A Novel, Non-immunogenic Fyn SH3-derived Binding Protein with Tumor Vascular Targeting Properties*

Dragan Grabulovski, Manuela Kaspar, and Dario Neri

From the ETH Zürich, Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, Wolfgang-Pauli-Strasse 10, CH-8093 Zürich, Switzerland

The generation of novel binding molecules based on protein frameworks (“scaffolds”) represents an emerging field in protein engineering, with the potential to replace antibodies for many research and clinical applications. Here, we describe the design, construction, characterization, and use of a novel human Fyn SH3 phage library, containing $1.2 \times 10^9$ individual clone members. We also present the isolation and in vitro characterization of Fyn SH3-derived proteins binding to the extra-domain B of fibronectin, 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 SH3 WT nor the D3 mutant was immunogenic in mice after four intravenous injections. The extra-domain B 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.

At present, antibodies represent 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. However, some applications of antibody technology present 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. Therefore, several research groups have recently focused on the development of small globular proteins as scaffolds for the generation of a novel class of versatile binding proteins. Typically, portions of the surface (e.g. loops) of a protein framework with suitable biophysical properties are combinatorially mutated to produce a protein library, which can then be screened for binding specificities of interest (1–4). Until now, more than 40 scaffolds have been used for the generation of protein binders (5). Being mainly a topic of academic interest at first, the development and use of non-antibody classes of proteins are now being pursued by small and medium size biotechnology companies. Kunitz-type domain mutants are currently being studied in phase I clinical trials for hereditary angiodema, open heart surgery, and cystic fibrosis indications (6). The small size of globular domain-binding proteins typically leads to rapid clearance from circulation, which can be prevented either by chemical modification with polymers or by fusion to an albumin-binding polypeptide (7). However, it is likely that the most crucial determinant for the success or failure of this novel class of biotherapeutics may be related to their in vivo immunogenicity profile. Because it has been reported that even fully human proteins can induce neutralizing antibodies in the patients after repeated administration (8), similar problems could arise with globular proteins, particularly if they are of non-human origin.

In previous works, the Src homology 3 (SH3) domains of Hck and Abl kinase have been used for the generation of binding proteins (9, 10). SH3 domains are ~60-residue protein modules found in proteins generally involved in the regulation of dynamic processes occurring at the plasma membrane, such as the organization of the cytoskeleton, the transduction of extracellular signals, and the internalization of membrane receptors (11, 12). Typically, SH3 domains bind to proline-rich peptides, containing a PXXP core-binding motif (13), but examples of unconventional SH3 binding sites have also been described (14). So far, SH3-derived proteins have been used only for the generation of binders against known SH3 ligands, such as human immunodeficiency virus-1 Nef protein (9) or synthetic peptides (10).

Fyn is a 59-kDa member of the Src family of tyrosine kinases (15, 16). The Fyn SH3 domain comprises 63 residues (amino acids 83–145 of the sequence reported by Refs. 17 and 18), and its amino acid sequence is fully conserved among man, mouse, rat, and monkey (gibbon). Fyn SH3 domains from chicken and Xenopus laevis differ from their human counterpart at one and two amino acid positions, respectively. Fyn SH3 is composed of two antiparallel β-sheets and con-
tains two flexible loops (called RT and n-Src loops) to interact with other proteins (Fig. 1a).

In the present article we describe the design, construction, and characterization of a human Fyn SH3 phage library containing more than 1 billion individual clones as well as the isolation and in vitro characterization of proteins binding to a pharmaceutically relevant target protein, the extra-domain B (EDB) of fibronectin, a marker of angiogenesis (19). Fibronectin is a large glycoprotein that is present in large amounts in plasma and body tissues. EDB is a 91-amino acid type III homology domain that becomes inserted into the fibronectin molecule by a mechanism of alternative splicing at the level of the primary transcript (20). EDB is essentially undetectable in healthy adult individuals but is abundant in many aggressive solid tumors and displays either predominantly vascular or diffusial stromal patterns of expression, depending on the tumor type (19, 21–27). Three therapeutic derivatives of the human monoclonal antibody L19, specific for the EDB domain, are currently being investigated in clinical trials for the targeted delivery of bioactive compounds in cancer patients (28–31). Here we present the first non-antibody, but Fyn SH3-derived, protein binding to EDB, and characterize the ability of D3 to selectively accumulate at the tumor site. Furthermore, we could demonstrate that neither Fyn SH3 WT nor the Fyn SH3-D3 mutant was immunogenic in mice after repeated intravenous injections.

**EXPERIMENTAL PROCEDURES**

**Media and General Procedures**—Unless stated otherwise, growth media, helper phage, and general protocols were essentially used as the protocols for antibody phage display described in (32).

**Library Construction and Cloning**—The gene encoding the Fyn SH3 (amino acid residues are numbered according to the sequence reported by Refs. 17 and 18) domain was amplified from a human cDNA library (human fetal MTC panel, brain (K1425-1)) (AMS Biotechnology (Europe) Ltd.) using the primers hFynSH3 domain ba (5′-AT CGC GGA TCC GGA GTG ACA CTC TTT TGT GCC CTT TAT-3′) and hFynSH3 domain fo (5′-GA AAG ACA GCT ATG ACC ATG ATT AC-3′) to verify the correct size of the insert. 16 clones were selected at random and sequenced to check for the absence of frameshifts and pervasive contamination. The percentage of clones expressing soluble Fyn SH3 mutants was determined by dot blot analysis of bacterial supernatants (ELISA system; Perbio) using anti-Myc HRP antibody conjugate (Roche) as a detecting agent. Peroxidase activity was detected using the ECL plus Western blotting detection system (Amersham Biosciences).

**Phage Display Selections and ELISA Screening**—The EDB of fibronectin (23) was expressed and purified as described previously. EDB was biotinylated using EZ-linkTM sulfo-NHS-SS-biotin (Perbio) according to the manufacturer’s instructions. The biotinylated EDB (final concentration 10−6 μl) was captured on avidin- (first and third round) or streptavidin-coated (second round) Maxisorp wells (Nunc). After blocking with 1% bovine serum albumin (Sigma) in PBS, 1 ml containing 1012 pfu/ml ampicillin (Applichem), 1% glucose (Sigma) (2xYT-AMP-GLU) into 96-well plates (NunclonTM surface, Nunc). The plate was incubated at 37 °C for 30 min. After spinning the plate at 1800 × g for 10 min and aspirating the supernatants off, the bacterial pellet was resuspended in 200 μl of 2xYT, 100 μg/ml of ampicillin (Applichem, 1% glucose (Sigma) (2xYT-AMP-GLU)) into 96-well plates (NuncTM surface, Nunc). The plate was incubated for 3 h at 37 °C in a shaker incubator. After removing 40 μl of each well for a glycerc stock, 40 μl of 2xYT-AMP-GLU containing 4 × 106 transforming units of helper phage was added to each well, and the plate was incubated at 37 °C for 30 min. After spinning the plate at 1800 × g for 10 min, the DNA was isolated, and used for phage production according to standard protocols (32).

**Library Characterization**—A total of 30 clones were tested by PCR using the primers LMB3long ba (5′-CAG GAA ACA GCT ATG ACC ATG ATT AC-3′) and EDSeqlong fo (5′-GAG ATTGGTGGAAGCCCGC-3′) to verify the presence of the insert. 16 clones were selected at random and sequenced to check for the absence of frameshifts and pervasive contamination. The percentage of clones expressing soluble Fyn SH3 mutants was determined by dot blot analysis of bacterial supernatants (ELISA system; Perbio) using anti-Myc HRP antibody conjugate (Roche) as a detecting agent. Peroxidase activity was detected using the ECL plus Western blotting detection system (Amersham Biosciences).
ampicillin, 33.3 μg/ml kanamycin (Applichem) and grown overnight at 30 °C. After spinning, phage supernatants were used for 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 of PBS, 10% milk, and 80 μl of phage supernatants were applied. After incubating for 1 h and washing, detection was made with anti-M13-HRP antibody conjugate (Amersham Biosciences). Peroxidase activity was detected by adding BM blue POD substrate (Roche), and the reaction was stopped by adding 1 M H2SO4. The DNA of positive clones was sequenced as described above.

**Subcloning, Expression, and Purification of D3 Monomer and Dimer**

**Expression and Purification of Fyn SH3 WT**—For expression of one binding clone, called D3, its sequence was subcloned into the pQE-12 vector (Qiagen). Of the glycerol stock described above, a single colony was picked for the PCR amplification of the D3 sequence using the primers LMB3long ba and FDseqlong fo, resulting in the PCR product a. For the monomer, the PCR product a was used for another PCR using the primers hFyn SH3 domain ba and Fyn SH3 cloning into pQE-12-fo (5' -ATC CCA GGC TTA GTG ATG ATG GTG ATG ACC CTG GAT AGA GTG ACC TGG AGC CAC-3'), introducing BamHI/HindIII restrictions sites and a hexa-His tag. For the dimer, PCR product a was used for two independent PCRs. In the first one, the primers 52.ba (5' -GAC TAA CGA GAT CGC GGA TCC GGA GTG ACA CTC TTT GTG GCC CCT TAT-3') and 47.fo (5' -TCC GCC ACC GCC AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC CTG GAT AGA GTA TCG AGC CAC-3') and in the second PCR, the primers 48.ba (5' -GTT GGA GCC GGT TCA GGC GGA GGT GCC TCT GCC GTG GGC GGA GGA GTG ACA CTC TTT GTG GCC CCT TAT-3') and 51.fo (5' -ATC CCA AGC TTA GTG ATG ATG GTG ATG CAG ACT CTC TTC TGA GAT GAG TTT TTT TCC ACC CTG GAT AGA GTC 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 (GGGGSGGGSGGGG) between the two domains and a Myc and hexa-His tag at the C-terminal-end of the protein. Sequencing revealed an additional insertion of an amino acid in the linker region occurred during the PCRs, leading to the following sequence, GGGGGSGGGSGGGG. Both, D3 monomer and dimer, were ligated into BamHI/HindIII double-digested and ligated into BamHI/HindIII double-digested pQE-12 vector. After transformation of TG1 cells, colonies of the constructs were inoculated in 100 ml of 2xYT medium containing 100 μg/ml ampicillin and 0.1% (w/v) glucose and grown at 37 °C in a rotary shaker at 200 rpm at A_{600} of 0.6; protein expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (Applichem). After 16 h at 30 °C in a rotary shaker (200 rpm), the bacterial cells were harvested by centrifugation and resuspended in 4 ml of 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 rpm. 1 ml of Ni^{2+}-NTA slurry (Qiagen) was added to the cleared lysate to capture the His_{6}-tagged proteins (incubation at 4 °C for 1 h while shaking on a rota-shaker). The resin was washed two times with 5 ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0), and the protein was eluted with 2 ml of 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 20 μl of protein solution.

**Specificity ELISA**—The biotinylated target proteins EDB (23), human domain A1 of tenasin-C (35), murine domain C of tenasin-C (35), human domains C-D-6 of tenasin-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 described previously. Mouse serum albumin was purchased from Sigma and biotinylated using EZ-link™ sulfo-NHS-SS-biotin (Pernio) according to the 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 of PBS, 4% milk, and 50 μl of purified D3 dimer (3 μM) were added. After incubating for 1 h 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 by adding 1 M H2SO4.

**Size Exclusion Chromatography (SEC)**—SEC of purified Fyn SH3 WT, D3 monomer, and D3 dimer was performed on an AKTA FPLC system using a Superdex 75 column (Amersham Biosciences).

**Surface Plasmon Resonance Experiments**—Affinity measurements were performed using a BIAcore 3000 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 (23) immobilized. The running buffer was PBS, 0.1% NaN_{3}, and surfactant P20 (Biacore). The interactions were measured at a flow of 20 μl/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 (36) (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 20 μl/min and injections of different concentrations of 7B89. All kinetic data of the interaction (separate k_{on}/k_{off}) were evaluated using BIA evaluation 3.2RC1.

**Immunohistochemistry**—Female Sv129 mice (Charles River) received subcutaneous injection of 3 x 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, 10-μm sections were cut, fixed with ice-cold acetone, and double fluorescence staining for EDB and CD31 was performed. D3 monomer and rat anti-mouse 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.
Radioiodination of Fyn SH3 WT, D3 Monomer, and D3 Dimer—125–200 μg of protein was combined with 200 μCi of 125I (Amersham Biosciences) and with filtered chloramine T (Sigma) solution (5 mg/ml; 0.25 μg of chloramine T per μg of protein was used) for 2 min followed by separation from unincorporated iodine using a PD-10 disposable gel filtration column (GE Healthcare). The binding activity after labeling was evaluated by loading an aliquot of radiolabeled sample onto 500 μl of EDB-Sepharose resin (37) 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 as licensed3. E9 murine teratocarcinomas were implanted as described previously (23, 37) in 129Sv mice (8–12-week-old, female). 125I-Labeled protein (5.6–10 μg; 9–13 μCi) in 100 μl of saline solution, radiolabeled on the same day, was injected intravenously. Mice were sacrificed at 4 and 24 h (Fyn SH3 WT only 24 h), and organs were weighed and radioactivity was counted. Three animals were used for each time point (except Fyn SH3 WT for 24 h and D3 monomer for 4 h; four animals). Targeting results of representative organs are expressed as % ID of protein/g of tissue (±S.E.), and tumor:organ ratios were determined.

Immunovincidity of Fyn SH3 WT, D3, and a scFv Antibody Fragment—Sv129 mice were injected four times (every third day) with 20 μg of Fyn SH3 WT or D3, respectively (5 mice per group). One day after the fourth 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 of blood/mice diluted in 50-μl of Heparin (Bichsel)). As a positive control, four mice were injected at equal time points and equal molar dosages (60 μg) with the human scFv antibody fragment clone E1 (anti-human α5-macroglobulin, expression and purification as described in (38)). 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 h at room temperature. After washing, the presence of murine antibodies in the blood samples was 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) was added. The negative controls were performed using the anti-mouse IgG HRP antibody conjugate without adding any blood sam-

3 Tumor Targeting, Bewilligung 198/2005, issued to D. Neri by the Veterinäramt des Kantons Zürich.
vector as a monomer and as a genetically fused homodimer, expressed, and purified (Fig. 3a). The expression yields ranged between 7 and 94 mg/liter of bacterial culture under non-optimized conditions in shake flasks (Fig. 1c). D3 dimer bound EDB in a highly specific manner and did not cross-react with any of the other five structurally related fibronectin type 3 homology repeats of tenasin-C or to mouse serum albumin as determined by ELISA (Fig. 3b). The size exclusion chromatograms (Fig. 3c) showed a delayed elution time for D3 monomer compared with Fyn SH3 WT. The D3 dimer eluted later than expected, too. The binding properties of both D3 proteins were analyzed by real-time interaction analysis on a BIACore chip coated with biotinylated EDB (Fig. 3d), 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. 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 (36) (Fig. 3e). This analysis revealed a $K_D$ value of $1.0 \times 10^{-7}$ M, which was comparable to the $K_D$ value measured for the interaction between D3 monomer and biotinylated EDB. Furthermore, immunohistofluorescence experiments performed with cryosections of F9 teratocarcinoma confirmed the ability of D3 to stain tumor neo-vascular structures in a fashion similar to the one of an $\alpha$-CD-31 antibody used as a positive control (Fig. 4).

**Biodistribution Studies in Tumor-bearing Mice**—We used mice bearing subcutaneously 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. 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. 4 and 24 h after intravenous injections 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 Table 1. Only D3 monomer and D3 dimer selectively accumulated in the tumor, whereas 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 with the one of D3 monomer.

**Immunogenicity Studies in Mice**—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 intravenously four times either with Fyn SH3 WT or D3 every third day. One day after the fourth 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 (39). 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 or against D3 was detectable (ELISA signals $< 0.03$), but all mice had developed antibodies against the human scFv antibody fragment (Fig. 5).
FIGURE 3. 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 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 3 homology repeats. 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 an EDB-coated chip. e, BiACore sensogram of 7B89, a fibronectin fragment containing the EDB, injected at different concentrations on a D3-coated chip.
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 a high level in soluble form; it is monomeric; it is stable ($T_m$ 70.5 °C) (40); it does not contain any cysteine residues; and it is of human origin with an amino acid sequence, which is completely conserved from mouse to man. We designed and constructed a new Fyn SH3 phage library with two randomized loops, containing more than 1 billion individual clones. One of these clones (D3) exhibited a specific recognition of the pharmaceutical target EDB 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 but at later time points compared with the Fyn SH3 WT. Chromatographic fractions were submitted to SDS-PAGE (Fig. 3a, lanes 3 and 5) and BLIcore analysis (data not shown), confirming the identity of the protein in the chromatographic peak. The delayed elution may be because of interactions with the column matrix.

**TABLE 1**

| Biodistribution experiments of radiolabeled proteins in F9 tumor-bearing mice. The results are expressed as % ID/g ± S.E. Tumor to organ ratios (T:O) are also reported. ND indicates not determined. |
|---|---|---|---|
|  | 4 h | 24 h |
| D3 monomer | % ID/g | % T:O | % ID/g | % T:O |
| Tumor | 5.60 ± 0.39 | 0.69 ± 0.12 | 0.39 | 0.69 | 0.12 |
| Liver | 2.09 ± 0.10 | 0.09 ± 0.00 | 2.14 ± 0.17 | 2.6 | 0.07 ± 0.00 | 9.9 |
| Lung | 4.10 ± 0.48 | 1.4 | 0.16 ± 0.01 | 4.3 |
| Spleen | 2.34 ± 0.09 | 2.4 | 0.08 ± 0.01 | 8.6 |
| Heart | 6.75 ± 0.19 | 0.8 | 0.23 ± 0.01 | 3.0 |
| Kidney | 1.88 ± 0.08 | 3.0 | 0.13 ± 0.02 | 5.3 |
| Intestine | 5.15 ± 0.26 | 1.1 | 0.12 ± 0.01 | 5.8 |

| Fyn SH3 WT | % ID/g | % T:O |
| Tumor | ND | ND | 0.19 ± 0.04 |
| Liver | ND | ND | 0.08 ± 0.00 | 2.4 |
| Lung | ND | ND | 0.1 ± 0.01 | 1.9 |
| Spleen | ND | ND | 0.07 ± 0.01 | 2.7 |
| Heart | ND | ND | 0.06 ± 0.00 | 3.2 |
| Kidney | ND | ND | 0.25 ± 0.02 | 0.8 |
| Intestine | ND | ND | 0.29 ± 0.05 | 0.7 |
| Blood | ND | ND | 0.16 ± 0.01 | 1.2 |

| D3 dimer | % ID/g | % T:O |
| Tumor | 3.44 ± 0.34 | 2.62 ± 1.03 |
| Liver | 1.25 ± 0.18 | 2.7 | 0.14 ± 0.00 | 18.7 |
| Lung | 3.29 ± 0.51 | 1.0 | 0.55 ± 0.00 | 4.8 |
| Spleen | 1.68 ± 0.28 | 2.0 | 0.14 ± 0.01 | 18.7 |
| Heart | 1.62 ± 0.20 | 2.1 | 0.12 ± 0.00 | 21.8 |
| Kidney | 6.71 ± 0.60 | 0.5 | 0.65 ± 0.01 | 4.0 |
| Intestine | 1.79 ± 0.12 | 1.9 | 0.29 ± 0.05 | 9.0 |
| Blood | 3.45 ± 0.61 | 1.0 | 0.3 ± 0.01 | 8.7 |

**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 a high level in soluble form; it is monomeric; it is stable ($T_m$ 70.5 °C) (40); it does not contain any cysteine residues; and it is of human origin with an amino acid sequence, which is completely conserved from mouse to man. We designed and constructed a new Fyn SH3 phage library with two randomized loops, containing more than 1 billion individual clones. One of these clones (D3) exhibited a specific recognition of the pharmaceutical target EDB 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 but at later time points compared with the Fyn SH3 WT. Chromatographic fractions were submitted to SDS-PAGE (Fig. 3a, lanes 3 and 5) and BLIcore analysis (data not shown), confirming the identity of the protein in the chromatographic peak. The delayed elution may be because of interactions with the column matrix.
The dissociation constant ($K_D$) of D3 monomer toward biotinylated EDB (8.5 × 10$^{-8}$ m) was satisfactory, considering that the D3 clone was isolated directly from a naive 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 × 10$^{-7}$ m) was observed for the interaction between D3 monomer and non-biotinylated 7B89, a fibronectin fragment containing the EDB. 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. 3b). For practical applications, the cloning and expression of a D3 dimer was sufficient for yielding a good binding avidity to the antigen and excellent 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 (Table 1). 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 Ref. 41).

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 therapeutical 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 of Fyn SH3 WT, D3, and of a human scFv antibody fragment in mice. After repeated intravenous injections, no antibodies against Fyn SH3 WT and D3 could be detected (ELISA signals < 0.03), whereas mice treated with scFv exhibited a strong antibody reaction against the human protein (Fig. 5). 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. Furthermore, the library of Fyn SH3 mutants described in this article may represent a rich source of antigen binding specificities. This single-pot library may provide useful reagents for many biochemical and biomedical applications as an alternative to more conventional IgG-based immunochemical technologies.

Acknowledgment—We thank Prof. Luciano Zardi for many helpful discussions.

REFERENCES

1. Nygren, P. A., and Uhlen, M. (1997) Curr. Opin. Struct. Biol. 7, 463–469
2. Binz, H. K., and Pluckthun, A. (2005) Curr. Opin. Biotechnol. 16, 459–469
3. Smith, G. (1998) Trends Biochem. Sci. 23, 457–460
4. Tomlinson, I. M. (2004) Nat. Biotechnol. 22, 521–522
5. Binz, H. K., Amstutz, P., and Pluckthun, A. (2005) Nat. Biotechnol. 23, 1257–1268
6. Hey, T., Fiedler, E., Rudolph, R., and Fiedler, M. (2005) Trends Biotechnol. 23, 514–522
7. Dennis, M. S., Zhang, M., Meng, Y. G., Kadkhodayan, M., Kirchofer, D., Combs, D., and Damico, L. A. (2002) J. Biol. Chem. 277, 35035–35043
8. Mirick, G. R., Bradt, B. M., Denardo, S. J., and Denardo, G. L. (2004) Q J Nucl. Med. Mol. Imaging 48, 251–257
9. Hiipakka, M., Poikonen, K., and Saksela, K. (1999) J. Mol. Biol. 293, 1097–1106
10. Panni, S., Dente, L., and Cesareni, G. (2002) J. Biol. Chem. 277, 21666–21674
11. Musacchio, A., Wilmanns, M., and Maraste, M. (1994) Prog. Biophys. Mol. Biol. 61, 283–297
12. Musacchio, A. (2002) Adv. Protein Chem. 61, 211–268
13. Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993) Science 259, 1157–1161
14. Karkkainen, S., Hiipakka, M., Wang, J. H., Kleinio, I., Vaha-Jaakkola, M., Renkema, G. H., Liss, M., Wagner, R., and Saksela, K. (2006) EMBO Rep. 7, 186–191
15. Cooke, M. P., and Perlmuter, R. M. (1989) New Biol. 1, 66–74
16. Resh, M. D. (1998) Int. J. Biochen. Cell. Biol. 30, 1159–1162
17. Semb, K., Nishizawa, M., Miyajima, N., Yoshida, M. C., Sukagewa, J., Yamanashi, Y., Sasaki, M., Yamamoto, T., and Toyoshima, K. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5459–5463
18. Kawakami, T., Pennington, C. Y., and Robbins, K. C. (1986) Mol. Cell. Biol. 6, 4195–4201
19. Castellani, P., Viale, G., Dorcaratato, A., Nicolo, G., Kaczmarek, J., Querze, G., and Zardi, L. (1994) Int. J. Cancer 59, 612–618
20. Zardi, L., Carnemollta, B., Sira, A., Petersen, T. E., Paolotta, G., Sebastio, G., and Baralle, F. E. (1987) EMBO J. 6, 2337–2342
21. Carnemollta, B., Balza, E., Sira, A., Zardi, L., Nicotra, M. R., Bigotti, A., and Natali, P. G. (1989) J. Cell Biol. 108, 1139–1148
22. Kaczmarek, J., Castellani, P., Nicolo, G., Spina, B., Allemanni, G., and Zardi, L. (1994) Int. J. Cancer 59, 11–16
23. Carnemollta, B., Neri, D., Castellani, P., Leprini, A., Neri, G., Pini, A., Winter, G., and Zardi, L. (1996) Int. J. Cancer 68, 397–405
24. Castellani, P., Borsi, L., Carnemollta, B., Biro, A., Dorcaratato, A., Viale, G. L., Neri, D., and Zardi, L. (2002) Am. J. Pathol. 161, 1695–1700
25. Hauptmann, S., Zardi, L., Sira, A., Carnemollta, B., Borsi, L., Castellucci, M., Klosterhalfen, B., Hartung, P., Weis, J., and Stocker, G. (1995) Lab. Invest. 73, 172–182
26. Birchler, M. T., Milisavljevic, D., Pfaltz, M., Neri, D., Odermatt, B., Schmidt, S., and Stoeckli, S. J. (2003) Laryngoscope 113, 1231–1237
27. Vogel, W., Berndt, A., Muller, A., Dahlse, R., Haas, K. M., Borsi, L., Zardi, L., and Kosmehl, H. (2003) Int. J. Mol. Med. 12, 831–837
28. Menrad, A., and Menssen, H. D. (2005) Expert Opin. Ther. Targets 9, 491–500
29. Berndorf, D., Borkowski, S., Sieger, S., Rother, A., Friebe, M., Viti, F., Hilger, C. S., Cyn, J. E., and Dinkelborg, L. M. (2005) Clin. Cancer Res. 11, S7053–S7063
30. Borsi, L., Balza, E., Carnemollta, B., Sassi, F., Castellani, P., Berndt, A., Kosmehl, H., Biro, A., Sira, A., Orecchia, P., Grassi, J., Neri, D., and Zardi, L. (2003) Blood 102, 4384–4392
31. Balza, E., Mortara, L., Sassi, F., Monteghirfo, S., Carnemollta, B., Castellani, P., Neri, D., Accolla, R. S., Zardi, L., and Borsi, L. (2006) Clin. Cancer Res. 12, 2575–2582
32. Viti, F., Nilsson, F., Demartis, S., Huber, A., and Neri, D. (2000) Methods Enzymol. 326, 480–505
33. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., and Winter, G. (1991) Nucleic Acids Res. 19, 4133–4137
34. Harrison, J. L., Williams, S. C., Winter, G., and Nissim, A. (1996) Methods Enzymol. 267, 83–109
35. Brack, S. S., Silacci, M., Birchler, M., and Neri, D. (2006) *Clin. Cancer Res.* **12**, 3200–3208
36. Carnemolla, B., Leprini, A., Allemanni, G., Saginati, M., and Zardi, L. (1992) *J. Biol. Chem.* **267**, 24689–24692
37. Neri, D., Carnemolla, B., Nissim, A., Leprini, A., Querze, G., Balza, E., Pini, A., Tarli, L., Halin, C., Neri, P., Zardi, L., and Winter, G. (1997) *Nat. Biotechnol.* **15**, 1271–1275
38. Ettorre, A., Rosli, C., Silacci, M., Brack, S., McCombie, G., Knochenmuss, R., Elia, G., and Neri, D. (2006) *Proteomics* **6**, 4496–4505
39. Halin, C., Rondini, S., Nilsson, F., Berndt, A., Kosmehl, H., Zardi, L., and Neri, D. (2002) *Nat. Biotechnol.* **20**, 264–269
40. Filimonov, V. V., Azuaga, A. I., Viguera, A. R., Serrano, L., and Mateo, P. L. (1999) *Biophys. Chem.* **77**, 195–208
41. Borsi, L., Balza, E., Bestagno, M., Castellani, P., Carnemolla, B., Biro, A., Leprini, A., Sepulveda, J., Burrone, O., Neri, D., and Zardi, L. (2002) *Int. J. Cancer* **102**, 75–85