Molecular Medicine 7(7): 461–469, 2001

Species-Crossreactive scFv Against the Tumor Stroma Marker "Fibroblast Activation Protein" Selected by Phage Display From an Immunized FAP$^{-/-}$ Knock-Out Mouse

Bodo Brocks,[1] Pilar Garin-Chesa,[2] Eva Behrle,[1] John E. Park,[2] Wolfgang J. Rettig,[2] Klaus Pfizenmaier,[1] and Dieter Moosmayer[1]

[1]Institut für Zellbiologie und Immunologie, Universität Stuttgart, Stuttgart, Germany
[2]Boehringer Ingelheim Pharma KG, Biberach (Riss), Germany

Accepted April 10, 2001

Abstract

Background: Fibroblast activation protein (FAP) is a type II membrane protein expressed on tumor stroma fibroblasts in more than 90% of all carcinomas. FAP serves as a diagnostic marker and is potential therapeutic target for treatment of a wide variety of FAP^{+} carcinomas. Murine tumor stroma models and FAP-specific antibodies are required to investigate the functional role of FAP in tumor biology and its usefulness for drug targeting. We here describe the development of antibodies with crossreactivity for human (hFAP) and murine FAP (mFAP), which share 89% amino acid identity.
Material and Methods: An FAP$^{-/-}$ mouse was sequentially immunized with recombinant murine and human FAP-CD8 fusion proteins. Immunoglobulin cDNA derived from hyperimmune spleen cells was used for the construction of a combinatorial single chain Fv (scFv) library. Phage display selection of FAP-specific scFv was performed on immobilized hFAP followed by selection on cells expressing murine FAP.
Results: High-affinity, species-crossreactive, FAP-specific scFv were isolated upon sequential phage display selection. A bivalent derivative (minibody MO36) constructed thereof was applied for immunohistochemical analyses and allowed detection of FAP expression on stroma cells of different human carcinomas as well as on murine host stroma in a tumor xenograft model.
Conclusions: MB MO36, derived from phage display selected species crossreactive scFv, is suitable for tumor stroma targeting and will be a valuable tool in the analyses of the functional role of FAP in tumor biology as well as in the evaluation of the suitability of FAP for drug targeting.

Introduction

The stroma compartment of solid tumors consists of nontransformed cell types, newly formed tumor blood vessels, and abundant stromal fibroblasts, which typically contribute 20–60% of the total tumor mass (1,2). The tumor stroma is considered to play a key role in nutrient and oxygen supply of tumors as well as in tumor invasion and metastasis (2–6). Because of the tumor stroma's essential function for the tumor along with its large structural contribution, it is considered not only as a diagnostic but also as a potentially important therapeutic target.

The tumor stroma-associated antigen fibroblast activation protein (FAP) is located on stromal fibroblast of over 90% of all epithelial tumors, like breast, lung, or colon carcinomas (1). FAP is a type II transmembrane protein with serine protease activity, originally identified by the monoclonal antibody F19 (6–9).

Radiolabeled mab F19 has been successfully used for detection of colon carcinoma metastases (2). This phase I clinical study provided evidence for the accessibility of the tumor stroma compartment via the circulation and thereby confirmed the feasibility of an antibody-mediated tumor stroma targeting concept for the treatment of solid tumors. An advantage of this approach compared to a direct targeting of the malignant cell is that stromal fibroblasts are genetically more stable than neoplastic cells. For this reason, the development of therapy resistance, caused by a loss of target antigen, seems less likely.

To study the physiologic function of FAP in vivo and its role in tumor formation and maintenance in more detail, the establishment of a murine tumor stroma model is of interest. For this purpose, the murine homologue of FAP was recently cloned and functionally characterized (10). Murine FAP (mFAP) shares 89% amino acid identity with human FAP (hFAP) and exhibits the same structural features. Human carcinoma xenografts in immunodeficient mice revealed characteristic histologic patterns of tumor stroma and malignant epithelial cells as seen in human biopsy material, with the tumor stroma

Address correspondence and reprint requests to: Klaus Pfizenmaier, Institut für Zellbiologie und Immunologie, Allmandring 31, 70569 Stuttgart, Germany.
Phone: 49-711-6856986; Fax 49-711-6857484; e-mail: klaus.pfizenmaier@po.uni-stuttgart.de
Present address for D. Moosmayer: Schering AG, 13342 Berlin, Germany
Present address for B. Brocks: Morphosys AG, 82152 Martinsried, Germany

cells being of murine origin as confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) of mFAP expression (10).

The existing FAP-specific reagents show no cross-reactivity among different species. Therefore, our intention was to generate recombinant antibodies with crossreactivity to both hFAP and mFAP to establish murine models for evaluation of potential therapeutic reagents for human cancers. Taking advantage of the great potential of recombinant antibody technology (for review see Hayden et al. [11], and Carter et al. [12]), the phage display technique was chosen to isolate scFv with the desired FAP specificity. To circumvent immunologic tolerance against murine antigenic determinants, a $FAP^{-/-}$ mouse (13) was used for immunization with recombinant hFAP.

Materials and Methods

Construction of a Combinatorial Murine scFv Phagemid Library from FAP-Immune Mice

Spleen cells from a hyperimmunized $FAP^{-/-}$ mouse (13) have been used as a source for mRNA extraction. The mouse was immunized using the following schema: three immunizations with 10–30 μg of a mouse CD8-hFAP complexed with the chimeric FAP-specific antibody cF19 (6), followed by two boosts of 10 μg each of a mouse CD8-mFAP. A rise in serum titer was followed by enzyme-linked immunosorbent assay (ELISA). Two and a half days after the last boost, spleen cells were taken for mRNA extraction. Random hexa-nucleotid–primed cDNA synthesis was performed using the first strand synthesis kit from Roche Diagnostics, Mannheim, Germany. For PCR amplification of the Ig V-regions, a primer set based on primers previously described by Zhou et al. (14) and Dattmajumdar et al. (15) was constructed (Tables 1 and 2).

PCR amplification was carried out in a total volume of 100 μl containing 1–2 μl of cDNA from the reverse transcription reaction mentioned above, 50 pmol of each primer, 200 μM of dNTPs, 10 μl of 10$\times$ PCR buffer, 2 mM of $MgCl_2$, and 1 U of Goldstar DNA polymerase (Eurogen-tec, Seraing, Belgium). Each variable heavy chain back primer (MHV.1-MHV.12) was combined with the forward primer MHC.F. Each κ light chain back primer (MKV.B1-MKV.B10) was combined with the forward primer MKC.F. The λ chain primer MLV.B was combined with the forward primer MLC.F. All primer pairs were used in separate reactions. For introduction of restriction sites, a second set of primers was constructed as follows: 5′ of each heavy chain back primer the sequence 5′-GAATAGGCCATGGCG-3′ (*Nco I* site underlined) and 5′ of each light chain back primer the sequence 5′-TACAGGATCCACGCTA-3′ (*Mlu I* site underlined) was added. To eliminate an internal *Hin*d III site in MHV.B1, the following primer was used for introduction of the *Nco* I restriction site: 5′-GAATAGGCCATGGCGATGTGAAGCT*G*CAGGAGTC-3′ (*Nco* I site underlined). For introduction of a *Hin*d III site, the following secondary heavy chain forward primer was used: GGCCAGTGGATAAAGCTTTGGGGGTGTCGTTTTGGC (*Hin*d III site underlined). 5′ of the forward primers for κ– and λ–chains the sequence 5′-GACAAGCTTGCGGCCGC-3′ (*Not* I site underlined) was added.

The PCR reaction was performed with 25 cycles using a HYBAID thermocyler. The thermal cycle was 95°C for 20 sec (denaturation), 52–55°C for 30 sec (annealing), and 72°C for 30 sec (extension). For introduction of restriction sites, a second PCR reaction was performed using 10 ng of each PCR frag-

Table 1. Primers for PCR amplification of murine VH regions.

Term	Nucleotide Sequence 5′ to 3′
MHV.B1	GATGTGAAGCTTCAGGAGTC
MHV.B2	CAGGTGCAGCTGAAGGAGTC
MHV.B3	CAGGTGCAGCTGAAGCAGTC
MHV.B4	CAGGTTACTCTGAAAGAGTC
MHV.B5	GAGGTCCAGCTGCAACAATCT
MHV.B6	GAGGTCCAGCTGCAGCAGC
MHV.B7	CAGGTCCAACTGCAGCAGCCT
MHV.B8	GAGGTGAAGCTGGTGGAGTC
MHV.B9	GAGGTGAAGCTGGTGGAATC
MHV.B10	GATGTGAACTTGGAAGTGTC
MHV.B11	GAGGTCCAGCTGCAACAGTC
MHV.B12	GAGGTGCAGCTGGAGGAGTC
MHC.F	GCCAGTGGATAGTCAGATGGGGGT GTCGTTTTGGC

Table 2. Primers for amplification of murine VL regions.

Term	Nucleotide Sequence 5′ to 3′
MKV.B1	GATGTTTTGATGACCCAAACT
MKV.B2	GATATTGTGATGACGCAGGCT
MKV.B3	GATATTGTGATAACCCAG
MKV.B4	GACATTGTGCTGACCCAATCT
MKV.B5	GACATTGTGATGACCCAGTCT
MKV.B6	GATATTGTGCTAACTCAGTCT
MKV.B7	GATATCCAGATGACACAGACT
MKV.B8	GACATCCAGCTGACTCAGTCT
MKV.B9	CAAATTGTTCTCACCCAGTCT
MKV.B10	GACATTCTGATGACCCAGTCT
MKC.F	GGATACAGTTGGTGCAGCATC
MLV.B	CAGGCTGTTGTGACTCAGGAA
MLC.F	GGTGAGTGTGGGAGTGGACTTGGGCTG

ment from the first PCR as template and primers as described above. Each primer pair was used in separate reactions and 10–12 thermal cycles. The annealing temperature for the first two cycles was 56°C, for the next two cycles 58°C, and the rest of the cycles 67°C. The temperature for denaturation and extension was chosen as described above.

For generation of a VH-library, the H-chains amplified in the second PCR were purified by agarose gel electrophoresis, digested with *Nco*I and *Hin*dIII, once more purified via an agarose gel, and ligated into the vector pSEX 81 (16). The transformation was performed with electrocompetent *Escherichia coli* XL-1 blue (Stratagene, La Jolla, CA USA). The L-chains from the second PCR were purified as described above, digested with *Mlu*I and *Not*I, and ligated into the pSEX 81-VH-library. For transformation, electroporation was used as described. Digestion with restriction enzymes, ligation, and transformation were performed according to protocols described previously (16).

Phage Display Selection

Phage display selection in immunotubes was essentially performed as described earlier (17). Purified CD8-FAP fusion protein (6) was produced in insect cells. Immobilization was done with 20 μg/ml of recombinant protein in 1 ml of ice-cold phosphate-buffered saline (PBS). For panning on whole cells, human fibrosarcoma cells (HT1080) and human embryonal kidney epithelial cells (HEK 293) stably transfected with a full-length human FAP cDNA (HT1080 hFAP$^+$) and murine FAP cDNA (HEK 293 mFAP$^+$), respectively, were used. The phages (10^{10}–10^{11} TU) were preadsorbed in 2% milk powder in PBS (MPBS) for 15 min at room temperature (RT). The mixture was clarified at 12,000 rpm in an Eppendorf centrifuge. The phage containing supernatant was then incubated with $\sim 5 \times 10^6$ control cells (HT1080 or HEK 293) for 30 min at RT. The cells were carefully sedimented by centrifugation (1200 rpm, 45 sec) in an Eppendorf centrifuge. The preadsorbed phage suspension was then incubated with 10^5–10^6 FAP$^+$ cells for 30–40 min at RT. After 10–15 wash steps with PBS + 0.1% bovine serum albumin (BSA), the phages were eluted with 0.1 M of HCl/glycine pH 2.2 + 0.1% BSA. Optional (off rate panning): The cells were incubated for 2 hr in presence of an excess of soluble CD8-hFAP (30 μg/ml) before elution.

Production and Purification of scFv

The scFv encoding regions were subcloned into the expression vector pOPE 101 (18). The applied scFv expression cassette contained between VH and VL a linker of 18 amino acids derived from the tubulin sequence (16,18). Transformed *E. coli* JM109 were induced with 30 μM of IPTG and incubated for 2–3 hr at 22–25°C. Harvested bacteria were sonified and clarified by centrifugation (30,000 g for 30 min). His-tagged scFv were enriched via Ni_2^+- or Zn_2^+-Fast Flow Sepharose (Pharmacia, Freiburg, Germany), basically as previously described (19).

The concentration of scFv in partially purified samples or crude bacterial lysates was determined by immunoblot analyses using a highly purified scFv as reference (19).

Sandwich ELISA

Maxisorb ELISA plates (Nunc, Rochester, NY, USA) were coated with rat anti-CD8 mab o/n at 4°C (200 ng/well). After blocking with Roti-Block (Roth, Karlsruhe, Germany) and washing, CD8-FAP (1 μg/ml) in MPBS was incubated with the immobilized ab for 1 hr at RT or o/n at 4°C. Serial dilution of scFv containing induced *E. coli* lysates in MPBS were added and incubated for 2 hr at RT. For detection, biotinylated c-myc–specific mouse mab 9E10 (Cambridge Research Biochemicals, Billingham, Cleveland, UK) was added followed by POD coupled streptavidin (Dianova, Hamburg, Germany), each incubated for 1 hr at RT. Optical density (405 nm) was measured 15–30 min after ABTS (Roche) substrate addition. For determination of functional affinity, half maximal binding concentration (EC_{50}) was calculated with the help of a hyperbolic curve fit algorithm using Sigma Plot software (Jandel Scientific, San Rafael, CA, USA).

The calculated EC_{50} from the sandwich ELISA corresponded well with the affinities determined by the method of Friguet et al. (20).

Competition ELISA for Epitope Characterization

A constant concentration (~5 nM) of scFv MO33 or MO36 was mixed with various concentrations (0.2–100 nM) of cF19. The mixtures were transferred to an ELISA plate previously coated with 100 ng of CD8-hFAP per well. After incubation of 1 hr, detection of bound scFv was done with mab 9E10 followed by POD conjugated goat-anti-mouse (Dianova), each for 1 hr at RT. Optical density was measured after addition of ABTS substrate as described above. All samples were analyzed in duplicate.

Minibody Construction and Expression

An expression cassette was constructed as described by Hu et al. (21), which encoded the following domains (5′-3′ orientation): signal sequence, scFv MO36, human hinge region (IgG1), linker, human CH3 (IgG1), c-myc tag, His_6 tag. This cassette was expressed and secreted in COS 7 cells using a standard eukaryotic expression vector. The selected minibody was designated MB MO36.

Cell-Binding Assay (Cell ELISA)

For cell-binding studies, crude bacterial lysate or scFv partially purified by IMAC (22) was used. For preadsorption, 10% MPBS was added to a final concentration of 2% milk and incubated for at least 15 min at RT. The mix was clarified by centrifugation (13,000 rpm, 5 min, 2–3 times) and 3 mM of azide was added. Dilutions were made in PFA

(PBS, 2% FCS, 3 mM of azide). One hundred microliters of scFv solution per well were mixed with 2–3 $\times$ 10^4 cells in a v-form tissue culture plate. After an incubation of 30–40 min at RT, the cells were washed three times with ice-cold PFA (centrifugation 1500 rpm, 5 min 4°C). For detection, mab 9E10 in PFA and goat anti-mouse (POD conjugated) in PBS + 0.1% BSA (without azide) were used. The incubation time of the detection antibodies was 30 min at RT each. Washing was performed as described above. The last washing steps were done with PBS + 0.1% BSA. For the colorimetric reaction TMB (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA substrate was added to the cells and the extinction measured at 630 nm after 15–60 min. The cell lines used for this assay were identical to those used for the phage display selection. As control for unspecific binding, the corresponding FAP-negative parental cell lines (HT1080, HEK 293) were used. Alternatively, cell binding of MB MO36 was analyzed using flow cytometry. In this case, detection of cell-bound MB MO36 was done using a FITC-labeled anti-human IgG serum (Dianova). EC_{50} values were determined as described for the sandwich ELISA.

Murine Tumor Xenograft Model

Breast carcinoma cell line ZR75.1 cells (1×10^6) were injected subcutaneously in the flank of a NMRI nu/nu mouse. Tumors were allowed to grow until they reached a diameter of approximately 0.5 cm. The mice were killed, and the tumors were excised, frozen, and stored at −80°C until use.

Immunohistochemistry

Immunohistochemical stainings were performed on freshly frozen tissue sections by the avidin-biotin complex immunoperoxidase method: 5-μm thick frozen sections were cut, mounted on poly-L-lysine coated slides, air dried, and fixed in acetone (4°C for 10 min). Sections were treated with 0.3% H_2O_2 for 3 min to block endogeneous peroxidase followed by blocking with normal horse or goat serum for 30 min at RT. Slides were incubated at 4°C with purified MB MO36 (10–20 μg/ml) or negative control antibodies for 12–18 hr. Sections from mouse tissues were washed and incubated with biotinylated goat anti-human IgG (1:500, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature, followed by avidin-biotin-horseradish peroxidase (HRP) complex (1:100 dilution). For the detection of FAP expression in human tissues, mab 9E10 (hybridoma culture supernatant, 1:5) was applied for 1 hr at RT, followed by biotinylated horse anti-mouse IgG (1:100, Vector Laboratories) and the avidin-biotin-HRP complex. The final reaction product was visualized with the chromogen DAB. Sections were counterstained with Harris' hematoxylin.

Results

Immunization of a FAP$^{-/-}$ Mouse With CD8-hFAP Fusion Protein

To circumvent immunologic tolerance to self-epitopes of FAP, we used a FAP$^{-/-}$ mouse (13) for immunization with recombinant CD8-hFAP and CD8-mFAP fusion protein.

The serum of the immunized "knock-out" mouse contained a high antibody titer (1:20,000) against hFAP as revealed by ELISA (data not shown). Even though the mouse was immunized using CD8-hFAP in an immobilized complex with the chimeric FAP-specific antibody cF19γ1, no response against this antibody could be detected. A control serum was negative for both antigens (data not shown).

Construction of a Combinatorial scFv

Splenic cDNA from the immunized mouse was used to construct a murine scFv library for phage display selection. For efficient PCR amplification of a representative murine V region repertoire, we developed a set of primers based on murine Ig primers previously described (14,15; Tables 1 and 2). The applied primer set comprised 11 5′-primers for amplification of the VH regions, 9 5′-primers for Vk, and 1 for Vl. The 3′-primers were annealed to the constant regions as described (23). To avoid bias during the amplification process, each of the nondegenerated primer pairs was used in separate reactions. For each reaction, 25 PCR cycles were sufficient for an efficient amplification (data not shown). In a second PCR, restriction sites for cloning of the VH and VL regions were introduced.

In a first cloning step, a VH sublibrary was constructed (1×10^7 independent clones). This sublibrary was randomly combined with the light chain repertoire resulting in a combinatorial library of approximately 1×10^8 independent clones. To check for correct expression of the scFv-pIII fusion protein, randomly picked clones were induced with IPTG and analyzed in an immunoblot using the mab 10C3, which is specific for the pIII minor coat protein of the filamentous phage M13 (24). About 80% of the analyzed clones produced fusion protein of the expected size of 116 kDa (data not shown). Diversity of the library was checked by *Bst*NI fingerprints of PCR amplified VH and VL regions. From 14 clones checked, only 2 light chain restriction pattern were indistinguishable in a high-resolution agarose gel. All heavy chain patterns were different (data not shown).

Phage Display Selection on Immobilized CD8-hFAP Fusion Protein

The first round of panning was carried out using CD8-hFAP immobilized in immunotubes. For detection of specific binders, scFv-pIII fusion proteins were produced in *E. coli* in microtiter plates and subsequently an antigen-specific ELISA was performed as described previously (25). About 25% of the

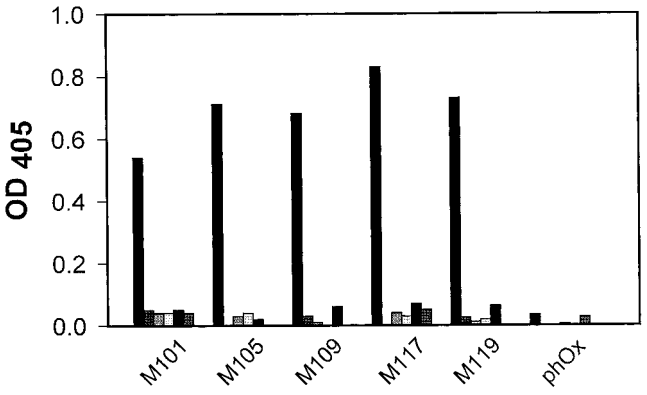

Fig. 1. Ag specificity of scFv-pIII fusion proteins from the first panning round. Supernatants of induced microcultures assayed by ELISA as described in Materials and Methods. ELISA wells were precoated with 100 ng/well of the indicated antigens. Antigens (from left to right): CD8-hFAP, tetanus toxoid, HSA, chymotrypsinogen, human serum, and milk proteins.

analyzed clones bound specifically to CD8-hFAP (Fig. 1). Sequence analyses showed that all positive clones consist of different heavy and light chains. The majority of the heavy chains belong to VH subgroup 2A (data not shown).

Selection on mFAP Positive Cells

With the intention to isolate scFv with reactivity toward both human and murine FAP, further panning rounds were performed on murine FAP. To obtain scFv with favorable specificity and affinity for cell-bound mFAP and to avoid selection of scFv, which recognize denatured antigen, we established a selection strategy using a suspension of mFAP-overexpressing HEK 293 cells. After two rounds of selection on cells, the phages eluted from $mFAP^+$ cells were significantly enriched in comparison to phages eluted from $mFAP^-$ control cells (Table 3). In the later selection round, we chose stringent selection conditions to enrich for scFv with slow off-rates. Therefore, bound phages were incubated for 2 hr in the presence of an excess of soluble CD8-hFAP at RT. The scFv clones selected with this procedure were screened for FAP-specific binders using an ELISA as described above. Seventy percent of the analyzed clones (20 of 28) showed specific binding to CD8-hFAP. *Bst*NI-fingerprints and sequencing revealed that one clone (MO36) was predominant. However, another clone (MO33) could be identified as well.

For further analyses, the V regions of both clones were subcloned into the plasmid pOPE101 for periplasmic expression of scFv. Soluble scFv could be produced in amounts of 5–10 mg/ of *E. coli* culture.

Sequence Analyses of scFv MO33 and MO36

Amino acid sequences of V regions of MO33 and MO36 are shown in Figure 2. According to Kabat et al. (26), V_H MO36 belongs to subgroup II(A) and MO33 to subgroup II(B). Although no significant

Table 3. Phage panning on hFAP coated immunotubes and cells overexpressing mFAP

Panning Round	Enrichment Factor	Percent Binders
I. hFAP coated antigen	≥8[#]	25
II. $mFAP^+$ cells	7	ND
III. $mFAP^+$ cells	100	70

Panning was carried out on recombinant human FAP coated on immunotubes (coated with 20 μg/tube) or on HEK 293 cells overexpressing mFAP (round II, 10^6 cells; round III, 10^5 cells in a total volume of 1 ml). In parallel, panning was done on noncoated tubes or control cells, respectively. To determine the enrichment factor, the phage output titer from panning on antigen was divided by the output titer of the control. Percentage of binders was determined after ELISA screening using CD8-hFAP fusion protein.

[#]Plates of negative control showed no colonies

H-CDR homologies could be observed, both clones—as well as mAk F19 (27)—share the joining region JH4 (28). V_K MO36 was assigned to subgroup III. V_L MO33 could be identified as λ-chain (26).

Binding of scFv MO33 and scFv MO36 to Human and Murine Recombinant FAP

To determine the antigen binding characteristics of the selected scFv, we developed a sandwich ELISA in which CD8-hFAP or CD8-mFAP is presented in

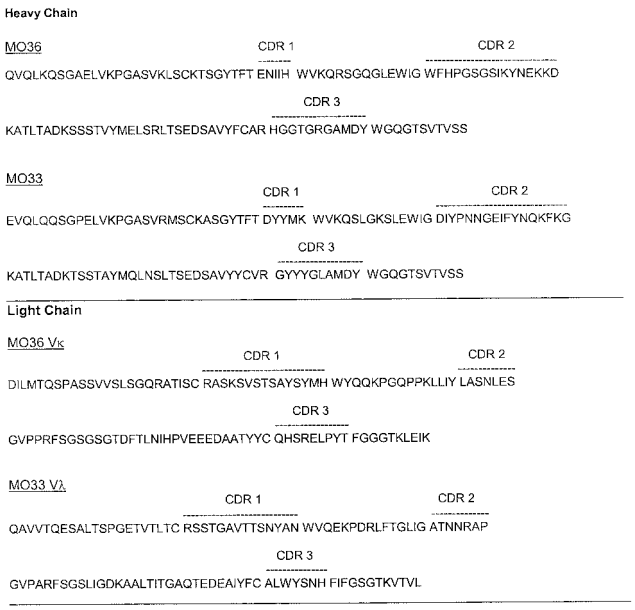

Fig. 2. Sequence analysis of V_H and V_L regions of clone MO36 and MO33. Amino acid sequences of V_H and V_L regions of clone MO36 and MO33 are shown. CDR, complementarity determining regions.

its native form via an immobilized rat anti-CD8 mab. Variable concentrations of scFv MO33 and scFv MO36 were incubated with this complex (Fig. 3). The apparent affinity for CD8-hFAP and CD8-mFAP was determined for both scFv by calculation of half maximal binding concentration using a hyperbolic curve fit algorithm. For scFv MO36, the binding characteristics on murine and human FAP were nearly indistinguishable. The apparent affinity for both antigens was around 8–9 nM (Fig. 3A). For scFv MO33, a significant difference in the apparent affinity for CD8-hFAP (5 nM) and CD8-mFAP (100 nM) was measured (Fig. 3B). Comparable affinities could also be measured for both scFv by the method of Friguet et al. (20) using soluble CD8-hFAP as competitor (data not shown). Due to limited availability of CD8-mFAP, the latter experiment could not be performed with the murine homologue.

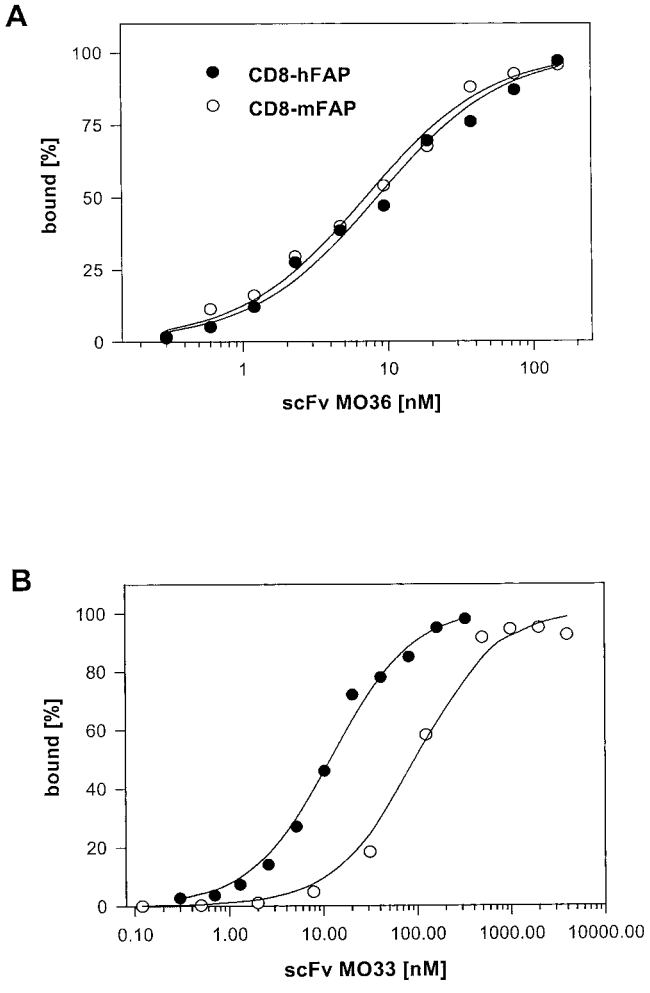

Fig. 3. Sandwich ELISA for affinity determination of scFv MO36 (A) and scFv MO33 (B). Bacterial lysates of scFv MO36 and partially purified scFv MO33 were diluted and incubated on human or murine CD8-FAP fusion protein immobilized via a CD8-specific rat mab. For detection, biotinylated c-myc–specific mab 9E10, POD-streptavidin, and ABTS substrate (measured at 405 nm) were used.

Binding of scFv MO33 and scFv MO36 on Cells

To study the binding characteristics on cells, a nonradioactive cell-binding assay using cells in suspension was performed (Fig. 4). EC_{50} was determined as described for the sandwich ELISA. Both scFv showed comparable binding characteristics on cell-bound FAP and on CD8-fusion protein. Again, the highest apparent affinity was revealed for scFv MO36 with an EC_{50} of 1.5 nM on mFAP overexpressing cells.

Competition Studies With the FAP-Specific Chimeric mab cF19γ1

Epitope analyses of human FAP using different mab against FAP revealed that the antigenic determinant recognized by mab F19 is immunodominant (29). In the present study, this could be confirmed using scFvs from the first panning round in a competition assay with cF19γ1. Two of five analyzed scFv from the selected panel could be competed by F19 (data not shown). Moreover, although scFv MO33 can be completely competed by cF19γ1, no competition was observed for scFv MO36 (Fig. 5). Together, these findings suggest that the epitope recognized by scFv MO33—but not the one recognized by scFv MO36—is overlapping or at least in close proximity to the F19 epitope. However, owing to the fact that scFv MO33, but not F19, binds to mFAP, the respective epitopes cannot be entirely identical.

Construction of a Bivalent Minibody Version of scFv MO36

ScFv MO36 was also expressed as a minibody derivative (MB MO36) in mammalian cells. MB MO36 bound to mFAP- and hFAP-expressing cells, as shown by flow cytometry with a high apparent affinity (EC_{50} of 1.7 nM and 3.8 nM, respectively; Fig. 6) and thus is a genuine species crossreactive FAP-specific reagent. Moreover, in addition to detection via the human CH3 region, the construct can be analyzed via a c-myc tag allowing immunohistochemical detection in human as well as in murine tissue.

The MB MO36 Defined Epitope Is Expressed in Human Cancer

The avidin-biotin-immunoperoxidase procedure was used to determine the distribution of the antigen recognized by MB MO36 in several human cancers including breast carcinoma (10× analyzed), colorectal carcinoma (4×), non-small lung carcinoma (4×), ovarian carcinoma (2×), kidney carcinoma (2×), as well as head and neck carcinoma (2×). To circumvent background staining by human immunoglobulins potentially present in the tissue, detection was performed via the myc tag. The construct showed specific staining in the stromal fibroblasts of the malignant tissues examined (Figs. 7A and 7C). Additionally, in a xenograft model, stromal fibroblasts of murine origin induced by transplantation of a human breast carcinoma cell line could be specifically detected by

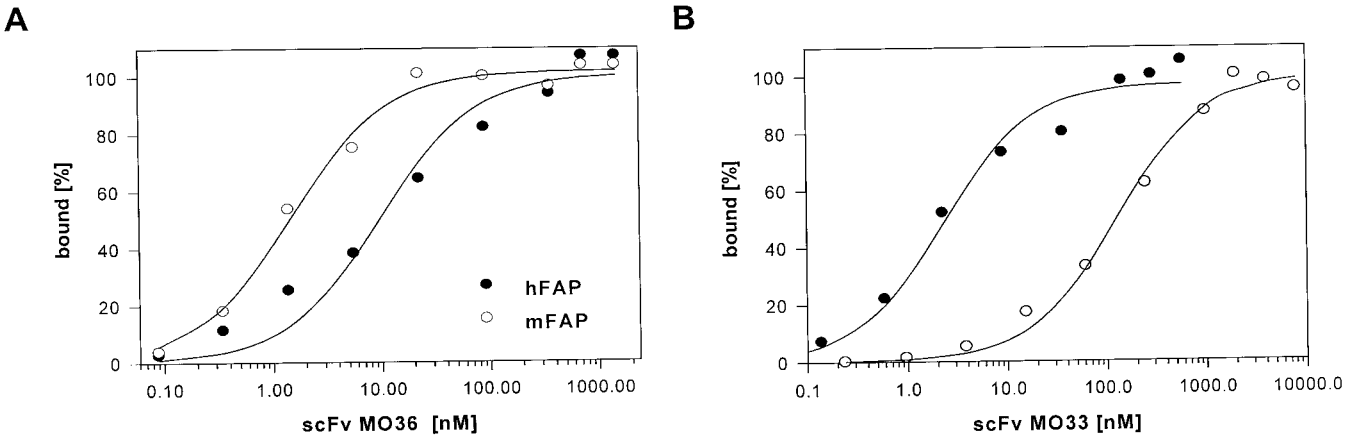

Fig. 4. Quantitative cell-binding assay for scFv MO36 (A) and scFv MO33 (B). Bacterial lysates of scFv MO36 and partially purified scFv MO33 were incubated in different dilutions on 2×10^4 cells ($hFAP^+$ or $mFAP^+$). For subtraction of unspecific binding, all dilutions were also incubated on nontransfected control cells. For detection anti–c-myc mab 9E10, POD-conjugated anti-mouse IgG-Fc detection antibody and TMB substrate (measured at 630 nm) were used.

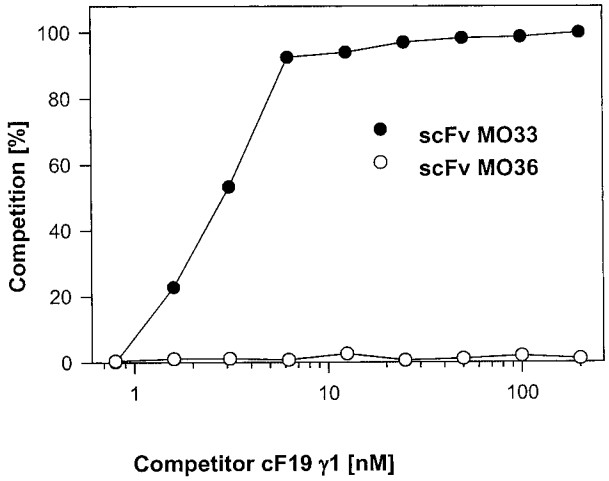

Fig. 5. Competition of scFv MO33 and scFv MO36 by the chimeric mab cF19γ1. Diluted bacterial lysate (1:50) of the scFv were mixed with variable concentrations of cF19γ1. The mixtures were incubated on CD8-hFAP in an ELISA. One hundred percent competition reflects a reduction of specific ELISA signal to background level ($OD_{405\ nm} \approx 0.03$) whereas 0% competition reflects the ELISA signal without competitor ($OD_{405\ nm} \approx 1$).

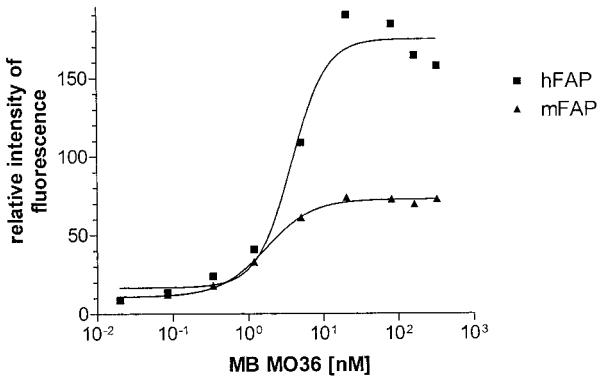

Fig. 6. Determination of the apparent affinity of MB MO36 for cell expressed human and murine FAP by FACS analyses. Stable hFAP (■) and mFAP (▲) transfectants were incubated with different concentrations of MB MO36 and subsequently with an anti–c-myc mab (9E10) and a FITC-labeled anti-mouse IgG-Fc specific serum. For each dilution, specific fluorescence intensity of 10,000 cells was determined. Mean fluorescence intensity (arbitrary units) is plotted over minibody concentration and apparent affinity is approximated from the determined concentration yielding half maximum binding to each of the transfectants.

MB MO36 (Fig. 7D). Moreover, in murine embryonic tissue, subsets of primitive mesenchymal cells were stained by MB MO36 (data not shown), which is in accordance with previous immunohistochemical data on embryonal FAP expression (13).

Discussion

In this study, we successfully used an immunized $FAP^{-/-}$ mouse in combination with the phage display system for generation of antibodies with cross-reactivity for hFAP and mFAP. By the depletion of the mFAP gene locus in this mouse, an immunologic tolerance against epitopes shared by hFAP and mFAP could be circumvented. In a similar approach, phage display selected antibodies against murine prion proteins (PRP), using an immunized $PrP^{-/-}$ mouse, (30) were obtained.

In the present study, phage display selection was applied because of its advantageous features in comparison to hybridoma technology. Thus, the scFv gene cassette isolated by phage display can be used directly for the construction of recombinant antibody derivatives (11,12,31). Further, in contrast to scFv cloned from hybridoma cell lines, phage display-derived scFvs are more readily produced in an active form in bacteria. For example, two FAP-specific scFvs from hybridoma, scFv F19 and scFv

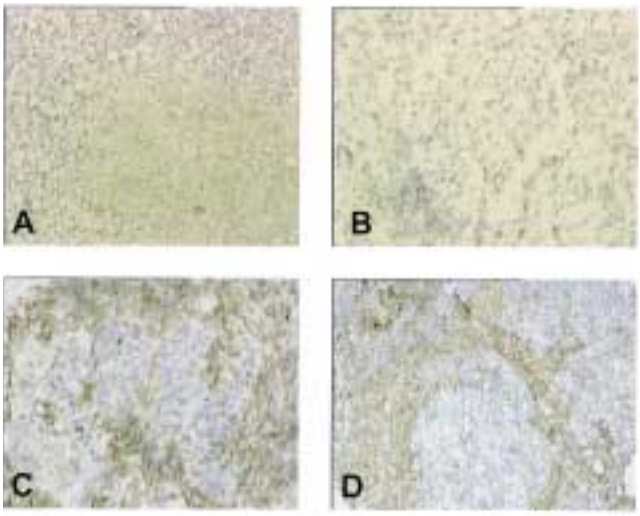

Fig. 7. Immunohistochemical detection of MB MO36 epitopes in human cancer. (A, B) Breast carcinoma. **(C)** Ovarian carcinoma. **(D)** Xenograft from the breast carcinoma cell line ZR75.1 in an NMRI nu/nu mouse. Fresh frozen sections were incubated with MB MO36 **(A, C, D)** or, as a negative control, without minibody **(B)**. Detection on samples A and B was done with an anti–c-myc antibody followed by biotinylated horse anti-mouse IgG and the avidin-biotin complex immunoperoxidase procedure. On samples C and D, biotinylated goat anti-human IgG and the avidin-biotin complex immunoperoxidase procedure were used. Counterstaining was done with Harris' hematoxylin.

FB52, were expressed in *E. coli* XL 1 Blue in amounts of only ~200–300 μg of soluble protein per liter of culture (B. Brocks, unpublished data), whereas phage display selected scFv MO33 and MO36 (see below) can be produced in amounts of up to 10 mg/l in the same host. This finding is in accordance with results obtained by other groups (32). Most relevant in the present case, the selective concept of phage display appeared essential for efficient isolation of scFv with the particular feature of species crossreactivity and preferential binding to cell-bound FAP antigen, as crossreactive antibodies were only rarely obtained by conventional hybridoma technology.

However, despite these theoretical advantages, phage display does not always work with the expected efficiency, even when immunized animals have been used. For example, in an earlier study that compared antibodies derived by phage display and hybridoma technique using splenic material from identical mice, the hybridoma-derived antibodies showed larger diversity and favorable biological properties. Moreover, three to four rounds of selection were necessary in this previous study to obtain specific binders (33).

To obtain a library that enabled us to develop efficient selection strategies, care was taken to achieve a high clonal diversity (10^8 clones) and functional expression (about 80%) of the phage display library. The general suitability of this library was apparent; 25% of the scFv clones selected in the first round of phage display were highly specific for FAP and apparently composed of independent V-region sequences, evident from a distinct restriction enzyme pattern. The applied combination strategy of sequentially immunizing and selecting with hFAP and mFAP, including the final panning on mFAP-overexpressing cells (which excluded scFvs selectively binding hFAP, denatured antigen, or epitopes only existing on the CD8-hFAP fusion protein) were likely essential steps in the successful isolation of a scFv with equal reactivity to hFAP and mFAP.

Owing to the high homology of human and murine FAP (89%) and the fact that the selection of hFAP-specific scFv was very efficient, we expected to isolate a wide range of antibodies with crossreactivity to both species in the $FAP^{-/-}$ mouse. However, contrary to these theoretical considerations, clone MO36 was isolated twice in two independent phage display selections and scFv MO33 was isolated only once. For this reason, we suppose that despite the FAP gene knock-out situation, only a limited antibody response against mFAP domains was elicited. A significant homology of mFAP to other structurally related murine proteins, like dipeptidyl peptidase, which shows 52% amino acid identity (10), may cause cross tolerance and thus restrict the mFAP-specific immune response.

The selected scFv MO33 and scFv MO36 showed different FAP binding characteristics. For scFv MO33 binding studies with CD8-FAP (natively immobilized in a capture ELISA) and cell membrane-bound FAP revealed affinities for hFAP in a range of 3–5 nM, whereas the affinity measured for mFAP was in a range of 100–130 nM. For this reason, it is likely that scFv MO33 originally was raised against a human epitope, which is not fully conserved on mFAP. Furthermore, competition experiments showed that the human epitope recognized by MO33 is in close vicinity or related to the immunodominant epitope of F19. In contrast to scFv MO33, the predominantly selected scFv MO36 exerts similar apparent binding affinities in the low nM range for both hFAP and mFAP, as shown by sandwich ELISA and cell-binding studies. It therefore appears likely that the epitope recognized by MO36 is highly conserved between species. Interestingly, unlike scFv MO33, scFv MO36 could not be competed by F19, suggesting that the epitope significantly differs from that recognized by F19 and MO33.

The minibody format of MO36 was suitable for immunohistochemical investigations of FAP expression in human as well as in murine tissues. This antibody format is advantageous for tumor targeting because of its bivalency, high tumor uptake, and favorable blood clearance, thereby supporting a selective accumulation in the tumor tissue (21). The minibody derivative of scFv MO36 (MB MO36) possesses the expected high affinity and displays a greater stability as compared to the parental scFvs (data not shown). We therefore consider MB MO36 suitable for tumor stroma targeting. Thus, this re-

combinant antibody will likely be a valuable tool in the analyses of the functional role of FAP in tumor biology and in the evaluation of FAP as a therapeutic target.

Acknowledgments

We gratefully acknowledge the skilled technical assistance of Elke Gerlach and Gabriele Sauter. We thank Uwe Bamberger for help with animal work. This work was supported by a research grant (Zentrales Schwerpunktprojekt Bioverfahrenstechnik, Universität Stuttgart, B3.6U/E) from the Bundesministerium für Bildung und Forschung and Boehringer Ingelheim Pharma GmbH, Germany.

References

1. Garin-Chesa P, Old LJ, Rettig WJ. (1990) Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody-target in human epithelial cancers. *Proc. Natl. Acad. Sci. USA* **87:** 7235–7239.
2. Welt S, Divgi CR, Scott AM, et al. (1994) Antibody targeting in metastasing colon cancer: a phase I study of monoclonal antibody F19 against a cell-surface protein of reactive stromal fibroblasts. *J. Clin. Oncol* **12:** 1193–1203.
3. Hanahan D, Folkman J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86:** 353–364.
4. Barinaga M. (1997) Designing therapies that target tumor blood vessels. *Science* **275:** 482–484.
5. Zetter BR. (1997) On target with tumor blood vessel markers. *Nature Biotechnol.* **15:** 1243–1244.
6. Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ, Rettig WJ. (1999) Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J. Biol. Chem.* **274:** 36505–36512.
7. Rettig WJ, Garin-Chesa P, Beresford HR, Oettgen HF, Melamed MR, Old LJ. (1988) Cell-surface glycoproteins of human sarcomas: differential expression in normal and malignant tissues and cultured cells. *Proc. Natl. Acad. Sci. USA* **85:** 3110–3114.
8. Rettig WJ, Garin-Chesa P, Healey J, et al. (1993) Regulation and heterodimeric structure of the firoblast activation protein in normal and transformed cells of mesenchymal and neuroectodermal origin. *Cancer Res.* **53:** 3327–3335.
9. Scanlan MJ, Raj BK, Calvo B, et al. (1994) Molecular cloning of fibroblast activation protein a, a member of the serine protease family selectively expressed in stromal fibroblasts of epithelial cancers. *Proc. Natl. Acad. Sci. USA* **91:** 5657–5661.
10. Niedermeyer J, Scanlan MJ, Garin-Chesa P, et al. (1997) Mouse fibroblast activation protein: molecular cloning, alternative splicing and expression in the reactive stroma of epithelial cancers. *Int. J. Cancer* **71:** 383–389.
11. Hayden MS, Gilliland LK, Ledbetter JA. (1997) Antibody engineering. *Curr. Opin. Immunol.* **9:** 201–212.
12. Carter P, Merchant MA. (1997) Engineering antibodies for imaging and therapy. *Curr. Opin. Biotechnol.* **8:** 449–454.
13. Niedermeyer J, Kriz M, Hilberg F, et al. (2000) Targeted distribution of mouse fibroblast activation protein. *Mol. Cell. Biol.* **20:** 1089–1094.
14. Zhou H, Fisher RJ, Papas TS. (1994) Optimization of primer sequences for mouse scFv repertoire display library construction. *Nucleic Acids Res.* **22:** 888–889.
15. Datmajumdar AK, Jacobson DP, Hood LE, Osman GE. (1996) Rapid cloning of any rearranged mouse immunoglobulin variable genes. *Immunogenetics* **43:** 141–151.
16. Welschof M, Little M, Dörsam H. (1997) Production of a human antibody library in the phage display vector pSEX81. In: Walker JM, ed: *Molecular Biology Based Methods in Clinical Diagnosis.* In the series *Methods in Molecular Medicine.* Totowa, NJ: Humana Press.
17. Welschof M, Terness P, Kyprijanov S, et al. (1997) The antigen binding domain of a human IgG anti F(ab')2 autoantibody. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 1902–1907.
18. Schmiedl A, Breitling F, Winter CH, Queitsch I, Dübel S. (2000) Effects of unpaired cysteins on yield, solubility and activity of different recombinant antibody constructs expressed in *E. coli. J. Immunol. Meth.* **242:** 101–114.
19. Rippmann FJ, Klein M, Hoischen, et al. (1998) Procaryotic expression of single-chain variable-fragment (scFv) antibodies: secretion in L-form cells of Proteus mirabilis leads to active product and overcomes the limitations of periplamic expression in Escherichia coli. *Appl. Environ. Microbiol.* **64:** 4862–4869.
20. Friguet B, Chaffotte AF, Djyvadi-Ohaniance L, Goldberg ME. (1985) Meaurement of true affinity constant in solution of antigen antibody complexes by enzyme-linked immunosorbent assay. *J. Immunol. Meth.* **77:** 305–319.
21. Hu S, Shively L, Raubitschek A, et al. (1996) Minibody: a novel engineered anti-carcinoembryonic antigen antibody fragment (single-chain Fv-CH3) which exhibits rapid, high-level targeting of xenografts. *Cancer Res.* **56:** 3055–3061.
22. Moosmayer D, Dübel S, Brocks B, et al. (1995) A single-chain TNF receptor antagonist is an effective inhibitor of TNF mediated cytotoxicity. *Therap. Immunol.* **2:** 31–40.
23. Dübel S, Breitling F, Fuchs P, et al. (1994) Isolation of IgG antibody Fv DNA from various mouse and rat hybridoma cell lines using the polymerase chain reaction with a simple set of primers. *J. Immunol. Meth.* **175:** 89–95.
24. Tesar M, Beckmann C, Röttgen T, Haase B, Faude U, Timmis KN. (1995) Monoclonal antibody against filamentous phage: An immunological tool to study pIII-fusion protein expression in phage display systems. *Immunotechnology* **1:** 53–64.
25. Mersmann M, Schmidt A, Tesar M, et al. (1998) High efficient monitoring of scFv selected from phage display repertoires using detection of scFv-pIII fusion proteins in a microtiter scale assay. *J. Immunol. Meth.* **220:** 51–58.
26. Kabat EA, Wu TT, Perry HM, Gottesmann KS, Foeller C. (1991) *Sequences of Proteins of Immunological Interest,* 5th ed. Bethesda, MD: US Department of Health and Human Services.
27. Mersmann M, Schmidt A, Wüest T, et al. (2001) Human antibody derivatives against the fibroblast activation protein for tumor stroma targeting of carcinomas. Int. J. Cancer 92:240–248.
28. Sakano H, Maki R, Kuroswa Y, Roeder W, Tonegawa S. (1980) Two types of somatic recombination are necessary fot the generation of complete immunoglobulin heavy-chain genes. *Nature* **286:** 676–683.
29. Rettig WJ, Su SL, Fortunato SR, et al. (1994) Fibroblast activation protein: purification, epitope mapping and induction by growth factors. *Int. J. Cancer* **58:** 385–392.
30. Williamson RA, Peretz D, Smorodinsky N, et al. (1996) Circumventing tolerance to generate autologous monoclonal antibodies to the prion protein. *Proc. Natl. Acad. Sci. U.S.A.* **93:** 7279–7282.
31. Brinkmann U. (2000) Recombinant antibody fragments and immunotoxin fusions for cancer therapy. *In Vivo* **14:** 21–27.
32. Kipriyanov SM, Moldenbauer G, Little M. (1996) High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. *J. Immunol. Meth.* **200:** 69–77.
33. Ames RS, Tornetta MA, Mc Millan FJ, et al. (1995) Neutralizing murine monoclonal antibodies against human IL 5 isolated from hybridomas and filamentous Fab display library. *J. Immunol.* **154:** 6355–6364.