Human Combinatorial Fab Library Yielding Specific and Functional Antibodies against the Human Fibroblast Growth Factor Receptor 3*

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The human combinatorial antibody library Fab 1 (HuCAL®-Fab 1) was generated by transferring the heavy and light chain variable regions from the previously constructed single-chain Fv library (Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellhöfer, G., Hoess, A., Wölle, J., Plückthun, A., and Virnæs, B. (2000) J. Mol. Biol. 296, 57–86), diversified in both complementarity-determining regions 3 into a novel Fab display vector, yielding 2.1 x 1010 different antibody fragments. The modularity has been retained in the Fab display and screening plasmids, ensuring rapid conversion into various antibody formats as well as antibody optimization using prebuilt maturation casettes. HuCAL®-Fab 1 was challenged against the human fibroblast growth factor receptor 3, a potential therapeutic antibody target, against which, to the best of our knowledge, no functional antibodies could be generated so far. A unique screening mode was designed utilizing recombinant functional proteins and cell lines differentially expressing fibroblast growth factor receptor isoforms diversified in expression and receptor dependence. Specific Fab fragments with subnanomolar affinities were isolated by selection without any maturation steps as determined by fluorescence flow cytometry. Some of the selected Fab fragments completely inhibit target-mediated cell proliferation, rendering them the first monoclonal antibodies against fibroblast growth factor receptors having significant function blocking activity. This study validates HuCAL®-Fab 1 as a valuable source for the generation of target-specific antibodies for therapeutic applications.

The development of the hybridoma technology opened the application of monoclonal antibodies for research and human therapy (2). A major drawback of these first generation monoclonal antibodies, especially for clinical application, was their murine origin, which often caused immune response in human and led to the generation of human anti-mouse antibodies (human anti-mouse antibody reaction), limiting efficacy in long term and repeated administration (3). Phage display and antibody library technologies have evolved as a powerful alternative for the generation of human antibody fragments for research, clinical, and therapeutic applications (for a review, see Ref. 4), since it was shown that peptides (5) and antibody fragments (6) can be displayed on the surface of filamentous bacteriophage and, function antibody fragments can be expressed in the periplasm of Escherichia coli cells (7, 8).

To date, a variety of different antibody libraries have been generated, which range from immune to naive and even synthetic antibody libraries. Immune libraries derived from IgG genes of immunized donors (9) are useful if immunized patients are available but have the disadvantage that antibodies can only be made against the antigens used for immunization. In contrast, antibodies against virtually any antigen, including self-, nonimmunogenic, or toxic antigens, can be isolated from naive or synthetic libraries. Naive libraries from nonimmunized donors have been generated by PCR-cloning Ig repertoire from various B-cell sources (10–13). Semisynthetic antibody libraries were built by in vitro assembly of PCR-amplified antibody genes derived from human germ line genes and randomized only in the CDR3 regions (14–16) or derived from a single V-gene with complete randomization of all CDRs (17, 18). The affinities of the antibodies derived from these libraries are assumed to be dependent on the size of the library (19). Therefore, many attempts have been undertaken to make the library size as big as possible, and site-specific recombination systems have been created to overcome the library size limitations given by the conventional cloning strategies (20, 21). Besides library generation, also the panning process itself limits the library size that can be handled conveniently. Therefore, it is important to generate libraries with a high quality of the displayed antibodies, thus emphasizing the functional library size and not only the apparent library size. With the recently described human combinatorial antibody library (HuCAL®)
concept, a synthetic library in the scFv format was created, focusing on a high number of correct antibody fragments (1, 22). HuCAL® is a fully human antibody library wherein each VH and VL subfamily frequently used in human is represented by a consensus framework, resulting in seven VH and seven VL master genes giving 49 different combinations. The master genes were optimized for expression and folding; furthermore, a high functional quality of the library is guaranteed by diversifying both CDR3 regions with trinucleotide mixtures (23), reflecting the natural amino acid composition of CDR3. The modular design of the library with unique restriction sites flanking the CDR and framework regions as well as compatible vector modules facilitate (i) conversion into different antibody formats, (ii) the addition of effector functions, and (iii) further antibody optimization by exchanging the CDR regions of selected binders by prebuilt CDR libraries (24). Here, we describe the generation of a second version of the HuCAL® library (HuCAL®-Fab 1), wherein we combined all of the characteristics of the HuCAL® concept with the Fab format. Whereas scFv fragments have a high tendency to form multimers (25–27), Fab fragments tend to stay completely monomeric, allowing selection for affinity in contrast to selection for avidity. The monomeric behavior even in crude periplasmic extracts allows a rapid off-rate screening for affinity ranking of the antibody fragments without time-consuming purifications. Furthermore, the Fab format ensures conversion into complete immunoglobulins without loss of function, thus being optimally suited for therapeutic applications.

HuCAL®-Fab 1 was challenged against FGFR3, so far resistant to generation of function-blocking antibodies. FGFR3 belongs to a family of highly homologous cell surface-expressed receptor tyrosine kinases, currently including five members (FGFR1 to 5) (reviewed in Ref. 28; Ref. 29 for FGFR5). The FGFRs are glycoproteins comprising two or three Ig-like extra-cellular domains, a hydrophobic transmembrane domain, and a cytoplasmic region that contains the tyrosine kinase domain (30–32). Binding of a complex of fibroblast growth factor (FGF) ligand and heparin to FGFR3 leads to its dimerization, resulting in autophosphorylation of the intracellular kinase domain and a downstream activation of intracellular signaling cascades (33). So far, more than 20 ligands (34) have been identified, varying in their specificity for individual FGFRs (e.g. FGF2 shows a very promiscuous binding pattern, whereas FGFR9 shows a high preference for FGFR2 and FGFR3) (35). FGFRs and their receptors regulate a multitude of cellular processes, including cell growth, differentiation, migration, and survival (reviewed in Refs. 36 and 37).

FGFR3 is mainly expressed during embryogenesis and development of bones, brain, lungs, and spinal cord (38, 39). Different pathological disorders such as dwarfism or tumor genesis were linked to increased FGFR3 activity (reviewed in Refs. 40 and 41). The transforming potential of FGFR3 harboring an activating mutation was especially shown for tumor genesis linked to increased FGFR3 activity (reviewed in Refs. 42, 43). So far, more than 20 ligands have been identified, varying in their specificity for individual FGFRs (e.g. FGF2 shows a very promiscuous binding pattern, whereas FGFR9 shows a high preference for FGFR2 and FGFR3) (35). FGFRs and their receptors regulate a multitude of cellular processes, including cell growth, differentiation, migration, and survival (reviewed in Refs. 36 and 37).

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system (Invitrogen) expressing the receptor in the absence of tetracycline (−tet) and lacking the receptor if tetracycline was added to the culture medium (+tet).

The mouse myeloid progenitor cell line FDCP-1 was cultured in Iscove's medium (Invitrogen) supplemented with 10% FCS, penicillin, streptomycin, glutamine, and 0.1 ng/ml IL-3. FDCP-1 cells transfected with full-length human FGFR1 (FGFR1) was a generous gift from Josef Schlessinger (New York University School of Medicine) or human FGFR3 were grown in the same medium, but IL-3 was substituted by 10 ng/ml FGF2 or FGF3, respectively.

Cloning of Fab Expression Vectors Containing Cysteines for Covalent Linkage of Heavy and Light Chains—Genes encoding the human CH1 (subtype IgG1; GenBank™ accession number A49444), C2, and C3 domains of IgG1 were obtained from pP1354 and pP1355 by PCR using Pfu polymerase. 5′-GTGAGCTTTATCTGACTGTCG-3′ and 5′-GATATCTGCAAGTTAACGGTTC-3′ were used as primers for C1, 5′-GAAATTACAGTACGGGCTG-3′ and 5′-TCTCTTCTAGCTGTTCTGACCCC-3′ for C2, and 5′-GGCGCCAGAATTACGGTTC-3′ and 5′-TCTCTTCTAGCTGTTCTGACCCC-3′ for C3.

The cloning procedure and expression in JM83 were performed as described (54) with vector pMORPH®X7_FabCys_FS. The expression vectors for heavy chain were cloned into the Fab format by cloning the VL and VH fragments into the expression vectors pMORPH®X7_FabCys_FS.

Thermal Stability of Disulfide-linked and Noncovalently Linked Fab Fragments—Control κ-scFv and κ-scFvS were converted into the Fab format by cloning the VL and VH fragments into the expression vectors for disulfide-linked Fab. pMORPH®X7_Fab_Cys had been ligated with a 2-fold molar excess of VL chains. The ligation and transformation of E. coli JM83 (54) were performed as described (54). Purification of the final library vectors were designated pMORPH®X7_FabCys_FS.

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Comparison of the OmBp and SII1 Signal Sequence for Light Chain Expression—The SII1 signal sequence for light chain expression in pMORPH®X7_Fab_Cys was replaced by the OmBp signal sequence from a derivative of pGl10 (57) via XbaI/EcoRV. The new expression vector was designated pMORPH®X7_Fab_Cys_OmBp.

Heavy and light chains of the control κ-Fab and λ-Fab were excised from pMORPH®X7_Fab_Cys and cloned into pMORPH®X7_Fab_Cys pBovAC2, respectively. Expression yields of these Fabs containing either the SII1 or the OmBp signal sequence were measured in E. coli JM83 (54, 22), crude periplasmic extracts were generated by osmotic shock (55), and Fab protein was analyzed by ELISA. 500 ng of the corresponding antigens were coated on Maxisorp plates (Nunc, Rochester, NY), and after blocking with 5% nonfat milk powder in TBS, 0.05% Tween 20 (Sigma), periplasmic extracts were added. Antibody activity was measured by ELISA development (OD at 370 nm).

Generation of HuCAL®-Fab 1—The phage used for cloning of the HuCAL®-Fab 1 library was designated pMORPH®18 (Fig. 1). Briefly, the vector contains a noncovalently linked κ- or λ-Fab with the PhoA signal sequence for heavy chain and the OmpA leader sequence for light chain expression. The C1 gene is directly followed by a truncated version of gene III with the first 249 codons deleted.

The whole VH chain (MunStyl fragment) was replaced by a 1205-bp Cloning of Fab Expression Vectors Containing Cysteines for Covalent Linkage of Heavy and Light Chains—Genes encoding the human CH1 (subtype IgG1; GenBank™ accession number A49444), C2, and C3 domains of IgG1 were obtained from pP1354 and pP1355 by PCR using Pfu polymerase. 5′-GTGAGCTTTATCTGACTGTCG-3′ and 5′-GATATCTGCAAGTTAACGGTTC-3′ were used as primers for C1, 5′-GAAATTACAGTACGGGCTG-3′ and 5′-TCTCTTCTAGCTGTTCTGACCCC-3′ for C2, and 5′-GGCGCCAGAATTACGGTTC-3′ and 5′-TCTCTTCTAGCTGTTCTGACCCC-3′ for C3.

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Biologically Active Fabs from HuCAL®-Fab 1 against FGFR3

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Expression and Stability Analysis of Disulfide-linked and Noncovalently Linked Fab Fragments—In parallel to constructing the HuCAL®-scFv library, all seven VH and seven VL master genes had been fused also to synthetic gene fragments encoding the CH1-CL domains, respectively (1). These constant domains had been synthesized without the C-terminal cysteines responsible for intermolecular disulfide bonding of IgG-type Fab fragments, since it is known that the Fab and the light chain do not dissociate into the component chains even when only noncovalently linked, as seen with mouse IgA or with mildly reduced fragments (60–63). Fig. 1, a and b, show the expression vectors encoding those domains were designated pMORPH®X7_FabCys_FS (key features in Fig. 1 a).

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Three scFv fragments previously selected from the HuCAL®-scFv against different antigens were then converted into both Fab formats. Western

In order to qualitatively compare binding epitopes of different HuCAL®-Fabs with FGFR3, the Fc fusion protein was captured as described before, followed by injection of a 1 µM concentration of the first HuCAL®-Fab until saturation of binding. Then a 1 µM concentration of the second HuCAL®-Fab was co-injected. We concluded that epitopes were independent if the signal increased for the second HuCAL®-Fab fragment. No increase of signal, therefore, meant overlapping or similar binding epitopes. To test antibodies for interference with FGF binding, FGFR3-Fc was captured as described before, followed by injection of 100 µg/ml heparin and 1 µg FGFR9 until saturation level. Then 1 µg purified HuCAL®-Fab was co-injected. The maximum binding capacity of the free FGFR3 surface was reached with 1 µg HuCAL®-Fab. Therefore, this binding signal was set to 100%. The signal resulting from HuCAL®-Fab binding to the FGFR3-FGFR9 complex in comparison with HuCAL®-Fab binding to FGFR3 without FGFR9 was calculated as a percentage. For stability tests of HuCAL®-Fab fragments, the Fabs were incubated at 37 °C for 0, 4, 8, and 13 days in 10% FCS, at a concentration of 1 µg/ml and injected to the FGFR3-Fc coated surface. The initial slope of the binding was determined for each time point and compared with the slope of the Fab not exposed to 37 °C, which was set to 100%.

RESULTS

Expression and Stability Analysis of Disulfide-linked and Noncovalently Linked Fab Fragments—In parallel to constructing the HuCAL®-scFv library, all seven VH and seven VL master genes had been fused also to synthetic gene fragments encoding the CH1 and CL domains, respectively (1). These constant domains had been synthesized without the C-terminal cysteines responsible for intermolecular disulfide bonding of IgG-type Fab fragments, since it is known that the Fab and the light chain do not dissociate into the component chains even when only noncovalently linked, as seen with mouse IgA or with mildly reduced fragments (60–63). Fig. 1, a and b, show the expression vectors encoding those domains were designated pMORPH®X7_FabCys_FS (key features in Fig. 1 a).

Three scFv fragments previously selected from the HuCAL®-scFv against different antigens were then converted into both Fab formats. Western Blot analysis under reducing and non-reducing conditions of crude periplasmic extracts (55) from E. coli expression cultures clearly demonstrated the formation of the disulfide bond between heavy and light chain only for the three Fab fragments cloned into pMORPH®X7_FabCys_FS (data not shown).

For comparison of the expression yields, the three disulfide-linked and the three noncovalently linked Fab fragments were expressed in 1 liter shaking flask cultures and purified from crude periplasmic extracts by StreptTactin affinity chromatography (53, 56). 0.5–4.4 mg of noncovalently linked and 0.2–2.8 mg of disulfide-linked Fab fragments were obtained. In all three cases, the expression yield was higher with the noncovalently linked Fab fragments (1.3–4-fold). SDS-PAGE and Coomassie Blue staining demonstrated that heavy and light chains were equally represented with a purity of >90% (data not shown).

Purified Fab fragments of two disulfide-linked and the corresponding unlinked Fab fragments (1 Fab with κ– and 1 Fab with λ– light chain) were incubated at temperatures from 4 to 70 °C in order to analyze their thermal stability. Relative binding activities were recorded in Biacore. As shown in Fig. 2, the binding activity stayed unchanged up to an incubation temperature of 60 °C for both the noncovalently linked and the disulf-
fide-linked Fab fragments. Only when raising the incubation temperature to 70 °C was the binding activity of the unlinked Fab fragments reduced between 5- and 25-fold, whereas the binding activity of disulfide-linked Fab fragments was reduced less than 2-fold. Long term storage at 37 °C in buffer containing 10% FCS showed no difference between both antibody formats (data not shown).

Taken together, the expression and stability analysis of disulfide-linked and noncovalently linked Fab fragments show that unlinked Fab fragments constantly reach better expression yields than disulfide-linked Fab fragments and are sufficiently stable for standard applications. Therefore, we decided to use the noncovalently linked Fab format for library construction.

Influence of the Signal Sequence on Light Chain Expression—For a further improvement of the expression rate of the noncovalently linked Fab fragments, we analyzed the influence of the signal sequence for VL. The initial Fab expression vector, pMORPH®X7_Fab_FS, contained the StII signal sequence for the light chain (64, 65), and preliminary experiments indicated that the amount of light chain might limit expression of Fab fragments (data not shown). Since the OmpA signal peptide (66) is an often used leader for protein expression in bacteria (67), the StII signal sequence was replaced by the OmpA leader, thus creating the expression vector pMORPH®X9_Fab_FS (see Fig. 1c).

Two Fab genes (one with κ and one with λ-light chain) were excised from pMORPH®X7_Fab_FS by EcoRV/EcoRI and cloned into pMORPH®X9_Fab_FS by EcoRV/EcoRI without transfer of the light chain signal sequence. Expression yields of the StII-Fab fragments and their OmpA counterparts were compared by measuring the binding activity of crude extracts in ELISA on the corresponding specific antigens (Fig. 3). The signal for the Fab fragments originating from the pMORPH®X9_Fab_FS vector was about 2-fold higher than for the Fab fragments originating from the pMORPH®X7_Fab_FS vector, which can only be attributed to a superior expression rate of Fab fragments equipped with the OmpA signal sequence. Quantitative Western Blot analysis of these crude extracts confirmed this result (data not shown). Therefore, replacement of the StII leader by the OmpA signal peptide for the light chain allows for a further improvement of Fab expression.

This demonstrates that the noncovalently linked HuCAL®-Fab format using the OmpA signal sequence for light chain and the PhoA signal sequence for heavy chain expression guarantees good expression and stability, thus being ideal features for generating a Fab library.
Generation of the HuCAL®-Fab 1 Library—The expression vectors pMORPH®X9_Fab_F5, having an OmpA signal peptide for VL expression and lacking the cysteine for the VH-VL linkage, were used as starting material. A fragment (codon 250–406) of the mature gene III protein of bacteriophage M13 was used to replace the FLAG and Strep tag directly fused to the heavy chain to generate the display vector pMORPH®18. To create vectors for library cloning, for which preparation of the vector fragment is facilitated and in which VH and VL chains of parental binders are completely absent, we removed the VH gene and inserted the complete β-lactamase transcription unit (bla) as a dummy sequence. A fragment of the bacterial alkaline phosphatase gene (bap) served as a cloning stuffer for VL 

HuCAL®-VH1, -VH2, and -VH3 diversified in L-CDR3 were amplified by PCR from precursor sublibraries of the HuCAL®-scFv library (1) and mixed according to their variability. VH chains of VH1, VH2, and VH3 also diversified in L-CDR3 were obtained from precursor sublibraries of the HuCAL®-scFv library (1) by restriction digest and also mixed according to their variability. VL1 and VL2 gene segment libraries were inserted into the Fab phage display vector, yielding 4.1 × 10⁸ independent transformants for the VL1 and 1.6 × 10⁸ independent transformants for the VL2 sublibrary. Thus, in each case, library size was at least 30-fold larger than in the corresponding scFv library, ensuring full coverage of the initial diversity. Quality control by sequencing of 54 clones revealed more than 90% correct clones.

The HuCAL® VH gene segment libraries diversified in the H-CDR3 region were prepared by restriction digest of all six HuCAL® VH master gene sublibraries except VH4, which was omitted because only very few binders could be selected from this VH family during various pannings (1). During the construction of the HuCAL®-scFv library, each VH-gene segment had been diversified separately with two different H-CDR3 library cassettes and combined with either the VL1 or the VL2 libraries, generating 20 different sublibraries (1).

From all 20 of these sublibraries, we prepared VH gene segments, combined VH chains from VL1 and VL2 sublibraries diversified by the same H-CDR3 cassette, and ligated the 10 different generated VH pools separately with the VL1 and the VL2 libraries obtained above (for details, see “Materials and Methods”). Thus, 20 different sublibraries were generated with in total 2.1 × 10¹⁰ different Fab fragments. This final library was designated HuCAL®-Fab 1.

VH and VL regions of 207 clones were sequenced in order to analyze the library quality. 138 clones (67%) were fully correct. Selection of FGFR3-specific Antibodies from HuCAL®-Fab 1—HuCAL®-Fab 1 was used to select specific Fab fragments against the receptor tyrosine kinase FGFR3 from two separate cloning strategies. In panning A, three selection rounds were performed. Phage were incubated with immobilized FGFR323–374DHis in the first and third round, and antigen-expressing 293T cells were used in the second round. 293T cells express endogenous human FGFR3 on the cell surface. In panning B, the first and third selection rounds were performed on FGFR3-Fc captured by an anti-Fc antibody alternating with a selection round on RCJ-FGFR3Q230H cells (−tet) expressing high amounts of recombinant human FGFR3Q230H on the cell surface. Individual Fab antibodies were produced in E. coli, and periplasmic preparations were tested in FACS for FGFR3 binding on RCJ-FGFR3 cells. In these cells, the regulated expression of recombinant FGFR3 is induced in the absence of tetracycline (−tet), whereas it is suppressed in the presence of tetracycline (+tet). Positive clones were subsequently tested on RCJ-FGFR3 cells cultured with tetracycline (+tet) to confirm FGFR3 specificity. In total, 1076 clones of panning A were tested in FACS, and 210 (19.5%) bound specifically to RCJ-FGFR3 (−tet) (Table I). The VH gene segment of 69 FACS-positive clones was sequenced, yielding 15 unique binders (clone 1-15). In panning B, 1632 clones were screened for FGFR3 binding in FACS. Of these clones, 521 binders were FGFR3-specific (31.9%). Then 120 clones were selected and sequenced, revealing another 34 unique binders. In total, 49 FGFR3-specific HuCAL®-Fab fragments were obtained, of which 37 were chosen for detailed analysis (see below). The unique clones from panning A and B represented all VH families of HuCAL®-Fab 1, with a bias on VH1, from which 36 unique clones were selected. Furthermore, two clones with VH2, five clones with VH3, three clones with VH5, and three clones with VH6 frameworks were isolated. Additionally, a high variety in length distribution of the H-CDR3, ranging from 8 to 20 amino acids, was found.

Characterization of Primary Hits—In order to test the specificity of the binders for FGFR3, purified Fab fragments were tested in ELISA (Fig. 4). We used FGFR1 as a representative of the FGFR family in order to analyze the FGFR3 binding clones for their antigen selectivity, since we could not exclude having generated clones that cross-react with FGFR1 because of an overall homology of 62% of both family members. All anti-FGFR3 Fab fragments in general showed strong binding to FGFR3 but differed in their cross-reactivity with FGFR1 (e.g., MSPRO2, -12, -21, -24, and -26 exclusively bound to FGFR3; MSPRO11 additionally showed weak binding to FGFR1; and MSPRO28 and -29 strongly cross-reacted with FGFR3-Fc and FGFR1-Fc in ELISA).

This difference in cross-reactivity to FGFR1 and -3 could similarly be shown in FACS analyses using RCJ cells express-
ing FGFR1 or FGFR3, respectively. Examples for a specific (MSPRO24) and a cross-reactive (MSPRO28) HuCAL®-Fab are given in Fig. 5. Overall, we found that all FGFR3-specific Fab fragments that were cross-reactive with FGFR1-Fc in ELISA also bound to FGFR1-expressing RCJ cells. Surprisingly, some Fab fragments that bound only weakly to FGFR1-Fc in ELISA now caused a clear shift on RCJ-FGFR1 (−tet) cells in FACS, indicating that under our conditions FACS analysis is the more sensitive assay. Based on the FACS analysis, 10 Fab fragments (MSPRO11, 22, 26, 27, 28, 29, 51, 52, 53, 54) of 37 selected clones (MSPRO1–15, MSPRO20–33, and MSPRO52–59) were cross-reactive for FGFR3 and FGFR1, and 27 antibodies were specific for FGFR3.

In parallel, purified Fab fragments were tested in FACS analysis on RCJ cells expressing the mutated form of FGFR3, FGFR3G380R. FGFR3-specific Fab fragments bound to RCJ-FGFR3G380R (−tet) expressing the mutant FGFR3G380R but did not bind the RCJ-FGFR3G380R (+tet) cells in which expression of FGFR3G380R was suppressed. In each case, the Fab antibody bound equally well to RCJ cells expressing FGFR3 (WT) and to RCJ cells expressing FGFR3G380R (data not shown).

**Inhibition of FGFR3-mediated Cell Signaling**—In order to analyze the inhibitory potential of FGFR3-specific HuCAL®-Fab fragments, they were tested in the FDCP-1-FGFR3 proliferation assay. Cell survival and growth of FDCP-1 cells normally depend on the presence of IL-3 in the culture medium. However, IL-3 can also be substituted by receptor tyrosine kinase ligands in FDCP-1 cells expressing the corresponding recombinant receptor tyrosine kinase (68). FDCP-1 cells expressing FGFR3 were grown in the presence of the FG9 ligand. HuCAL®-Fab fragments were added to these cells for 48 h. Then cells were stained with XTT to analyze the amount of living cells in a microtiter plate reader. In total, 25 of the 37 analyzed HuCAL®-Fab fragments inhibited FDCP-1-FGFR3 proliferation in the presence of FG9. None of the HuCAL®-Fab fragments affected FDCP-1-FGFR3 proliferation in the presence of IL-3 (data not shown), demonstrating that the inhibitory effect of HuCAL®-Fab fragments is not mediated by nonspecific cytotoxic effects. IC50 values were calculated to compare the activity of individual HuCAL®-Fab fragments. Inhibition curves of two different purified HuCAL®-Fab fragments are shown in Fig. 6. A summary of those 10 HuCAL®-Fab fragments showing the best binding and inhibition characteristics is given in Table II. Eight of 10 HuCAL®-Fab fragments show an IC50 value below 100 nM, ranging from 19 nM for the best clone (MSPRO59) to 70 nM (MSPRO24).

**Determination of Affinity to FGFR3**—The affinity of the Fab fragments was analyzed in Biacore on captured FGFR3-Fc fusion protein. Overall, relative affinities ranged from 1.5 nM to 1.5 μM. The data for 10 HuCAL®-anti-FGFR3 Fab fragments with relative affinities below 50 nM are shown (Table II). The monovalent affinities of 8 HuCAL®-Fab fragments measured in Biacore were 10 nM or below. For 9 of these 10 HuCAL®-Fabs, we also calculated the affinities in FACS using indirect labeling and Scatchard analysis (58). In Scatchard analysis, the amount of bound antibody to antigen expressed on cells is measured via indirect fluorescence labeling. In Biacore analysis, however, the association and dissociation rate constants for the antibody interaction with immobilized antigen are determined. Taking into account that the principles of the two binding assays are completely different, the obtained affinities of six of these Fab fragments are in good agreement, differing by a factor ranging from 1 to maximally 3 (Table II). However, for the three anti-FGFR3 Fab fragments, MSPRO21, -28, and -29, the FACS experiments resulted in significantly lower values of the monovalent Kd below 1 nM. We presume that these Fabs bind to epitopes that undergo conformational changes upon coating of the recombinant protein to the Biacore chip in contrast to the native protein expressed on the cell surface as it is detected in FACS analysis. Therefore, we consider the FACS data to be more relevant.

Comparing the IC50 values with the apparent KD of the selected 10 HuCAL®-Fab fragments showed that the IC50 values were not as good as the apparent Kd. The difference ranged from a factor of 3.3 (MSPRO29) to even a factor of 55 (MSPRO11). This might reflect the fact that the HuCAL®-Fab fragments block different epitopes on FGFR3. Therefore, a characterization of the binding epitopes was performed.

**Characterization of the Binding Epitope on FGFR3**—In a first set of experiments, FGFR3-specific HuCAL®-Fab fragments were tested for identical (or overlapping) binding epitopes on FGFR3. FGFR3-Fc was captured via an anti-Fc antibody to the Biacore chip surface and saturated with the first anti-FGFR3 HuCAL®-Fab fragment. Then a second anti-FGFR3 HuCAL®-Fab fragment was added. An additional increase in the binding signal could only occur if the epitope on FGFR3 of the second Fab fragment was not already blocked by the first Fab fragment. In contrast, no increase in RU was seen if binding of the first Fab fragment interfered with that of the second Fab fragment. Fig. 7 shows the experiment for MSPRO11 in comparison with MSPRO2, -12, and -21. MSPRO11 did not block binding of MSPRO2 and -12, whereas binding of MSPRO11 interfered with MSPRO21. All of the previously characterized inhibitory FGFR3-specific HuCAL®-Fab fragments were similarly tested. Finally, we identified two groups of inhibitory clones (Table III). Binding of the noninhibitory FGFR3-specific Fab fragments did not interfere with binding of any of the inhibitory Fab fragments (data not shown).

In a second set of BiACore experiments, competition of HuCAL®-Fab fragments and FG9 for binding to FGFR3 was tested. An example with MSPRO11 and a control Fab fragment is shown in Fig. 8. HuCAL®-Fab fragments were applied to the FGFR3-Fc coated surface (thick line) or FGFR3-Fc surface previously saturated with FG9/heparin (dotted line). If FG9/heparin interfered with binding of the Fab fragment to FGFR3, a smaller increase of RU compared with binding to the free receptor was measured, as is shown for MSPRO11 (Fig. 8A). All of the 10 selected inhibitory HuCAL®-Fab fragments showed a significantly reduced binding to the receptor if FGFR3 was occupied with the ligand. MSPRO6 and other FGFR3-specific binders that did not inhibit FDCP-1-FGFR3 proliferation did not show reduced binding capacity to the FGFR3-FG9-heparin complex (Fig. 8B).

**Stability of HuCAL®-Fab Fragments**—The stability of anti-FGFR3 HuCAL®-Fab fragments in vitro was analyzed. Fab fragments at a concentration of 1 μM were incubated with 10% FCS at 37°C for up to 13 days. The remaining relative binding activities of the antibodies MSPRO2, 11, 12, 21, 26, 28, and 29 were evaluated in Biacore studies and compared with freshly purified Fabs, where the initial slope of binding was set to 100% (Fig. 9). The remaining inhibitory potential was also
tested in FDCP-1-FGFR3 proliferation assays (data not shown). None of the tested HuCAL®-Fab fragments showed a significant loss of activity after 4 days at 37 °C either in Biacore experiments or in FDCP-1-FGFR3 proliferation assays. After 8 days, all tested Fabs retained more than 80% activity, as determined by Biacore. After 13 days of incubation, the remaining biochemical activity measured in Biacore ranged from 58 to 88%. These results were confirmed in the proliferation assays, which revealed only a weak decrease of FGFR3 neutralizing activity after 13 days at 37 °C. These data confirm the high stability of noncovalently linked Fab fragments as already determined in the preexperiments with some control HuCAL®-Fab fragments.

DISCUSSION

The HuCAL® concept is based on covering the essential features of the human antibody repertoire with a minimal number of different sequences, which are designed to facilitate extensive engineering with standard techniques. A fully synthetic human antibody library in the scFv format, with a diversity of $2 \times 10^6$, has recently been constructed, and it could be demonstrated that antibodies with high affinity and speci-
binding or VH/VL domain orientation. Thus, we decided to optimize Fab expression using a set of Fab fragments converted from scFv previously selected from the HuCAL®-scFv library. The Fd chain and the light chain of Fab fragments created by papain digestion of IgG1 are covalently linked by a disulfide bridge located at the beginning of the hinge region, and consequen
tly antibody Fab libraries constructed so far encode this disulfide bridge (13, 20, 70, 73). It is known, however, that the two chains of the Fab fragment self-associate and also interact noncovalently with high affinity (60–62). We rationalized that the removal of the two cysteines might improve expression yield but decrease stability. Indeed, we found that for all three antibodies tested, final yields after purification were up to 4-fold higher without the intermolecular disulfide bond. However, the stability of purified, noncovalently linked Fab fragments against thermal denaturation was reduced only above 60 °C under the experimental conditions. Incubation of Fab fragments at 37 °C in 10% FCS for up to 13 days did not lead to any significant reduction of binding activity, regardless of the presence of the C-terminal disulfide bridge. We, therefore, decided to construct the HuCAL®-Fab 1 library without the C-terminal cysteines in the constant domains.

During phage display of Fab fragments, only the Fd chain is fused to the phage coat protein, whereas the light chain gets attached to Fd after folding in the E. coli periplasm by noncovalent interactions only, if the C-terminal cysteines are missing. Therefore, it cannot be excluded that some light chains might get exchanged between different phages in a phage preparation, which would abolish the genotype-phenotype linkage. The noncovalent Fd-light chain interaction was found to be extremely strong (74), however, and extensive usage of the HuCAL®-Fab library proved that it is a reliable source for the isolation of binders against a variety of targets.

The HuCAL® master genes had been constructed originally in both the scFv and the Fab format (see Ref. 1). For the Fab format, the StII signal sequence derived from the E. coli heat-stable enterotoxin II (65) had been used to direct secretion of the light chain to the periplasm, mainly because StII has already been successfully used for functional Fab secretion (75). Preliminary expression experiments had shown, however, that light chains equipped with StII are less well expressed than light chains preceded by the OmpA signal sequence (66). We found that the replacement of StII by OmpA leads to about 2-fold higher expression yields and therefore decided to use the OmpA signal sequence for all Fab light chains. Preliminary expression experiments had shown, however, that light chains equipped with StII are less well expressed than light chains preceded by the OmpA signal sequence for all Fab light chains. The HuCAL®-Fab 1 library was finally constructed by inserting the diversified variable regions, transferred from the HuCAL®-scFv libraries, into the phage display vector pMORPH®18 containing the HuCAL® master genes in the Fab configuration. We omitted the VH4 master gene, since the extensive usage of the HuCAL®-scFv library has shown that this particular gene is only very rarely obtained after selection (1). A detailed analysis (76) has shown that the VH4 domain is less stable and not as stable enterotoxin II (65) had been used to direct secretion of the light chain to the periplasm, mainly because StII has already been successfully used for functional Fab secretion (75). Preliminary expression experiments had shown, however, that light chains equipped with StII are less well expressed than light chains preceded by the OmpA signal sequence (66). We found that the replacement of StII by OmpA leads to about 2-fold higher expression yields and therefore decided to use the OmpA signal sequence for all Fab light chains. Preliminary expression experiments had shown, however, that light chains equipped with StII are less well expressed than light chains preceded by the OmpA signal sequence for all Fab light chains. The HuCAL®-Fab 1 library was finally constructed by inserting the diversified variable regions, transferred from the HuCAL®-scFv libraries, into the phage display vector pMORPH®18 containing the HuCAL® master genes in the Fab configuration. We omitted the VH4 master gene, since the extensive usage of the HuCAL®-scFv library has shown that this particular gene is only very rarely obtained after selection (1). A detailed analysis (76) has shown that the VH4 domain is less stable and not as

![Graph](image1.png)  
**Fig. 6. Inhibitory effect of selected HuCAL®-Fabs on FGFR3-mediated cell proliferation.** FDCP-1-FGFR3 cells were grown in the presence of FGF9. Increasing amounts of the indicated HuCAL®-Fabs were added. After 2 days, proliferation was determined by adding XTT reagent (ΔA<sub>415</sub>). Inhibition of proliferation (IC<sub>50</sub> values) was calculated by a sigmoidal curve fit of the data.

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<th>TABLE II</th>
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<td><strong>Inhibition of FGFR3-dependent cell proliferation (IC&lt;sub&gt;50&lt;/sub&gt;) by selected HuCAL®-Fabs and affinity to FGFR3 (K&lt;sub&gt;D&lt;/sub&gt;)</strong></td>
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All values are calculated from three independent experiments except the numbers marked by an asterisk, which are derived from single measurements. ND, not determined.
RU

Fig. 7. Example of competition for FGFR3 binding epitopes by HuCAL®-Fabs in Biacore. First, HuCAL®-Fab MSPRO11 bound to FGFR3 until saturation was reached. Then a second HuCAL®-Fab (either MSPRO12, MSPRO2, MSPRO21, or buffer) was injected (indicated by an arrow). An additional increase in response units resulted from binding to different, nonoverlapping epitopes (e.g. MSPRO11 and MSPRO2). No increase in response units (RU) meant that the two Fabs recognized the same or overlapping epitopes on FGFR3 (e.g. MSPRO11 and MSPRO21).

Table III

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Table III contains the competition of selected inhibitory HuCAL®-Fabs for the binding epitope on FGFR3. The table lists the Fabs that were used to test for binding competition.

HuCAL®-Fabs inhibiting FGFR3-dependent cell proliferation can be divided in two groups. Binders of the different groups do not compete for the same or overlapping epitopes on FGFR3 in Biacore analysis.

TABLE III

Competition of selected inhibitory HuCAL®-Fabs for the binding epitope on FGFR3

The facts that HuCAL®-Fab 1 is a fully human antibody library and that, due to the modularity of HuCAL®, conversion into full immunoglobulins as well as antibody optimization is rapidly feasible render this new library ideally suited for the selection of antibodies for therapeutic application.

We therefore tested HuCAL®-Fab 1 against FGFR3, a receptor tyrosine kinase involved in skeletal disorders like achondroplasia and in tumor genesis. A potential therapeutic antibody candidate should be fully human, display high affinity and specificity to its target, and efficiently block FGFR3 activity.

Several antibodies to FGFR3 have been described in the past. Monoclonal antibodies were derived from an in vitro immunization approach and used for immunohistochemistry (77). Otherwise, mainly polyclonal antibody sera were raised by immunizing rabbits with peptides or domains of the human FGFR3 (78, 79). A blocking antibody to human FGFR3, which inhibits its signaling activity, had not been described so far. Difficulties in making monoclonal antibodies in mice could be due to the high homology of the mouse and human FGFR3 amino acid sequence. Human FGFR3 has an overall sequence homology of 92% to the mouse equivalent (80). We overcome homology problems and succeeded in rapidly isolating a plethora of specific antibodies against FGFR3 using the synthetic yet fully human HuCAL®-Fab library.

The members of the FGFR family (FGFR1 to 4) show an overall amino acid identity of 48–69% and some subregions are up to 100% identical (31, 32, 81). Additionally, FGFR1 and FGFR3 have similar ligand-binding pockets, and some (but not all) FGF ligands can activate both receptors (81). Thus, generation of antibodies specific to FGFR3 could be severely hampered as seen with other anti-FGFR antibodies cross-reacting with at least one other member of the family (77, 82). We also generated a variety of FGFR1/FGFR3 cross-reactive binders, but additionally, Fab antibodies discriminating between FGFR1 and FGFR3 were identified.

Sequence analysis of H-CDR3 revealed a length variation from 8 to 20 amino acids, covering a broad range of naturally occurring CDR3 lengths (83). FGFR3 antibodies were found from all VH families present in HuCAL®-Fab 1, although the VH1 family was the dominant source for FGFR3 binders, providing 73% of the selected Fabs. We have seen such a prevalence of certain VH families over the others already in the HuCAL®-scFv library (22) and also in applying HuCAL®-Fab 1 to other antigens. The dominating VH family varies and is dependent on the antigen. As a result, we conclude that it is advantageous for a library to contain a set of different VH family frameworks to ensure successful selections against a variety of antigens.

With a unique, differential whole cell panning approach (22), we succeeded in isolating 25 of 37 analyzed antibodies from HuCAL®-Fab 1 that blocked FGFR3-mediated cell proliferation. Of these 25 inhibitory clones, 13 clones had an affinity below 50 nM, and of these 13 clones, 6 Fabs blocked only FGFR3 activity and not FGFR1.

Competition experiments revealed that we identified two groups of FGFR3-neutralizing antibodies binding to different regions on FGFR3 yet both efficiently blocking the binding of the ligand FGF9 to FGFR3. These antibodies will be useful for further research and could be developed for e.g. therapy of achondroplasia caused by the G380R mutation of FGFR, since this activated receptor isoform was described to be still predominantly ligand-dependent (51, 84). Additionally, it was speculated that disregulation of the wild type FGFR3 in mul-
Multiple myeloma supports tumor growth (42, 44). The inhibitory anti-FGFR3 antibodies could thus also represent a new treatment for this blood-borne malignancy.

For FGFR3, some mutations are described, leading to ligand-independent activation of FGFR receptors (e.g. the constitutively active receptor mutant FGFR3S249C (85, 45), which is present in about 30% of bladder carcinomas (45–47)). Antibodies, inhibiting such ligand-independent signaling of constitutively active mutated FGFR3 receptor forms, would be of special interest for cancer treatment. Therefore, experiments have been initiated to evaluate whether the HuCAL® anti-FGFR3 Fabs inhibit different activated receptor forms as well. We identified several HuCAL®-Fabs that may become promising candidates for a future therapeutic development program. To the best of our knowledge, these are the first human monoclonal antibodies that are able to specifically block FGFR3 activity. They display a high selectivity for FGFR3, without cross-reactivity to FGFR1 and have an affinity in the subnanomolar range and an IC50 in the low nanomolar range. The monomeric affinities (0.7–0.9 nM) of these nonoptimized HuCAL®-Fabs are similar to affinities of other therapeutic antibodies against receptor tyrosine kinases (e.g. VEGFR2, c-ErbB2, or EGFR), which are in the range of 50 pM to 1 nM for bivalently binding IgGs (86–89).

At least two different inhibitory binding regions were defined. It will have to be tested which group will be more efficient in blocking FGFR3 activity in vivo. In the future, the efficacy of the HuCAL® anti-FGFR3 Fabs will be tested in different in vitro and in vivo settings in order to characterize their therapeutic potential for achondroplasia and tumor treatment. As HuCAL® enables the rapid generation of different antibody formats via one-step cloning procedures (22), it will be a fast process to define the best format for each indication.

Here, we report the generation of HuCAL®-Fab 1, a fully human phage display library, applying the HuCAL® concept on the Fab format. HuCAL®-Fab 1 was challenged against the tyrosine kinase receptor FGFR3, playing a crucial role in achondroplasia and tumor genesis. For the first time, FGFR3-specific and -inhibiting antibodies have been isolated with the potential for further preclinical and clinical development.

Meanwhile, we have further developed our HuCAL®-Fab technology by constructing the HuCAL®-GOLD library. HuCAL®-GOLD is a Fab library, based on the CysDisplay™ technology (4, 90), with all six CDRs being diversified according to the natural composition of antibody CDR regions.

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Human Combinatorial Fab Library Yielding Specific and Functional Antibodies against the Human Fibroblast Growth Factor Receptor 3


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