cloning and characterization

The results described in the previous chapters showed that the isolation of Fyn SH3 variants binding to MSA with an apparent K_d of ~100 nM was possible. However, these results were achieved from a "RT-loop-library" via an affinity maturation step, resulting in Fyn SH3 variants with mutated residues in the RT- and n-Src loops. In order to create a new, large and highly diverse library of Fyn SH3 mutants, residues in the RT- and n-Src loops of the molecule were combinatorially mutated simultaneously (Fig. 2.12a). For library construction (Fig. 2.12b), the randomized gene fragment of library 3 (chapter 2.1.2) was amplified by PCR, thereby introducing appropriate restriction sites. A total of 10 µg of insert was ligated into 75 µg of double digested phagemid vector pHEN1 and electroporated into freshly prepared electrocompetent E.coli TG1 cells, resulting in a library containing a total of 1.2 x 10^9 individual clones.

Figure 2.12 Fyn SH3 wt - library cloning strategy. (a) Structure of the FynSH3 domain (pdb file 1M27 prepared with the software "Pymol" (www.pymol.org)). The RT-loop is colored in green and the Src-loop in red. (b) Library cloning strategy: mutations were introduced in the RT- and n-src loop regions by PCR using partially degenerate primers. PCR fragments were then assembled by PCR and cloned into the pHEN1 vector (262).
The quality and the functionality of the library were assessed by PCR colony screening, dot blot and DNA sequencing. PCR colony screening showed that 30/30 randomly picked clones from the library contained an insert of the correct size of approximately 400 bp (Fig. 2.13a). A dot blot experiment revealed that more than 70% of the analyzed library clones expressed a detectable amount of soluble Fyn SH3 mutants (Fig. 2.13b). Sequence analysis of 8 clones showed that all inserts were in frame and that mutations were introduced in the correct region of the sequence (Table 2.4).
Figure 2.13 Characterization of the library. (a) PCR colony screening of 30 clones. All the tested clones showed an insert with the correct size of approximately 400 bp. As control, a BirA insert (1200 bp) of a pHEN1 vector was amplified. (b) Dot Blot analysis of 58 induced supernatants of individual library clones. The soluble Fyn SH3 mutants were detected with the anti-myc-HRP antibody conjugate. More than 70% of the clones express a detectable amount of soluble protein. The positive control represents Fyn SH3 wt.

Table 2.4 Amino acid sequences in the loop regions of randomly selected sequences of FynSH3 mutants (RT / n-Src-loop library).

<table>
<thead>
<tr>
<th>Clone</th>
<th>sequence RT-loop 94-99</th>
<th>sequence n-Src-loop 113-116</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fyn SH3 wt</td>
<td>E A R T E D</td>
<td>N S S E - -</td>
</tr>
<tr>
<td>clone 1</td>
<td>T L Q L H C</td>
<td>T T Y A Q I</td>
</tr>
<tr>
<td>clone 2</td>
<td>W D S Y D A</td>
<td>P T K Y S S</td>
</tr>
<tr>
<td>clone 3</td>
<td>Y K P V T C</td>
<td>P F S Q V F</td>
</tr>
<tr>
<td>clone 4</td>
<td>W R L R G D</td>
<td>L M S C N T</td>
</tr>
<tr>
<td>clone 5</td>
<td>A R R G H T</td>
<td>E M G H A W</td>
</tr>
<tr>
<td>clone 6</td>
<td>C L D I Q H</td>
<td>D S P S A N</td>
</tr>
<tr>
<td>clone 7</td>
<td>L R K E L Q</td>
<td>T E S D K L</td>
</tr>
<tr>
<td>clone 8</td>
<td>F Q V S A V</td>
<td>A L G T Y Q</td>
</tr>
</tbody>
</table>
2.5 Generation of Fyn SH3 derived proteins binding to the extra-domain-B of fibronectin (EDB)

2.5.1 Isolation and in vitro characterization of Fyn SH3 mutants specific to the EDB of fibronectin, a marker of angiogenesis

Specific Fyn SH3 binding proteins to the extra-domain B (EDB) of fibronectin were isolated from the novel Fyn SH3 phage display library described in chapter 2.4. The target protein was biotinylated using sulfo-NHS-SS-biotin as biotinylation reagent prior to selections. The use of biotinylated target protein increases the probability that the domain retains its native conformation upon immobilisation on streptavidin-coated wells, compared to unspecific absorption on plastic. Three rounds of panning with EDB as target and with about $10^{12}$ phage particles were performed. The phage titers after each round were $7 \times 10^6$ (first round), $2 \times 10^6$ (second round) and $1 \times 10^7$ (third round). After the third round of panning, two unique clones were enriched, named B11 and D3, giving a positive signal in a phage ELISA screening experiment. The amino acid sequences of the loop regions are shown in Table 2.5. Both clones were further characterized and subcloned into a cytosolic expression vector, expressed and purified (Fig. 2.14a). Both variants gave a strong binding signal (Fig. 2.14b) in a soluble ELISA experiment, using the purified proteins as primary binding reagents.

![Figure 2.14 SDS PAGE and ELISA of Fyn SH3 binding proteins to EDB. (a) SDS-PAGE analysis of Fyn SH3 wt, D3 and B11 is shown. (b) Using the purified proteins, a soluble ELISA was performed against biotinylated EDB (without His tag) with anti-His HRP immunonjugate as secondary binding reagent. (MPBS = no antigen, wells were only blocked with milk, PBS)](image)

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D3 was chosen for further characterization and cloned as a genetically fused homo-dimer in order to increase the apparent affinity by avidity effects. The cloning strategy is depicted in Fig. 2.15. The expression yields of all constructs ranged between 7 and 94 mg/L of bacterial culture, under non-optimized conditions in shake flasks (Table 2.5).

**Figure 2.15 Cloning strategy for D3 dimer.** The gene coding for D3 was used for two independent PCRs. The fragments were assembled by PCR, yielding a D3 dimer with BamHI/HindIII restriction sites with a 14 amino acid linker (GGGGSGGGGSGGGG) between the two domains and a Myc and hexa-His tag at the C terminal-end of the protein.

**Table 2.5 Sequences of EDB binding clones.** The amino acid sequences of the loop regions are shown, including Fyn SH3 wt. Expression yields are also indicated (expression under non-optimized conditions in shake flasks).

<table>
<thead>
<tr>
<th>Clone</th>
<th>RT-loop</th>
<th>n-Src-loop</th>
<th>Expression yield (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fyn SH3 wt</td>
<td>EARTED</td>
<td>NSSEE -</td>
<td>60</td>
</tr>
<tr>
<td>D3</td>
<td>HAGSAG</td>
<td>KFGRGK</td>
<td>50</td>
</tr>
<tr>
<td>B11</td>
<td>RAYHST</td>
<td>RFGRFK</td>
<td>7</td>
</tr>
<tr>
<td>D3 dimer</td>
<td>as monomer</td>
<td>as monomer</td>
<td>94</td>
</tr>
</tbody>
</table>
The purified constructs were analyzed by size exclusion chromatography (Fig. 2.16c). The major peak showed a delayed elution time for D3 monomer compared to Fyn SH3 wt. Also, the D3 dimer eluted later than expected, thus suggesting a smaller size. Therefore, to ensure the correct molecular mass of the Fyn SH3 mutants, the chromatographic fractions under the peak were submitted to SDS-PAGE (Fig. 2.16a, lanes 3 and 5), confirming the identity of the protein in the chromatographic peak. In addition, the oligomeric state of D3 mutants (monomer and dimer) was determined by dynamic light scattering, using the Fyn SH3 wt domains as reference (Fyn SH3 wt dimer was cloned and expressed the same way as described for D3 dimer). As it can be seen in Figure 2.17, the Fyn SH3 variants have the same size as the wild-type proteins, confirming that the mutants are present in a monomeric form, like the wild-type proteins.

In order to determine the specificity of D3, an ELISA experiment was performed using other structurally related fibronectin type III domains and MSA as target proteins. MSA was commercially available, the other domains were available in our lab as His-tagged proteins. Since D3 monomer also carries a His-tag, the experiment was performed with D3 dimer, which has a His- and a Myc-tag. Binding was detected with anti-Myc HRP antibody conjugate. D3 dimer bound EDB in a highly specific manner and did not cross-react with any of the 5 other structurally related fibronectin type III homology repeats of tenascin-C, nor to mouse serum albumin (Fig. 2.16b).

The binding properties of both D3 constructs were analyzed by real-time interaction analysis on a BIAcore chip coated with biotinylated EDB (Fig. 2.16d), revealing a dissociation constant \(K_D\) of \(8.5 \times 10^{-8}\) M for the monomer and an apparent \(K_D\) of \(4.5 \times 10^{-9}\) M for the dimer, at the antigen surface density used. In order to confirm the ability of D3 monomer to specifically recognize EDB in the context of the adjacent fibronectin domains, we performed BIAcore studies probing the biomolecular interaction of D3 with the recombinant EDB-containing fibronectin fragment 7B89 (263) (Fig. 2.16e). This analysis revealed a \(K_D\) value of \(1.0 \times 10^{-7}\) M, which is comparable to the \(K_D\) value measured for the interaction between D3 monomer and biotinylated EDB.
Figure 2.16 Fyn SH3 derived EDB binding proteins. (a) SDS PAGE of Fyn SH3 wt (lane 1), D3 monomer after purification (lane 2) and after collecting the peak of size exclusion chromatography (SEC) (lane 3), D3 dimer after purification (lane 4) and after SEC (lane 5). (b) Specificity of the anti-
EDB binding protein D3 (dimer). ELISA signals are shown on different fibronectin type III homology repeats. EDB, extradomain B of fibronectin; h A1 Tnc, human domain A1 of tenascin-C; mu D Tnc, murine domain D of tenascin-C; h C-D-6, human domains C, D and 6 of tenascin-C; MSA, mouse serum albumin; no antigen, wells only blocked with PBS, milk. (c) Size exclusion chromatograms of Fyn SH3 wt, D3 monomer and D3 dimer on a Superdex 75 column. For every construct, the major peak corresponds to the appropriate molecular weight of the corresponding proteins. (d) BIAcore sensograms of D3 monomer and dimer injected at different concentrations on a EDB coated chip. (e) BIAcore sensogram of 7B89, a fibronectin fragment containing the EDB domain, injected at different concentrations on a D3 coated chip.

Figure 2.17 Dynamic Light Scattering. (top) Size distributions of Fyn SH3 wt (red) and D3 monomer (green) are very similar. (bottom) The same observation was made when comparing Fyn SH3 wt dimer (red) with D3 dimer (green).
Furthermore, immunohistofluorescence experiments performed with cryosections of F9 teratocarcinoma confirmed the ability of D3 monomer to stain tumor neo-vascular structures, in a fashion similar to the one of an α CD-31 antibody used as positive control (Fig. 2.18). The staining of vascular structures was further confirmed by immunohistochemistry, using D3 dimer as primary binding reagent on F9 teratocarcinoma cryosections (Fig. 2.19).

**Figure 2.18 Immunohistofluorescence on F9 teratocarcinoma sections.** Left, the fluorescent pattern of D3, right the staining of α-CD31 antibody. As controls, no binding molecule was added. Bar represents 50μm.
Figure 2.19 Immunohistochemistry with D3 dimer on F9 teratocarcinoma sections. D3 dimer was used as primary binding reagent, followed by biotinylated anti-myc antibody and streptavidin-phosphatase as secondary and tertiary reagents. (top panel) Purified D3 dimer solution (17μM). (middle panel) D3 diluted in PBS, corresponding to a 0.17μM solution. (bottom panel) In the negative control, no D3 dimer was added.
2.5.2 Biodistribution studies in tumor bearing mice

We used mice bearing s.c. grafted F9 murine teratocarcinoma as a syngeneic tumor model for the biodistribution analysis of the performance of D3 monomer and dimer in the molecular targeting of angiogenesis. In order to demonstrate that selective tumor uptake was a consequence of a specific EDB recognition, we also studied the biodistribution properties of Fyn SH3 wt as a protein of irrelevant binding specificity in the mouse. Twenty-four hours after i.v. injection of $^{125}$I-labeled proteins, animals were sacrificed, organs were excised and weighed, and radioactivity was counted. The results expressed as % injected dose per gram of tissue (%ID/g), are summarized in Figure 2.20. Only D3 monomer and D3 dimer selectively accumulated in the tumor, while Fyn SH3 wt did not exhibit any preferential tumor uptake. As expected, the tumor targeting performance of the avid dimeric EDB binder was superior compared to the one of D3 monomer. Tumor:organ ratios ranged between 3.0 and 9.9 for D3 monomer, the values for D3 dimer were between 4.0 and 21.8.
Figure 2.20 Biodistribution experiments of radiolabeled proteins in F9 tumor-bearing mice. The results are expressed as % injected dose per gram of tissue (% ID/g) ± S.E. (top) D3 monomer, 24 h after injection. (middle) Fyn SH3 wt monomer, 24 h after injection (bottom) D3 dimer, 24 h after injection.
2.6 Immunogenicity studies

Because the Fyn SH3 sequence is identical in mouse and man, we used Sv129 mice to study the immunogenic potential of Fyn SH3 derived proteins. Mice were injected i.v. 4 times either with Fyn SH3 wt or D3, every third day. One day after the 4th injection, blood samples were analyzed by ELISA for the presence of an IgG response against the injected protein. This injection schedule typically exhibits a strong immunogenic reaction, when human proteins are administered to mice (182). We used a human scFv antibody fragment as positive control which was expected to be immunogenic in mice. Indeed, mice had to be sacrificed after the third injection because mice became sick and blood samples were taken at that time point for the scFv group. ELISA analysis of the blood samples revealed that no murine antibodies against Fyn SH3 wt nor against D3 were detectable (ELISA signals < 0.03), but all mice had developed antibodies against the human scFv antibody fragment (Figure 2.21).
Figure 2.21 Immunogenicity ELISA. ELISA signals represent the presence of murine antibodies after repeated i.v. injections in mice. Top, no anti Fyn SH3 wt were detectable in 5 mice (ELISA signal < 0.03). Middle, no anti D3 antibodies were detectable in 5 mice (ELISA signal < 0.03). Bottom, all 4 mice had developed anti human scFv antibodies. Positive controls represent anti-tag antibodies of the coated target proteins.
3 Discussion

The selection efficiency of any synthetic protein library crucially depends on the biophysical properties of the selected protein scaffold, as well as on the library design, size, quality and use. We have chosen the Fyn SH3 domain as a scaffold for the generation of binding proteins because of a number of attractive features: it is expressed in bacteria at high level in soluble, monomeric form (104), it is stable (T_M: 70.5° C) (105), it does not contain any cysteine residues, it is of human origin, with an amino acid sequence which is completely conserved from mouse to man. Moreover, the structural basis of its binding mode has been resolved which indicates that this particular domain, unlike other SH3 domains, does not necessarily need a PXXP core binding motif: an important prerequisite for the isolation of binders against a variety of different target epitopes.

Novel binding specificities can be introduced into a protein scaffold by randomizing those residues that are involved in a protein-protein interaction of the wild-type protein (10). The Fyn SH3 domain contains two flexible loops (RT and n-Src loop) to interact with other proteins, and therefore residues in this region were chosen to be randomized for the design of 3 test libraries. Protein expression was not impaired for the majority of the tested clones in any of the 3 libraries, as shown by dot blot analysis (Fig. 2.3). In a first attempt, we designed and constructed a Fyn SH3 phage library with a randomized RT loop. We reasoned that the randomization of solely this loop might be sufficient to yield high-affinity binding derivatives, since the RT loop is known to contribute a lot to SH3-mediated protein interactions (79,84). Model affinity selection experiments using mouse serum albumin (MSA) as target protein led to the successful isolation of two unique MSA binding clones (G4 and C4). Although it was possible to detect binding in a phage ELISA, ELISA experiments performed with the soluble protein of one of these clones (G4) failed to yield binding signals. This difference reflects the moderate binding affinities of the clone G4 from the primary phage repertoires of 10^7 individual clone members. It also reflects the different sensitivities of the assays, phage ELISA being more sensitive because of the amplified detection of the phage by virtue of the 3000 pVIII coat protein copies of the phage 'tail' and/or greater binding avidities afforded by multivalent display on phage.
In order to increase the affinity of G4 for MSA, two affinity maturation strategies of G4 were performed. Both led to the isolation of specific binding clones with higher affinities. One clone, E11, showed an apparent dissociation constant of 87 nM. We write apparent dissociation constant since the oligomeric state of E11 (and other MSA binders) has not been determined by size exclusion chromatography (SEC) (data not shown), because the MSA binders eluted at later time points than Fyn SH3 wt. Some clones had an elution volume of more than 30 ml (instead of the expected 14-15 ml for Fyn SH3 wt, see Fig. 2.16c), corresponding to an apparent molecular weight < 1 kDa. The retardation of the clones in the column might be due to unspecific interactions with the matrix of the column, and/or simply a general characteristic of such small domains to elute at different time points, although having a similar size. For example, several monomeric single domain antibodies (13 kDa) were shown to have apparent molecular weights ranging from about 10 to 22 kDa when analyzing their size exclusion chromatograms (Figure 2 in Ref. (19)). Mutants of the fibronectin type III domain have also been shown to elute after the wild-type protein (22). Dynamic light scattering is an appropriate experiment to examine the size and the oligomeric state of the MSA binding Fyn SH3 variants and other mutants (see below). Importantly, from the MSA project we learned that it was possible to isolate Fyn SH3 mutants binding specifically to MSA with dissociation constants in the range of \( \sim 100 \) nM, from libraries containing Fyn SH3 variants with a randomized RT- and n-Src loop. From these results we concluded that selections of binding proteins from naïve libraries containing Fyn SH3 derivatives with a randomized RT- and n-Src loop should be feasible. For these reasons, a novel library was constructed, containing more than 1 billion individual Fyn SH3 clones.

One clone (D3), exhibited a specific recognition of the pharmaceutical target EDB domain of fibronectin and an impressive ability to selectively localize at the tumor site in mice bearing murine F9 teratocarcinomas. The EDB binder D3 elutes as a single peak in size exclusion chromatography (SEC), but at later time points compared to the Fyn SH3 wt. Chromatographic fractions were submitted to SDS PAGE (Fig. 2.16a, lanes 3 and 5) and BLAcore analysis (data not shown), confirming the identity of the protein in the chromatographic peak. Dynamic light scattering experiments further confirmed the correct size and monomeric nature of the D3 mutants (Fig 2.17). As discussed above, the delayed elution in SEC may be due to interactions with the column matrix.
The dissociation constant (K_D) of D3 monomer towards biotinylated EDB (8.5 x 10^-8 M) is satisfactory, considering that the D3 clone was isolated directly from a naïve library and was not submitted to affinity maturation. Further mutagenesis of judiciously selected residues in the vicinity of the antigen binding site may yield EDB binders of even higher affinity. A similar K_D value (1.0 x 10^-7 M) was observed for the interaction between D3 monomer and non-biotinylated 7B89, a fibronectin fragment containing the EDB domain. This finding indicates that the presence of the adjacent domains 7, 8 and 9 of EDB within the fibronectin molecule and the absence of biotin did not impair the binding affinity of D3. Importantly, D3 specifically recognized EDB and did not cross-react with other, structurally related proteins as shown by ELISA (Fig. 2.16 b). For practical applications, the cloning and expression of a D3 dimer was sufficient for yielding a good binding avidity to the antigen and good in vivo biodistribution properties in tumor-bearing mice. Twenty-four hours after injection of D3 monomer in tumor bearing mice, tumor to organ ratios ranged between 3.0 and 9.9, whereas the Fyn SH3 wt did not accumulate in the tumor (Figure 2.20). At the same time point, these ratios for D3 dimer ranged between 4.0 and 21.8. The absolute tumor value of D3 dimer reached 2.6% ID/g; a similar value (3.2% ID/g) was obtained in the same mouse model by the anti-EDB human antibody fragment scFv(L19), in the non-covalent homodimeric format (Table V in (177)).

Immunogenicity of protein drugs should be carefully assessed for all the scaffolds intended for therapy, as the final molecule has a framework with engineered regions, thus potentially introducing novel B- and T-cell epitopes. Ultimately, the immunogenic potential of a protein for therapeutic applications can only be studied in the clinical setting, because of immunological differences among animal species. Because the Fyn SH3 sequence is identical in mouse and man, we investigated the immunogenic potential Fyn SH3 wt, D3 and of a human scFv antibody fragment in mice. After repeated i.v. injections no antibodies against Fyn SH3 and D3 could be detected (ELISA signals < 0.03), while mice treated with scFv exhibited a strong antibody reaction against the human protein (Fig. 2.21). We have chosen an injection schedule, which in our experience typically exhibits a strong immunogenic reaction, when human proteins are administered to mice (182). The complete identity of Fyn SH3 in mouse, rat, gibbon and man, encourages us to pursue clinical developments of D3 derivatives for the targeted delivery of bioactive agents to the tumor neo-vasculature of...
patients with cancer, in full analogy to derivatives of the L19 antibody, which are currently being investigated in clinical trials.

The data presented in this thesis open the way on one hand the development of D3 derivatives as discussed above (e.g., D3 cytokine fusion proteins) and on other hand the use of the Fyn SH3 domain as a scaffold for the generation of new binding proteins, in general. In future, it will be important to perform selections against a variety of different target proteins to prove that this single-pot library may provide useful reagents for many biochemical and biomedical applications. Recently, phage ELISA positive clones have been obtained for another 2 different target proteins, glutathione S transferase and tumor necrosis factor alpha (*unpublished data*). These findings further suggest that the Fyn SH3 domain might be well suited for the isolation of binders against many different target proteins. Targets containing an exposed peptide stretch (e.g., extracellular loops of transmembrane proteins) might represent particularly attractive targets, since SH3 domains are known to bind well to (proline-rich) peptidic stretches of their corresponding natural target proteins (84).

Several other binding proteins based on scaffolds described in the literature (e.g., ankyrin repeat proteins, affibody molecules, kunitz type domains etc.) have dissociation constants in the low picomolar range towards their targets (see Introduction). In addition, the biophysical properties of some derivatives have been described to be very favorable (e.g., ankyrin repeat proteins and avimcrs) (29,65). Proving that such high affinities and good biophysical properties can be achieved also with the Fyn SH3 domain is a very important goal. Until now, we have been able to isolate binding proteins exhibiting dissociation constants between 26 nM (MSA-binder isolated by Bertschinger et al. (146) using covalent DNA display and the affinity maturation Sublib1 described in this thesis) and 85 nM with clone D3 against EDB. Applying different affinity maturation approaches of selected Fyn SH3 variants, such as randomizing residues in the vicinity of the loops, error-prone PCR, randomization of certain residues within the loops and the use of more stringent selection protocols will show whether higher affinities can be achieved. If needed, the biophysical properties of Fyn SH3 clinical candidates can be engineered as well, e.g., in full analogy to what Jespers et al. (19) have impressively shown for domain antibodies: randomization of residues in the CDRs can yield aggregation resistant domain antibodies using phage display technology in combination with heat denaturation steps. The same strategy - randomizing selected residues of the Fyn
SH3 variant and performing selection strategies for the desired effect—might also work for the Fyn SH3 domain.

Overall, the presented data in this thesis encourage us to pursue the development of this promising domain as a scaffold for the generation of new binding proteins, thus providing useful reagents for many biochemical and therapeutic applications as an alternative to more conventional IgG-based immunochemical technologies.
4 Materials and methods

_Fyn SH3 wt cloning, expression and purification_

The gene encoding the Fyn SH3 (amino acid residues are numbered according to the sequence reported by (101) and (100)) domain was amplified from a human cDNA library [human fetal MTC panel, brain (#K1425-1) (AMS Biotechnology (Europe) Ltd., Switzerland)] using the primers hFynSH3 domain ba [5'-AT CGC GGA TCC GGA GTG ACATTC TTG GCC CTT TAT-3'] and hFynSH3 domain fo [5'-GA AGATCT CTG GATAGAGTC AAC TGG AGC CAC ATA-3'] (all primers were purchased from Operon Biotechnologies). The resulting DNA fragment was cloned in the vector pQE-12 (Qiagen) using the restriction sites BamHI and BglII (New England Biolabs). The correct insertion was verified by DNA sequencing (Big Dye Terminator v3.1 Cycle Sequencing kit; ABI PRISM 3130 Genetic Analyzer; Applied Biosystems). After transformation of TG1 cells, colonies of the constructs were inoculated in 100 ml 2xYT medium containing 100 μg/ml ampicillin and 0.1% (w/v) glucose and grown at 37°C in a rotary shaker at 200 r.p.m. at an OD (600 nm) of 0.6, protein expression was induced by the addition of 1 mM IPTG (Applichem, Germany). After 16 hours at 30°C in a rotary shaker (200 r.p.m.), the bacterial cells were harvested by centrifugation and resuspended in 4 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH = 8.0). 1 mg/ml lysozyme was added and the cells were incubated on ice for 30 min. After cell lysis by sonication, the lysates were centrifuged for 25 min at 11,000 r.p.m. 1 ml of Ni²⁺-NTA slurry (Qiagen) was added to the cleared lysate to capture the 6xHis tagged proteins (incubation at 4°C for 1 h while shaking on a rotary shaker). The resin was washed 2 times with 5 ml wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH = 8.0) and the protein was eluted with 2 ml elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH = 8.0). After elution, proteins were dialyzed against PBS. SDS PAGE (Invitrogen) analysis was performed with 20μl of protein solution.
Library cloning strategy is depicted in Fig. 2.2. The gene encoding Fyn SH3 wt was used as a template for all three libraries.

First library (RT loop randomized):

Two independent PCRs were performed. The first was done with the primer EDBHis ba [5'-GAA AAG TGC CAC CTG ACG TCT AA-3'] and the partially degenerate primer FYN-SH3-LOOPRT fo [5'-TTC TCC TTT GTG AAA ACT CAG GTC MNN MNN MNN MNN MNN MNN MNN AT A AAG GGC CAC AAA GAG-3'], the second with the primer FYN-SH3-LOOPRT ba [5'-GAC CTG AGT TTT CAC AAA GGA GAA-3'] and EDBHis fo [5'-CGGTCTGGTTATAGGTACA TTGAGC-3']. Full-length FynSH3 mutants were assembled by PCR.

Second library (n-Src loop extended and randomized):

Two independent PCRs were performed. The first was done with the primer EDBHis ba [5'-GAA AAG TGC CAC CTG ACG TCT AA-3'] and the partially degenerate primer FYN-SH3LOOP2ND fo [5'-GGA GCG GGC TTC CCA CCA ATC TCC MNN MNN MNN MNN MNN MNN AAA TAT TTG AAA TTT TCC TCC TTT-3'], the second with the primer FYN-SH3-LOOP2ND ba [5'-GGAGATTTGGTGGAAGCCCGCCT-3'] and EDBHis fo [5'-CGGTCTGGTTATAGGTACA TTGAGC-3']. Full-length FynSH3 mutants were assembled by PCR.

Third library (RT and n-Src loop randomized, the latter extended):

Three independent PCRs were performed. The first was done with the primer EDBHis ba [5'-GAA AAG TGC CAC CTG ACG TCT AA-3'] and the partially degenerate primer FYN-SH3-LOOPRT fo [5'-TTC TCC TTT GTG AAA ACT CAG GTC MNN MNN MNN MNN MNN MNN AT A AAG GGC CAC AAA GAG-3'], the second with the primer FYN-SH3-LOOPRT ba [5'-GAC CTG AGT TTT CAC AAA GGA GAA-3'] and the partially degenerate primer FYN-SH3LOOP2ND fo [5'-GGA GCG GGC TTC CCA CCA ATC TCC MNN MNN MNN MNN MNN MNN AAA TAT TTG AAA TTT TCC TCC TTT-3'], whereas the third was done with the primer FYN-SH3-LOOP2ND ba [5'-GGAGATTTGGTGGAAGCCCGCCT-3'] and EDBHis fo [5'-CGGTCTGGTTATAGGTACA TTGAGC-3']. Full-length FynSH3 mutants were assembled by PCR.
The randomized inserts of all three libraries were independently double digested with EcoRI/BglII (New England Biolabs) and cloned into EcoRI/BglII-digested pQE-12 vector. The resulting ligation products were electroporated into electrocompetent *Escherichia coli* TG1 cells.

The percentage of clones expressing soluble Fyn SH3 variants was determined by dot blot analysis of bacterial cell lysates (ELIFA system; Perbio) using anti-His-HRP antibody conjugate (Roche) as detecting agent. Peroxidase activity was detected using the ECL plus Western blotting detection system (Amersham Biosciences).

Bacterial cell lysates were prepared the following way: Transformed bacteria of all three test libraries (see above) were plated on agar plates containing 100 μg/ml ampicillin and 1% (w/v) glucose and grown overnight in an incubator at 37°C. The next day, colonies were picked from the agar plate and grown in a round bottom 96-well plate (Nunc, cat. no. 163320) in 200 μl 2xYT medium containing 100 μg/ml ampicillin and 0.1% (w/v) glucose. Protein expression was induced after growth for 3 hours at 37°C and 200 r.p.m. by adding 1 mM IPTG (Applichem, Germany). Proteins were expressed overnight in a rotary shaker (200 r.p.m., 30°C). Subsequently, the 96-well plate was centrifuged at 1800g for 10 min and the supernatant was discarded. The bacterial pellets were resuspended in 60 μl lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH = 8.0) containing 1 mg/ml lysozyme and left for 30 min on ice. Afterwards, the bacterial cells were lysed by sonication in a water bath (6 bursts à 10 sec) and then centrifuged at 1800g for 10 min. The supernatant (1:4 diluted in PBS) was used for the dot blot experiment.

**Construction of the RT-loop library**

The first test library described in the previous section was amplified using SH3(NcoI) ba [5'-CAT GCC ATG GGC GGA GTG ACA CTC TTT GTG GCC CTT TAT-3'] and SH3(NotI) fo [5'-TTT TCC TTT TGC GGC CGC CTG GAT AGA GTC AAC TGG AGC CAC ATA-3']. The randomized insert was double digested with NcoI/NotI (New England Biolabs) and cloned into NcoI/NotI-digested pHEN1 phagemid vector (262). The resulting ligation product was electroporated into electrocompetent *Escherichia coli* TG1 cells according to Viti et al. (108), thereby obtaining a library of 1,2 x 10⁷ Fyn SH3 mutant clones. The library was stored
as glycerol stocks, rescued and used for phage production according to standard protocols (108).

**RT-loop library characterization**

A total of 10 clones were tested by PCR using the primers LMB3long ba \([5’-\text{CAG GAA ACA GCT ATG ACC ATG ATT AC-3’}]\) and FDseqlong fo \([5’-\text{GAC GTT AGT AAATGA ATT TTC TGT ATG AGG-3’}]\) to verify the correct size of the insert. 15 clones were selected at random and sequenced as described before. The percentage of clones expressing soluble Fyn SH3 mutants was determined by dot blot analysis of bacterial supernatants (ELIFA system; Perbio) using anti-myc-HRP antibody conjugate (Roche) as detecting agent. Peroxidase activity was detected using the ECL plus Western blotting detection system (Amersham Biosciences). The display of the Fyn SH3 mutant – pIII fusion protein on the phage surface was evaluated by western blot. Different amounts of purified phage were loaded on SDS gel and then transferred to NC membrane (Protran BA 85; Schleicher & Schuell, Dassel, Germany). As detecting reagents anti-pIII mouse mAB (MoBiTec, Göttingen, Germany) and anti-mouse HRP immunoglobulins were used. Peroxidase activity was detected using the ECL plus Western blotting detection system (Amersham Biosciences).

**Phage display selections (RT loop library) against MSA and ELISA screening**

Unless stated otherwise, growth media, helper phage, and general protocols were essentially used as the protocols for antibody phage display described in (108).

An immunotube (Nunc) was coated with MSA (Sigma) at a concentration of 100µg/ml in PBS, overnight at room temperature. The immunotube was then rinsed with PBS and blocked for 2 h at room temperature with 2% skimmed milk in PBS (MPBS 2%). After rinsing with PBS, \(>10^{12}\) phage particles in MPBS 2% were added to the immunotube. The immunotube was first incubated on a shaker for 30 min and then for 1.5 h standing upright at room temperature. Unbound phage were washed away by rinsing the immunotube ten times with PBS 0.1% Tween 20 and ten times with PBS. The bound phage was eluted specifically with
1% MSA solution in PBS for 30 min. The eluted phage was used to infect exponentially growing E. coli TG1. Phage production was performed according to standard protocols (108). After three rounds of panning, monoclonal phage ELISA was performed essentially as described (264). Individual colonies from the plates of phage-infected TG1 were inoculated into 200μl 2xYT-, 100μg/ml ampicillin (Applichem), 1% Glucose (Sigma) (2xYT-AMP-GLU) into 96-well plates (Nunclon™ Surface, Nunc). The plate was incubated for 3 h at 37°C in a shaker incubator. After removing 40μl of each well for a glycerol stock, 40μl 2xYT-AMP-GLU containing 4x10⁹ t.u. of helper phage were added to each well and the plate was incubated at 37°C for 30 min. After spinning the plate at 1800g for 10 min and aspirating the supernatants off, the bacterial pellet was resuspended in 200μl of 2xYT, 100μg/ml ampicillin, 33.3μg/ml kanamycin (Applichem) and grown overnight at 30°C. After spinning, phage supernatants were used for ELISA screening using biotinylated MSA as target. MSA was biotinylated using EZ-Link™ Sulfo-NHS-SS-Biotin (Perbio) according to manufacturer’s instructions and was captured (concentration: ~3 x 10⁻⁷ M) on streptavidin coated wells (StreptaWells, High Bind, Roche). After blocking with PBS, 2% Milk (Rapilait, Migros, Switzerland), 20 μl PBS, 10% Milk and 80 μl of phage supernatants were applied. After incubating for 1 hour and washing, detection was made with anti-M13-HRP antibody conjugate (GE Healthcare). Peroxidase activity was detected by adding BM blue POD substrate (Roche) and the reaction was stopped adding 1 M H₂SO₄. Absorbance was measured using the VersaMax microplate reader (OD₄₅₀nm - OD₆₅₀nm). The DNA of positive clones was sequenced as described above.

Construction and characterization of the affinity maturation libraries Sublib1 and Sublib2

One MSA binding clone G4 was affinity matured using its sequence and creating 2 sublibraries (Sublib1 and Sublib2). Out of the glycerol stock of G4 described above, a single colony was picked for the PCR-amplification of the G4 sequence using the primers LMB3long ba [5'-CAG GAA ACA GCT ATG ACC ATG ATT AC-3'] and FDseqlong fo [5'-GAC GTTAGTAAATGAATTTC TGT ATGAGG-3'], resulting in the template for the libraries.
Two independent PCRs were performed. The first was done with the primer LMB3long ba [5' - CAG GAA ACA GCT ATG ACC ATG ATT AC-3' ] and the partially degenerate primer FYN-SH3_LOOP2ND fo [5' - GGA GCG GGC TTC CCA CCA ATC TCC MNN MNN MNN MNN MNN MNN MNN CAA TAT TTG AAA TTT TTC TCC TCC TTT-3' ], the second with the primer FYN-SH3_LOOP2ND ba [5' - GGAGATTTGTTGGGAAGCCCGCT-3' ] and FDseqlong fo [5' - GAC GTT AGT AAA TGA ATT TTC TGT ATG AGG-3' ]. Full-length FynSH3 mutants were assembled by PCR.

Two independent PCRs were performed. The first was done with the primer LMB3long ba [5' - CAG GAA ACA GCT ATG ACC ATG ATT AC-3' ] and the partially degenerate primer Fyn-SH3_sub_113-16/119/132 fo [5' CC AGTTGT CAAGGAGCGGGCTTC CCAMNN ATC TCC MNN MNN MNN MNN MNN MNN MNN MNN MNN MNN MNN CAA TAT TTG AAA TTT TTC TCC 3' ], the second with the primer Fyn-SH3_sub_113-116/119/132 ba [5' GCC CGC TCC TTG ACA ACT GGA GAG ACA GGT NNK ATT CCC AGC AAT 3' ] and FDseqlong fo [5' - GAC GTT AGT AAA TGA ATT TTC TGT ATG AGG-3' ]. Full-length FynSH3 mutants were assembled by PCR.

The resulting inserts of both sub-libraries were further amplified using LMB3long ba [5' - CAG GAA ACA GCT ATG ACC ATG ATT AC-3' ] and FDseqlong fo [5' - GAC GTT AGT AAA TGA ATT TTC TGT ATG AGG-3' ]. After double digestion with NcoI/NotI (New England Biolabs) and cloning into NcoI/NotI-digested pHEN1 phagemid vector (262), the resulting ligation products of both sublibraries were electroporated into electrocompetent Escherichia coli TG1 cells according to Viti et al. (108), thereby obtaining a library of 2.0 x 10^7 (Sublib1) and 4.0 x 10^7 (Sublib2) Fyn SH3 mutant clones. The library was stored as glycerol stocks, rescued and used for phage production according to standard protocols (108). For the characterization of the libraries, PCR colony screening and dot blot experiments were performed as described above under the section „RT-loop library characterization”.

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Phage display selections (Sublib1 and Sublib2) against MSA and ELISA screening

The affinity maturation selections and screenings were essentially performed as described in "Phage display selections (RT loop library) against MSA and ELISA screening". For each sublibrary, an immunotube (Nunc) was used. In addition, a third immunotube was used for mixing the 2 sublibraries. Except for the third immunotube, bound phage was eluted in 1 ml of 100mM triethylamine solution and inverting the tube for 5 min. Triethylamine was neutralized by adding 0.5ml 1 M Tris-HCl pH 7.4. Bound phage in the third tube were incubated for 5 min with a 1µM MSA solution in PBS, washed again with PBS, 0.1% Tween 20 and PBS, and then eluted specifically with a 0.5% MSA solution on PBS (30min). The eluted phage of all three immunotubes were used to infect exponentially growing E.coli TG1, separately. Phage production was performed according to standard protocols (108). After three rounds of panning, monoclonal phage ELISA was performed as described before. The DNA of positive clones was sequenced as described above.

Subcloning, expression and purification of affinity matured MSA binders

For expression of selected affinity matured MSA binding clones, their sequence was subcloned into the pQE-12 vector (Qiagen). Out of the glycerol stocks prepared during the ELISA screening as described above, a single colony was picked for the PCR-amplification of the sequences using the primers LMB3long ba [5’-CAG GAA ACA GCT ATG ACC ATG ATT AC-3’] and FDseqlong fo [5’-GAC GTT AGT AAT GA TTT TTC TGT ATG AGG-3’], resulting in PCR-product that was used for another PCR using the primers hFynSH3 domain ba [5’-AT CGC GGA TCC GGA GTG ACA CTC TTT GTG GCC CTT TAT-3’] and FYN- SH3_cloning_into_pQE12 fo [5’ ATC CCA AGC TTA GTG ATG GTG ATG GTG ATG ACC CTG GAT AGA GTG GTG ACC TAA GTG AGC CAC 3’], introducing BamHI/HindIII restrictions sites and a hexa-His tag. All clones were BamHI/HindIII (New England Biolabs) double-digested and ligated into BamHI/HindIII double-digested pQE-12 vector. After transformation of TG1 cells, colonies of the constructs were induced for protein expression, lysed and purified as described before for the Fyn SH3 wt protein.
Fifty μl of the purified proteins B1 (2 μM), E11 (65μM), C6 (28μM), C3 (100μM), H9 (35μM), D12 (220μM), G4 (300μM) and FynSH3 wt (37μM) were separately added together with 50μl of MPBS 4% into the wells of a MaxiSorp plate (Nunc) that had been previously coated with 100μl of a MSA, HSA, RSA, BSA and Ovalbumin (OVA) solution (all purchased from Sigma; coating concentration: 100μg/ml in PBS). After 2h at RT, the plate was washed 3 times with PBS, followed by the addition of 100μl anti His-HRP immunoconjugate (Sigma) diluted 1:1000 in 2% MPBS. The 96-well plate was left for 1 h at RT and then washed 3 times with PBS, 0.1% Tween 20 and 3 times with PBS. Colorimetric detection was made by the addition of 100μl of BM Blue POD substrate (Roche) and the reaction was stopped with 60μl 1 M H₂SO₄. Absorbance was measured using the VersaMax microplate reader (OD₄₅₀nm – OD₆₅₀nm).

Surface plasmon resonance experiments with MSA

Affinity measurements were performed using a BIAcore3000 instrument (Biacore). For the interaction analysis between MSA and E11, a CM5 chip (Biacore) was used with 18680 RU MSA immobilized. The running buffer was PBS, 0.1% NaN₃, Surfactant P20 (Biacore). The interactions were measured at a flow of 20μl/min and injections of different concentrations of E11. All kinetic data of the interaction (separate kₜₚ/kₜₑ) were evaluated using BIAevaluation 3.2RC1.

Construction and characterization of the RT- / n-Src loop library

The third test library described in the previous section was amplified using SH3(NcoI) ba [5'-CAT GCC ATG GGC GGA GTG ACA CTC TTT GTG GCC CTT TAT-3'] and SH3(NotI) fo [5'-TTT TCC TTT TGC GGC CGC CTG GAT AGA GTC AAC TGG AGC CAC ATA-3']. The randomized insert was double digested with NcoI/NotI (New England Biolabs) and cloned into NcoI/NotI-digested pHEN1 phagemid vector (262). The resulting ligation product
was electroporated into electrocompetent *Escherichia coli* TG1 cells according to Viti et al. (108), thereby obtaining a library of $1.2 \times 10^9$ Fyn SH3 mutant clones. The library was stored as glycerol stocks, rescued and used for phage production according to standard protocols (108).

The quality and functionality of this new library were assessed by DNA sequencing (8 clones) PCR colony screening (30 clones) and dot blot experiment (58 clones), performed as described above under the section "RT-loop library characterization".

**Phage display selections (RT- / n-Src loop library) against EDB and ELISA screening**

The EDB domain of fibronectin (169), was expressed and purified as previously described. EDB was biotinylated using EZ-Link™ Sulfo-NHS-SS-Biotin (Perbio) according to manufacturer’s instructions. The biotinylated EDB (final concentration $10^{-6}$ M) was captured on avidin- (first and third round) or streptavidin-coated (second round) Maxisorp wells (Nunc). After blocking with 1% BSA (Sigma) in PBS, 1 ml containing $10^{12}$ transforming units (t.u.) of Fyn SH3 phage library in 1% BSA were added to the wells (aliquoted in 8 wells) and incubated for 45 minutes at room temperature on a shaker. The wells were rinsed 10 times with PBS, 0.1% Tween20 (Sigma) and 10 times with PBS. Bound phage was eluted by incubation with 100 mM triethylamine (Fluka) for 5 min. The eluted phage was used to infect exponentially growing E.coli TG1. Phage production was performed according to standard protocols (108). After three rounds of panning, monoclonal phage ELISA was performed as described above. In ELISA screening, biotinylated EDB ($10^{-6}$ M) was captured on streptavidin coated wells (StreptaWells, High Bind, Roche) and after blocking with PBS, 2% Milk (Rapilait, Migros, Switzerland), 20 μl PBS, 10% Milk and 80 μl of phage supernatants were applied. After incubating for 1 hour and washing, detection was made with anti-M13- HRP antibody conjugate (GE Healthcare). Peroxidase activity was detected by adding BM blue POD substrate (Roche) and the reaction was stopped adding 1 M H$_2$SO$_4$. Absorbance was measured using the VersaMax microplate reader (OD$_{450nm}$ – OD$_{650nm}$). The DNA of positive clones was sequenced as described above.
For expression of B11 and D3, their sequence was subcloned into the pQE-12 vector (Qiagen) as described before for the MSA binders.

**ELISA of purified B11 and D3 proteins**

Fifty μl of the purified proteins B11 (10 μM) and D3 (170μM), were separately added together with 50μl of MPBS 4% into streptavidin coated wells (StreptaWells, High Bind, Roche) that had been coated with biotinylated EDB (without His tag) (10^6 M) or MPBS 4%. After incubating for 1 hour and washing, detection was made with the addition of 100μl anti His-HRP immunoconjugate (Sigma). The 96-well plate was left for 1 h at RT and then washed 3 times with PBS, 0.1% Tween 20 and 3 times with PBS. Colorimetric detection was made by the addition of 100μl of BM Blue POD substrate (Roche) and the reaction was stopped with 60μl 1 M H₂SO₄.

**Cloning, expression and purification of D3 and Fyn SH3 wt dimer**

Using the D3 sequence as template (first PCR product obtained when cloning D3 into the pQE-12 vector, see above) two independent PCRs were performed: in the first one, the primers 52. ba [5'-GAC TAA CGA GAT CGC GGA TCC GGA GTG ACA CTC TTT GTG GCC CTT TAT-3'] and 47. fo [5'-TCC GCC ACC GCC AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC CTG GAT AGA GTC AAC TGG AGC CAC-3'] and in the second PCR the primers 48. ba [5'-GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT GGA GGA GGA GTGACA CTC TTT GTG GCC CTT TAT-3'] and 51. fo [5'-ATC CCA AGC TTA GTG ATG GTG ATG GTG ATG CAG ATC CTC TTC TTA ACC CTG GAT AGA GTG AAC TGG AGC CAC-3'] were used. The two fragments were PCR assembled yielding a D3 dimer with BamHI/HindIII restriction sites, with a 14 amino acid linker (GGGGSGGGGSGGGG) between the two domains and a myc and hexa-His tag at the C-terminal end of the protein. The insert was BamHI/HindIII (New
England Biolabs) double-digested and ligated into BamHI/HindIII double-digested pQE-12 vector. After transformation of TG1 cells, expression and purification was made as described before for the MSA binders. For the cloning of Fyn SH3 wt, the same procedure was made using the Fyn SH3 wt as template.

Specificity ELISA (EDB)

The biotinylated target proteins EDB (169), human domain A1 of Tenascin C (199), murine domain C of Tenascin C (199), human domains C-D-6 of Tenascin C were available in our laboratory and expressed as (His)$_6$ tagged proteins in E.coli TG1 from pQE-12 based expression vector, purified and biotinylated as previously described. Mouse serum albumin was purchased from Sigma and biotinylated using EZ-Link™ Sulfo-NHS-SS-Biotin (Perbio) according to manufacturer's instructions. Target proteins were added to streptavidin coated wells (StreptaWells, High Bind, Roche) and after blocking with PBS, 2% Milk (Rapilait, Migros, Switzerland), 50 µl PBS, 4% Milk and 50 µl of purified D3 dimer (3µM) was added. After incubating for 1 hour and washing, detection was made with anti-myc-HRP antibody conjugate (Roche). Peroxidase activity was detected by adding BM blue POD substrate (Roche) and the reaction was stopped adding 1 M H$_2$SO$_4$. Absorbance was measured using the VersaMax microplate reader (OD$_{450nm}$ - OD$_{650nm}$).

Size exclusion chromatography (SEC)

SEC of purified Fyn SH3 wt, D3 monomer, D3 dimer was performed on an ÄKTA FPLC system using a Superdex 75 column (Amersham Biosciences).

Dynamic Light Scattering

Dynamic Light Scattering measurements were performed using the Zetasizer Nano-ZS instrument (Malvern), according to predefined settings for protein size measurements. The
results represent average sizes of 30 (Fyn SH3 wt and D3 monomer) and 60 (Fyn SH3 wt and D3 dimer) independent runs, respectively.

**Surface plasmon resonance experiments (EDB)**

Affinity measurements were performed using a BIAcore3000 instrument (Biacore). For the interaction analysis between biotinylated EDB and D3 monomer and D3 dimer, a streptavidin SA chip (Biacore) was used with 600 RU biotinylated EDB (169) immobilized. The running buffer was PBS, 0.1% NaN₃, Surfactant P20 (Biacore). The interactions were measured at a flow of 20μl/min and injections of different concentrations of D3 monomer and dimer. For the interaction analysis between D3 monomer and 7B89, a fibronectin fragment containing the EDB domain, ((263), kindly provided by Philogen, Italy) a CM5 chip (Biacore) was used with 3300 RU D3 monomer immobilized. The running buffer was HBS-EP (Biacore). The interactions were measured at a flow of 20μl/min and injections of different concentrations of 7B89. All kinetic data of the interaction (separate $k_{on}/k_{off}$) were evaluated using BIAevaluation 3.2RC1.

**Immunohistofluorescence**

Female Svl29 mice (Charles River) received subcutaneous injection of $3 \times 10^6$ F9 teratocarcinoma cells in the right flank. After excising, the tumors were embedded in cryoembedding compound (Microm), frozen in chilled isopentane and stored at -80°C. Then, 10μm sections were cut, fixed with ice cold acetone and double fluorescence staining for EDB and CD31 was performed. D3 monomer and rat antimouse CD31 (BD Pharmingen) were used as primary binding reagents. As secondary detection antibodies, we used for EDB anti His Alexa Fluor 488 conjugate (Qiagen) and for CD31 donkey anti rat Alexa 594 (Molecular Probes). Slides were mounted with Glycergel mounting medium (Dako) and analyzed with a Zeiss Axioskop 2 mot plus.
**Immunohistochemistry**

Immunohistochemistry was performed using the same cryosections as described above. Pre-blocked D3 dimer (17 μM or 170 nM) in 2% BSA and biotinylated anti-Myc antibody were used as primary binding reagents, followed by the Streptavidin-Alkaline-Phosphatase complex (Biospa). Fast Red TR Salt (Sigma) was used to develop the red staining. Slides were counterstained with hematoxylin (Sigma), mounted with Glycergel mounting medium (Dako) and analyzed with a Zeiss Axiovert S100 TV microscope.

**Radioiodination of Fyn SH3 wt, D3 monomer and D3 dimer**

125μg -200μg Protein were combined with 200μCi of $^{125}$I (Amersham) and with filtered Chloramin T (Sigma) solution (5mg/ml; 0.25μg Chloramin T per μg of protein was used) for 2 min followed by separation from unincorporated iodine using a PD-10 disposable gel filtration column (Pharmacia). The binding activity after labeling was evaluated by loading an aliquot of radiolabeled sample onto 500 μl of EDB-Sepharose resin (170) on a pasteur pipette, followed by radioactive counting of the flow-through, wash and eluate fractions. Binding reactivity, defined as the ratio between the counts of the eluted protein and the sum of the counts (flow-through, wash, eluate and column) was 87% for the D3 monomer and between 58 and 68 % for the dimer (36% for the non-binding Fyn SH3 wt).

**Biodistributions of tumor-bearing mice injected with radiolabeled Fyn SH3 wt, D3 monomer and D3 dimer**

Biodistribution studies were performed under a license (Tumor targeting, Bewilligung 198/2005) issued to D.N. by the Veterinäramt des Kantons Zürich. F9 murine teratocarcinomas were implanted as described previously (169,170) in 129Sv mice (8-12 weeks old, female). $^{125}$I-labeled protein (5.6 - 10μg (9 – 13 μCi)) in 100μl of saline solution, radiolabeled on the same day, was injected i.v. Mice were sacrificed at 4 and 24 h (Fyn SH3 wt only 24 h) and organs were weighed and radioactively counted. 3 animals were used for each time point (except Fyn SH3 wt 24h and D3 monomer 4h: 4 animals). Targeting results
of representative organs are expressed as %ID of protein /g of tissue (± SE) and tumor:organ ratios were determined.

Immunogenicity of Fyn SH3 wt, D3 and a scFv antibody fragment

Sv129 mice were injected 4 times (every third day) with 20 µg of Fyn SH3 wt or D3, respectively (5 mice per group). One day after the 4th injection blood samples were taken for examining the presence or absence of murine anti-Fyn SH3 wt and anti-Fyn SH3 D3 antibodies (75-150 µl blood per mouse, diluted in 50 µl Heparin (Bichsel, Switzerland)). As a positive control, 4 mice were injected at equal time points and equal molar dosages (= 60 µg) with the human scFv antibody fragment clone E1 (anti human α2-macroglobulin described in (153); expression and purification as described in (153)). However, because mice became sick after the third injection of scFv fragments, they were sacrificed and blood samples were taken after the third injection. Blood samples were analyzed in ELISA: Maxisorp wells (Nunc) were coated overnight with 100 µl of antigen solution (Fyn SH3 wt: 20 µg/ml, D3: 20 µg/ml or scFv: 60 µg/ml in PBS). After washing and blocking, 50 µl of PBS, 4% Milk and 50 µl of blood samples (1:4, 1:10, 1:50 and 1:100 diluted in PBS) were added and incubated for 1 hour at room temperature. After washing, the presence of murine antibodies in the blood samples were detected by adding anti mouse IgG HRP antibody conjugate (Sigma). As controls for the coating efficiency of the antigens, anti His HRP antibody conjugate (Sigma) (Fyn SH3 wt and D3) or anti-myc HRP antibody conjugate (Roche) were added. The negative controls were performed using the anti mouse IgG HRP antibody conjugate without adding any blood samples. Peroxidase activity was detected by adding BM blue POD substrate (Roche) and the reaction was stopped adding 1 M H2SO4. Absorbance was measured using the VersaMax microplate reader (OD450nm – OD650nm).
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6 Curriculum vitae

Personal details

First Name    Dragan
Last Name     Grabulovski
Date of birth March 9, 1978
Place of birth Winterthur, Switzerland
Address       Imfeldstrasse 84a
              CH-8037 Zürich
Phone         + 41 43 343 13 24
E-mail        d.grabulovski@alumni.ethz.ch
Nationality   Swiss, Macedonian
Marital Status Unmarried

Education

Nov 2004 – May 2007  PhD thesis: „The SH3 domain of Fyn Kinase as a Scaffold for the Generation of New Binding Proteins“, Institute of Pharmaceutical Sciences, ETH Zürich, Supervisor: Prof. Dr. Dario Neri

Graduated: Didaktischer Ausweis ETH in Pharmazie
Studies in Pharmacy, ETH Zürich  
Graduated: **Eidg. Dipl. Apotheker ETH**  
(average mark: 5.8 out of 6)

Diploma thesis: „Random synthesis: Isolation, structure elucidation and activity screening of tyrosine kinase inhibitors produced by γ-irradiation“, Supervisor: Prof. Dr. Gerd Folkers, ETH Zürich

Semester thesis: „Cloning and expression of mutants of L19-IFNγ“, Supervisor: Prof. Dr. Dario Neri, ETH Zürich

**Swiss Matura B**, Kantonsschule Rychenberg, Winterthur

**Practical trainings**

Mar 2004  
Compulsory practical training as teacher in biology, Kantonsschule Wettingen (Dr. S. Ginsburg)

Aug 2000 – Aug 2001  
Compulsory one-year practical training, Adler Apotheke, Winterthur (Dr. U. Reinhard)

**Working experience**

As of May 2007  
**Co-founder and CSO of Covagen AG**, a spin-off company of ETH Zürich
Jan 2006 – May 2007 Saturday’s Service as pharmacist, Apotheke im Shoppingcenter, 8957 Spreitenbach (Dr. R. Gysler)

Nov 2004 – Mar 2006 Sunday’s Service as pharmacist, Apotheke im Bahnhof, 8400 Winterthur (H. Sonanini)

Jan 2004 – Oct 2004 Pharmacist (80%), Apotheke im Bahnhof, 8400 Winterthur (H. Sonanini)

Oct 2001 – May 2003 Saturday’s and Sunday’s service as cand.pharm. (Apotheker-Assistent)
- Apotheke Seuzach, 8472 Seuzach (Dr. P. Steigrad)
- Apotheke im Bahnhof, 8400 Winterthur (H. Sonanini)

Awards

Feb 2006 Venture Competition 2006, ETH Zürich & McKinsey: One of the ten best business ideas and business plans, in team with Dr. Julian Bertschinger

Nov 2004 ETH medal for Diploma thesis

Nov 2003 2nd place Amedis Förderpreis 2003 for the Diploma thesis

Publications & Patents

Feb 2007
*these authors contributed equally to the work

Feb 2007

May 2006
Patent application EP 06017336.6, Grabulovski D. and Neri D.

Mar 2005

Languages

Macedonian Native Speaker
German Fluent
English Fluent
French Good knowledge
Serbian / Croatian Good knowledge
Spanish Basic knowledge
Bulgarian Basic knowledge

Hobbies
Sports (Basketball, Snowboard, Swimming)

Zurich, May, 2007
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