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Isoenzyme-directed selection and characterization of anti-creatine kinase single chain Fv antibodies from a human phage display library

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Abstract

Epitopes differing among isoenzymes of creatine kinase (CK) are apparently limited in number and poorly immunogenic in vivo. Especially for the BB-CK isoenzyme, very few monoclonal antibodies (mAb) are available. Here, we use in vitro selection with a synthetic human phage display antibody library and develop isoenzyme competition and peptide panning strategies to obtain human single chain Fv (scFv) antibodies against specific CK isoenzymes. We isolated and characterized seven scFv clones that recognize native as well as denatured cytosolic BB-CK in ELISA, immunoblot, immunofluorescence histochemistry and surface plasmon resonance (SPR) spectroscopy. To a variable but minor degree, they also react with cytosolic MM-CK, but not with mitochondrial CK isoenzymes. Epitope mapping revealed that the scFv antibodies recognize different BB-CK epitopes, including the N-terminus and the isoenzyme-specific box, a highly conserved sequence of unknown function for which no mAb were available so far. With a K_D of $3.5–9.6 \times 10^{-7}$ M, the isolated scFv compare favorably with mouse mAb and may overcome certain of their limitations. Our results demonstrate the advantages of in vitro antibody selection for the generation of isoenzyme-specific antibodies.

Keywords: Creatine kinase; Epitope mapping; Human synthetic phage display antibody library; Single chain Fv fragment; Surface plasmon resonance

1. Introduction

By classical methods of immunization of animals, valuable antisera as well as monoclonal antibodies (mAb) for a large number of proteins have been produced over the last decades. However, the use of single-chain Fv fragments (scFv) in combination with human synthetic phage display libraries, containing V genes rearranged in vitro or in vivo, has created numerous advantages [1,2] (reviewed in Refs. [3,4]). Instead of immunoselecting antibodies in vivo, scFvs displayed at the surface of phages are selected in vitro under controlled conditions [5,6]. Thus, antibodies can be generated that allow detection of epitopes that are poorly immunogenic in vivo. Any amount of soluble scFv fragments is then conveniently produced by recombinant techniques in *E. coli*. Thus, scFv antibodies can be subcloned for intracellular expression [7] and are easily mutated or modified, e.g. for subsequent affinity maturation [8]. Finally, human or humanized mAb for diagnostic and therapeutic purposes that are rather difficult to obtain from hybridoma cells can be readily selected from human scFv phage display antibody libraries [8,9]. An increasing number of highly specific scFv successfully isolated from phage display libraries suggest that it is the method of choice for tailor-made antibodies (e.g. Refs. [7–10], reviewed in Ref. [4]).

Isoenzymes of creatine kinase (CK) are key regulators of vertebrate energy metabolism [11,12] and have been recently involved in many human pathologies [13]. The cytosolic brain-type CK (BB-CK), for example, is up-regulated in wounded skin [14] and overexpressed in numerous malignant tumors [15]. In concert with its substrates creatine and phosphocreatine, BB-CK exerts cell-protective functions in many neurodegenerative and age-related diseases [15,16]. BB-CK is one out of five different CK isoenzymes that are assembled from four different subunits encoded by different genes [17]. Cytosolic MM-, MB-, and BB-CK are dimeric [18], while mitochondrial sMtCK and uMtCK form homodimers and homooctamers

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[12,19,20]. CK isoforms exhibit an extremely high homology in their sequence, and even more in their molecular structure. B-CK shows strong sequence identity with M-CK (77–82%) and mitochondrial CK (60–65%), depending on the species [17], and its fold revealed by the X-ray structure is almost identical to M-CK and MtCK [18–20]. This high homology has made it difficult to generate specific anti-human B-CK antibodies in another mammalian species like mouse or rabbit. In addition, the few epitopes differing between CK isoenzymes like the "isoenzyme-specific box" [17] are apparently poorly immunogenic for the in vivo production of poly- and monoclonal antibodies. Thus, only very few mAb recognizing native as well as denatured cytosolic B-CK were generated and applied for immunohistochemistry [21], immunoaffinity purification and immunoradiometric assays [22,23]. As far as determined, they recognize the N-terminal domain, or were explicitly generated with an N-terminal BB-CK peptide [21]. In vitro selection of scFv from phage display libraries could potentially circumvent the described problems. Moreover, there are multiple applications that would profit from new mAb recognizing different epitopes of cytosolic B-CK. These include immunohistochemistry in transgenic mice, the study of interaction epitopes between cytosolic CK and partner molecules, as well as diagnostic or even therapeutic purposes for these types of cancer overexpressing BB-CK.

We have focused on the selection of scFv clones from a human antibody phage display library [8] that recognize cytosolic BB-CK. The library is based on principles of protein design and contains $>3 \times 10^8$ different clones. Here, we report on the isolation and characterization of a panel of seven scFv binding to different epitopes of native as well as denatured human BB-CK. One epitope is the highly conserved isoenzyme-specific domain [17] that probably has a hitherto unrecognized function. Some of the scFv clones perform well in ELISA, immunoblotting, as well as in immunohistochemistry, thus demonstrating the advantages of in vitro selection for the generation of isoform-specific antibodies.

2. Materials and methods

2.1. Materials, bacterial strains, and proteins

The following *E. coli* strains were used: TG1 (K12, Δ(lac-pro), supE, thi, hsdΔ5/F′ traΔ36, $proA^+B^+$, lacIq, lacZΔM15) and HB2152 (K12, ara, D(lac-pro), thi/F′ $proA^+B^+$, lac^qZΔM15) (Amersham Pharmacia Biotech, Uppsala, Sweden). Highly purified recombinant human BB-CK, sMtCK, and uMtCK were prepared as described [18,19], peptides were synthesized by ANAWA (Wangen, Switzerland), and tissue extracts were prepared according to Schlegel et al. [24]. Protein was quantified with the Bio-Rad (Glattbrugg, Switzerland) reagent with BSA as a standard. If not mentioned otherwise, PBS buffer (50 mM Na-phosphate, 100 mM NaCl, pH 7.2) was used throughout the work.

2.2. Phage library

The phage display library of human recombinant antibodies in scFv format (ETH-2) has been kindly provided by Dr. D. Neri (Institute of Applied Biosciences, ETH Zürich, Switzerland). It is a modified version of the synthetic antibody library described in Ref. [8]. The library uses three antibody germline segments most frequently found in human antibodies (V_H: DP-47; V_k: DKP-22 and DPL-16; [25]) to generate a large repertoire of $>3 \times 10^8$ different scFv by randomizing the short complementary-determining regions. The ETH-2 library was cloned into phagemid vector pDN332 (derived from pHEN1; [2]) under the control of the *lac*Z promoter. The gene was fused at the 5′ end to a PelB leader (for secretion from *E. coli* into the medium) and at the 3′ end to a versatile tag (including hexa-His and FLAG-tag), an amber codon, and finally to the gene for the phage coat protein III [8].

2.3. Preparation of antigens for selection

The EZ-Link™ Biotin HPDP kit (Pierce, Rockford, USA) was used to biotinylate reduced forms of human BB-CK and huBpep (a 17-mer peptide corresponding to the isoenzyme-specific domain of human BB-CK; see Fig. 1b) at cysteine groups via disulfide bonds after the supplier's instructions. Thus, the protein/peptide can be cleaved away from HPDP-biotin by reducing agents like DTT. Concentrations were chosen to biotinylate BB-CK randomly in average at a single Cys residue or huBpep at the N-terminal Cys. Excess Biotin-HPDP and DTT were removed from BB-CK by dialysis and from huBpep by gel filtration chromatography with Superdex Peptide (Amersham Pharmacia Biotech). Peptide-and biotin-containing fractions were identified by dot blots with avidin-coupled horse radish peroxidase (Sigma, Buchs, Switzerland) or polyclonal anti-human BB-CK antibodies in combination with secondary HRPO-conjugated goat anti-rabbit IgG (Nordic, Tilburg, The Netherlands) and enhanced chemiluminescent substrate (Renaissance; NEN, Zaventem, Belgium).

2.4. Selection and amplification of specific scFv clones

The phage library was subjected to panning for binding using streptavidin-coated paramagnetic beads (Dynabeads M-280; Dynal, Skøyen, Norway) coated with nonlimiting amounts of biotinylated BB-CK or huBpep (10^{-7} to 10^{-8} M) according to described methods [8]. In five panning rounds, 10^{12} pfu phages were incubated with 6.7×10^7 coated Dynabeads in panning buffer (PBS with 2.5% milk powder) for 60 min at 22 °C on a rotary shaker. Binding to human BB-CK was competed with increasing amounts of

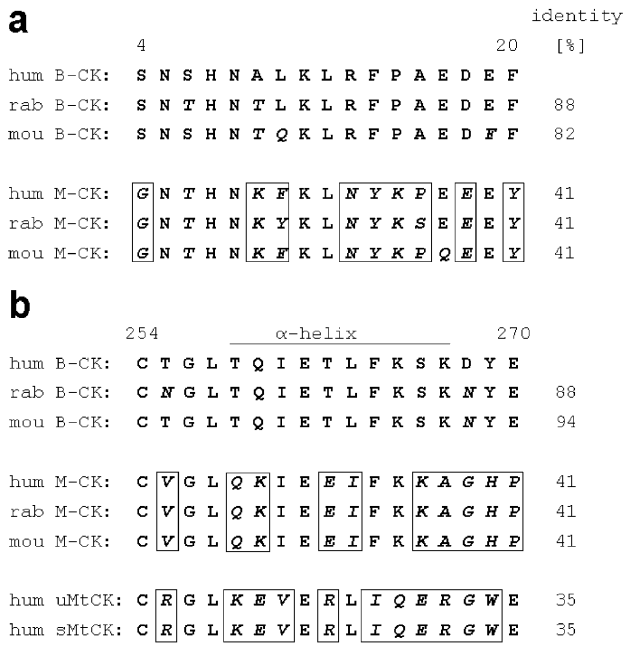

Fig. 1. Sequence comparison of peptide immunogens. Peptides used for the generation of (a) mouse mAb clone *21E10* (earlier work, see Ref. [28]), and (b) human scFv-Ab clones *p5-7* and *p5-8* (this work). Human, rabbit, and mouse sequences of different CK isoenzymes are presented. Amino acid substitutions compared to human BB-CK are given in italics and are boxed for other isoenzymes. The percentage of amino acid identity with the human peptide used to raise or select the mAb is indicated on the right. For a detailed sequence comparison of CK isoenzymes, see Ref. [17].

rabbit MM-CK (1×10^{-6} to 4×10^{-6} M) in panning buffer. Dynabeads were harvested with a magnetic particle concentrator and washed six times with PBS containing 0.1% Tween-20 and another four times with PBS, always using a magnetic particle concentrator for buffer exchange. Bound phages were recovered by addition of 20 mM DTT in PBS, reducing the S–S bonds that attach the antigen to the HPDP-biotin/avidin anchor. Phages were amplified and rescued from single colonies of infected *E. coli* TG-1 (an amber suppressor strain, allowing production of scFv fused to phage coat protein) with VCS-M13 helper phage (Stratagen, La Jolla, USA) as described [8]. Phages of the fourth and fifth selection rounds were used for expression and ELISA screening of specific binders.

2.5. Expression, purification, and gel filtration analysis of soluble scFv

Eluted phage was used to infect *E. coli* HB2152 (an amber non-suppressor strain). Single colonies from LB-Amp plates were grown in 200-μl 2 × TY medium supplemented with ampicillin and 0.1% glucose for 2 h at 37 °C, using 96-microwell plates (Nuncolon, Life Science Technologies, Basel, Switzerland) on a rotary shaker (30 rpm). Aliquots were stored in 2 × TY/40% glycerol at −20 °C. The cultures were induced by 3 mM isopropyl D-thiogalactoside (IPTG) to express soluble scFv and incubated for another 18–20 h at 30 °C. Microwell plates were centrifuged for 15 min at 1800 × *g* and protein concentration in the supernatants was determined with the Bio-Rad assay. For large-scale expression of selected scFv clones, aliquots from glycerol stocks were grown in 2 × TY/1% glucose for 3 h at 37 °C, transferred into 2-l Erlenmeyers containing 600-ml 2 × TY supplemented with ampicillin and 0.1% glucose, grown until 0.8 OD_{600} at 37 °C on a rotary shaker at 200 rpm, induced with 2.5 mM IPTG, incubated for another 16 h at 30 °C, and finally centrifuged 2 h at 3600 × *g*. ScFv were purified with Protein A Sepharose CL-4B (Amersham Pharmacia Biotech) [2]. The oligomeric state of scFv was determined by gel filtration chromatography of 30-μl samples with a Superose 12 column (Pharmacia) connected to HPLC (BioCad, Perkin-Elmer). Separations were done in 50 mM sodium phosphate, 300 mM NaCl, pH 7.0, at a flow rate of 0.75 ml min^{-1} and a temperature of 22 °C.

2.6. ELISA, immunoblot, and immunohistochemistry

Soluble ELISA of bacterial supernatants from single colonies was performed as described in Ref. [26]. Plates (96-well) were coated with 50 μg/ml human BB-CK, sMtCK or uMtCK (all highly pure, recombinant proteins), 150 μg/ml human muscle or human brain extract, or 60 μg/ml avidin coupled to biotinylated huBpep or huMpep (17-mers, see Fig. 1b). Supernatants of scFv-expressing *E. coli* HB2152 were adjusted to equal protein concentration before adding to the 96-well plates. After washing, bound scFv were detected with mouse anti-penta-His (1:10,000; Qiagen, Basel, Switzerland) and HRPO-conjugated goat anti-mouse IgG (1:30,000; Pierce) together with POD chemiluminescence ELISA substrate (Roche Biochemicals, Basel, Switzerland) by measuring the absorbance at 450 nm.

SDS-PAGE (12%) and semidry blotting were performed as described in Ref. [14]. Incubation with antibodies in TBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl) was as follows: scFv (10 μg/ml) for 60 min, mouse anti-penta-His (1:10,000; Qiagen) or mouse anti-FLAG (10 μg/ml; Sigma) for 30 min, and HRPO-conjugated goat anti-mouse IgG (1:30,000; Pierce) for 30 min. Blots were developed with enhanced chemiluminescence substrate (Renaissance; NEN) and exposed to X-ray film (Fuji Medical X-ray film, Fuji, Düsseldorf, Germany) for 2 s to 2 min.

Ethanol/acetic acid-fixed and paraffin wax-embedded samples of mouse skin (strains balb C, CL57BL/6, and B-$CK^{-/-}$uMtCK$^{-/-}$) as well as rat cerebellum were cut with a microtome into 5–7-μm-thin sections, processed, and mounted as described in Ref. [14]. Incubation with antibody diluted in PBS, 0.2% Tween-20 (octyphenol–polyethylenglycol), and 1% BSA was as follows: scFv (100 μg/ml, overnight at 4 °C), mouse anti-FLAG (10 μg/ml, 1 h at 22 °C; Sigma), and Cy3-conjugated goat anti-mouse IgG (1:200–1:500, 1 h at 22 °C; Pierce). Fluorescence images of sections were recorded on a fluorescence equipped

microscope (Carl Zeiss, Oberkochen, Germany) with an attached CCD camera (Hamamatsu 3CCD, Hamamatsu, Herrsching, Germany) using Plan-Neofluoar 10 × /0.3 objectives.

2.7. Surface plasmon resonance (SPR) spectroscopy

Binding kinetics, rate and equilibrium constants for the interaction between scFv and immobilized human BB-CK were determined in real time by SPR with a BIAcore 2000 instrument (Biacore, Uppsala, Sweden) as described [27]. The BIAcore signal is expressed in arbitrary response units (RU), which are proportional to the amount of material bound at a sensorchip surface. BB-CK or BSA (100–500 RU) were randomly linked to a carboxymethylated dextran-sensorchip (CM5; Biacore) using routine amine coupling [28] with 2 mg/ml BB-CK in acetate buffer pH 4.5 at 1 μl/min flow rate for 5–30 min. All SPR experiments were performed in PBS buffer containing 0.001% (v/v) detergent P-20. Programmed measurement cycles (sensorgrams, RU versus time) were routinely run with 300 RU immobilized CK at 20 μl/min and 25 °C. They consisted of association phase (or contact phase; injection of 1.8×10^{-6} to 1.8×10^{-7} M scFv), dissociation phase (injection of buffer), and surface regeneration (injection of 0.1 M acetic acid containing 0.5 M NaCl). In additional experiments, we varied the amount of immobilized CK (100 and 500 RU) and the flow rate (40 μl/min) to determine the influence of diffusion limitation or rebinding effects. The SPR signal obtained in parallel sensorgrams with immobilized BSA was subtracted from the binding kinetics to account for bulk refractive index changes and unspecific binding. The resulting kinetics was analyzed by nonlinear least-square fitting with BIAevaluation software to single-exponential rate equations (single-site interaction model), using a time interval from 10 to 90 s of association and dissociation kinetics to avoid artifacts due to mixing, bulk refractive index changes, and rebinding [29]. Off-rates (k_d) derived from dissociation kinetics and an M_r of 27 for scFv fragments were used to calculate on-rates (k_a) from association kinetics after $y = R_{eq}(1 - e - (k \cdot ac + kd) \cdot t)$. From these data, dissociation equilibrium constants (affinity constants) were calculated as $K_D = k_d/k_a$. For each scFv concentration, three different data sets of different experiments were pooled to calculate mean ± standard deviation (S.D.). To validate consistency of the equilibrium constants, they were independently calculated for clone *i5-2* from the concentration dependency of the extrapolated equilibrium response R_{eq} (Scatchard plot).

2.8. Two-site SPR binding assay for epitope mapping of scFv

Epitopes of the human BB-CK dimer recognized by the different scFv clones were compared by SPR. First, mouse anti-FLAG mAb (Sigma) were covalently linked to a CM5 sensorchip by amine coupling. Programmed sensorgrams run at 5 μl/min and 25 °C consisted of binding of a first mAb (scFv, 50 μg/ml) to the FLAG-tag, blocking of free FLAG-tag with FLAG-peptide (10 μg/ml; Sigma), binding of human BB-CK (1 mg/ml) to the first mAb, binding of a second mAb (50 μg/ml) to bound BB-CK, and surface regeneration (injection of 10 mM glycine pH 1.5). The duration of each association phase was 60 s. SPR signals obtained after 120-s dissociation of either BB-CK or the second mAb were taken as binding parameter of the first or second mAb, respectively. In control experiments, BB-CK and scFv/mAb were injected onto immobilized mouse anti-FLAG mAb blocked by FLAG-peptide, revealing neglectable background binding. First and second mAb were also injected in inverse order to check for consistency.

3. Results

3.1. Library panning and expression of soluble scFv

Recombinant phage expressing a library of scFv antibodies on their surface was produced by helper phage rescue and selectively enriched by panning (i) against immobilized native human BB-CK, competed by excess rabbit MM-CK, or (ii) against immobilized peptide corresponding to the isoenzyme-specific box of human B-CK (huBpep, residues 254–270; Fig. 1b). Soluble scFvs were produced by infection of *E. coli* HB2152 cells with phage obtained after four or five rounds of panning. Recognition of the amber stop codon between scFv and M13 gene III coat protein resulted in the production of soluble scFv by this strain. The soluble scFv were directed to the periplasmic space by a pelB leader sequence and incorporation of peptide epitopes (hexa-His-and FLAG-tag) at the C terminus of scFv allowed specific detection with anti-His or anti-FLAG M2 antibody. On induction with IPTG, an immunoreactive band at about 30 kDa was detected in periplasmic extracts of *E. coli* infected with phage. Soluble scFv were further purified by Protein A Sepharose, giving single bands on Coomassie-stained reducing SDS-PAGE (data not shown). Dimers or aggregates of scFv antibodies as frequently observed [30] were almost absent as shown by gel filtration chromatography (Fig. 2).

3.2. Specificity of selected scFv for native CK analyzed by ELISA

Several scFv clones obtained from competition screening against *i*ntegral BB-CK (*i* clones) or screening against *p*eptide huBpep (*p* clones) were ELISA-positive. They strongly bound to purified human BB-CK, human brain extract or huBpep (in case of *p* clones). For the seven clones selected, binding to rabbit MM-CK was either undetectable or very low (*i4-1*, *i5-2*, *i5-3*, *p5-7*, and *p5-8*) or only modest (*i4-4* and *i5-5*), as seen with purified protein or skeletal

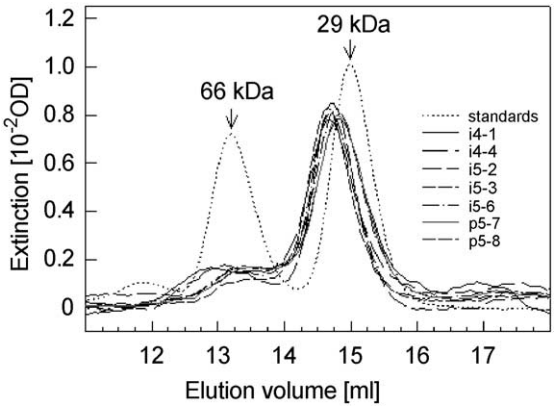

Fig. 2. Gel filtration chromatography of scFv. About 2 μg of protein (30 μl) of each scFv was separated on a Superose 12 column (Pharmacia) at a flow rate of 0.75 ml min^{-1} and 22 °C. As size standards, carbonic anhydratase (29 kDa) and bovine serum albumin (66 kDa) were used.

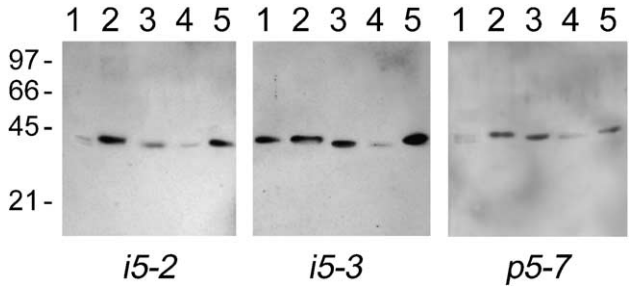

Fig. 4. Tissue specificity of phage display scFv; immunoblot of tissue extracts and purified CK probed with scFv-Ab clones *i5-2*, *i5-3*, and *p5-7*. Lanes: (1) human heart; (2) human brain; (3) human skeletal muscle; (4) rabbit MM-CK; (5) human BB-CK. Blots were probed with scFv antibody (10 μg/ml), followed by mouse anti-FLAG-tag mAb and rabbit anti-mouse Ab (conjugated with horse-radish peroxidase) as secondary and tertiary Ab, respectively. Exposure to film was 2 s (*i5-2*) or 20 s (*i5-3*, *p5-7*; for details see Materials and methods). M_r of molecular mass markers is indicated on the left.

muscle extract (data not shown). The latter two clones were selected despite their lower specificity for BB-CK, since a strong ELISA signal indicated rather high affinity. None of the selected scFv showed binding to human MtCK isoenzymes.

3.3. Specificity of selected scFv to denatured CK analyzed by immunoblots

Specificity of the selected, purified scFv to denatured proteins separated on SDS-PAGE was analyzed by Western blotting. Four clones (*i4-4*, *i5-2*, *i5-3*, and *p5-8*) were almost specific for the human B-CK subunit and showed only faint reactivity with the second cytosolic subunit, M-CK, from rabbit (Fig. 3). No cross-reactivity was seen with human mitochondrial MtCK isoenzymes or other proteins in human tissue extracts from brain, heart, and skeletal muscle (Fig. 4). Clones *i4-1*, *i5-5*, and *p5-7* recognized both, B-CK and M-CK, although *i4-1* and *p5-7* could distinguish the native dimeric forms of BB- and MM-CK

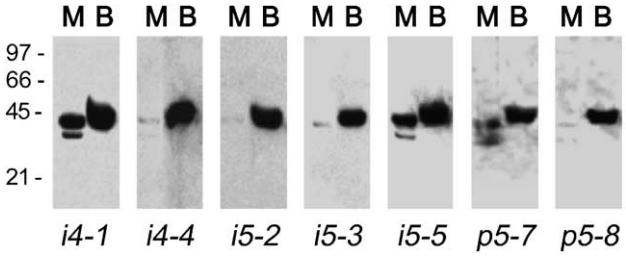

Fig. 3. CK isoenzyme specificity of phage display scFv; immunoblot of human BB-CK (lane B; 2 μg) and rabbit MM-CK (lane M; 2 μg) probed with a panel of seven different scFv-Ab (10 μg/ml) selected from the phage display library. Mouse anti-FLAG-tag mAb and rabbit anti-mouse Ab (conjugated with horseradish peroxidase) were used as secondary and tertiary antibodies, respectively (for details see Materials and methods). Exposure to film ranged from 20 s (*i4-4*) to 2 min (*p5-7*). M_r of molecular mass markers is indicated on the left.

in ELISA. By contrast, the cross-reaction of *i4-4* with MM-CK seen in ELISA was almost absent in the immunoblot. B-CK in human heart and brain was clearly detected by most scFv clones, e.g. *i5-3* (Fig. 4, lanes 1, 2 and 5), migrating slightly above the M-CK band (Fig. 4, lanes 3 and 4). Clones *i5-2* and *p5-7* differed in giving only a faint signal with B-CK in heart extracts. It is unknown whether this property is related to the presence of the MB heterodimer in this organ or to possible modifications in the recognized B-CK epitope. All scFv showed variable reactivity with denatured human MM-CK in skeletal muscle extracts (Fig. 4, lane 3), which was stronger than seen with native rabbit MM-CK (Fig. 4, lane 4; Fig. 2). Similar patterns of scFv reactivity were found for brain and muscle extracts from mouse and rat (data not shown), in agreement with the minimal sequence differences between these species (see Fig. 1 and Ref. [17]).

3.4. Binding parameters of selected scFv determined by SPR

Binding specificity, kinetic and equilibrium constants of purified scFv were determined by SPR spectroscopy. All selected scFv showed concentration-dependent binding to immobilized rabbit and human BB-CK (e.g. *i5-2*, Fig. 5), but not to a BSA control, which was then used to subtract the SPR signal due to bulk refractive index changes. Variation of immobilized BB-CK (100–500 RU) or flow rate (10–50 μl/min) had no systematic influence on association of dissociation kinetics, indicating that mass transport limitation and rebinding during dissociation did not affect our measurements. For clone *i5-2*, the dissociation equilibrium constant (K_D) was independently derived from on- and off-rates (k_a, k_d; see Table 1) and from Scatchard plot analysis. Both approaches gave similar results, indicating the consistency of the values obtained. The K_D of scFv clones ranged from 3.5×10^{-7} to 9.6×10^{-7} M, which is

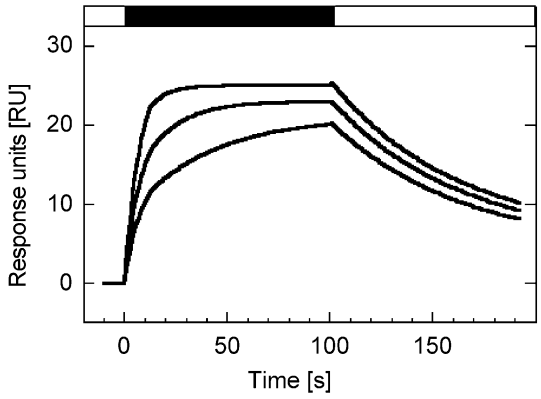

Fig. 5. Association and dissociation kinetics of phage display scFv on immobilized human BB-CK. Association and dissociation of clone *i5-2* at different concentrations (1.85×10^{-6}, 7.4×10^{-7}, and 1.85×10^{-7} M) was measured by surface plasmon resonance spectroscopy (BIAcore) to determine on- and off-rates (k_a and k_d) and to calculate affinity (K_D, as shown in Table 1). For details see Materials and methods.

only slightly lower than the affinity of *21E10* [21], an anti-BB-CK mAb derived from the murine secondary immune response (Table 1). Somewhat higher affinity was observed for *i4-4* obtained from panning against integral native BB-CK with competition by MM-CK as compared to other scFv.

3.5. Epitopes recognized by scFv determined by SPR

Since we had used integral human BB-CK for panning, we examined whether the selected scFv recognize similar or different epitopes. In particular, we analyzed whether they cluster with mAb *21E10* (produced against the N-terminal peptide of human BB-CK; see Fig. 1a and Ref. [21]) or with the isoenzyme-specific box scFv (*p5-7* or *p5-8*). A two-site SPR binding assay (Fig. 6a) clearly showed that all scFv obtained against BB-CK cluster with *21E10* and therefore with the N-terminal domain of BB-CK (Fig. 6b). *i5-2* and *i5-3* bind an epitope that is very close or identical to the N-terminal peptide recognized by *21E10* (see Fig. 1a). Since

Table 1
Kinetic and equilibrium constants for interaction of scFv with human BB-CK

Clone (scFv or mAb)	k_a (on-rate) [$\times 10^4$ M^{-1} s^{-1}]	k_d (off-rate) [$\times 10^{-2}$ s^{-1}]	K_D (affinity) [$\times 10^{-7}$ M]
i4-1	n.d.	n.d.	n.d.
i4-4	5.5	1.9	3.5 ± 1.2
i5-2	5.0	4.4	8.8 ± 2.1
i5-3	4.9	4.5	9.2 ± 2.2
i5-5	n.d.	n.d.	n.d.
p5-7	5.7	5.5	9.6 ± 1.9
p5-8	2.6	1.5	5.8 ± 1.4
21E10	9.8	1.5	1.5 ± 0.4

Kinetic constants were determined by surface plasmon resonance spectroscopy (BIAcore); K_D was calculated ($K_D = k_d/k_a$) and verified by Scatchard plot analysis (see Materials and methods). n.d., not determined. K_D values are given as mean ± standard deviation ($n = 3$).

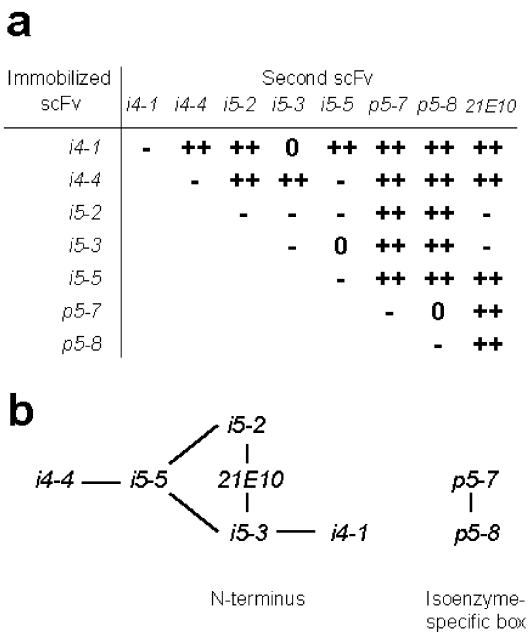

Fig. 6. Pairwise epitope mapping of phage display scFv and BB-CK. (a) The panel of seven different scFv-Ab selected from the phage display library, as well as the mouse mAb *21E10* [28], was used for pairwise epitope mapping with intact human BB-CK using surface plasmon resonance spectroscopy (BIAcore). The first scFv immobilized on anti-FLAG mAb was used to trap BB-CK. The second mAb was then tested for binding to the scFv–BB-CK complex (see Materials and methods), and the results classified as follows: (++) binding (50–100% of max. RU), (0) reduced binding (20–50% of max. RU), and (–) low or no binding (0–20% of max. RU). (b) Diagram of competing pairs of scFv. Pairs of scFv that show reduced binding as indicated by (–) or (0) are connected by lines. Note: The diagram reveals two clusters, the first containing *21E10* (binding to the N terminus), and the second containing scFv clones selected against the isoenzyme-specific box peptide.

binding sites of *i4-1* and *i5-5* overlap with *i5-3*, they probably also bind near the N terminus. *p5-7* and *p-8* form an independent group, binding to the isoenzyme-specific box.

3.6. Specificity of selected scFv in immunohistochemistry

Paraffin-embedded samples of rat cerebellum (Fig. 7a–c) and murine skin (Fig. 7d–f) were analyzed by fluorescence-immunohisotochemistry using the scFv clone *i4-4*. All tissues revealed specific staining of cells known to express BB-CK (the molecular layer in rat cerebellum, especially Bergmann glia cells, as well as epidermis, hair follicles, and panniculus carnosus in mouse skin) or MM-CK (Purkinje cells in rat cerebellum, panniculus carnosus in mouse skin) [14,31]. Control staining with the secondary mAb (anti-FLAG) and tertiary antibody gave negative results. When scFv clone *i4-4* was applied to skin sections of B-CK/uMtCK double knockout (k.o.) mice, no B-CK signal was detectable in epidermis and hair follicles (see arrows in Fig. 7f, lower part) as compared to controls (Fig. 7f, upper part). The remaining faint staining in the subepidermal layers of

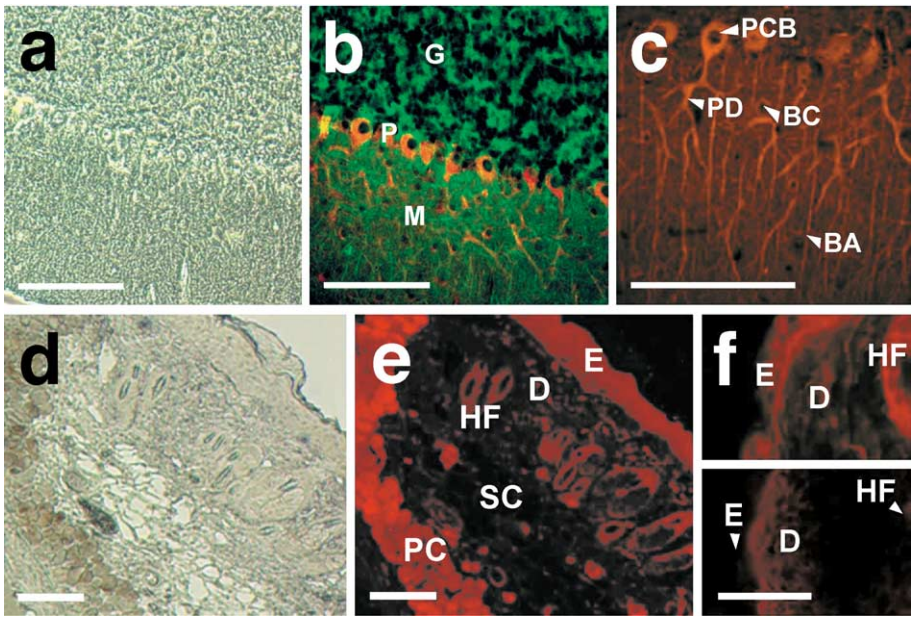

Fig. 7. Immunohistochemistry using phage scFv. (a, d) Phase contrast and (b, c, e) immunofluorescence microscopy images of paraffin sections of rat cerebellum (a–c) and murine skin of strain BALB/C (d, e), strain CL57 (f, upper half), and B-CK/uMtCK double knockout mice (f, lower half). Sections were stained for BB-CK with scFc clone *i4-4* (10 μg/ml), mouse anti-FLAG mAb, followed by a Texas Red-conjugated secondary antibody (red channel in immunofluorescence), and for glia filament acidic protein (marker for astrocytes and some other neural cell types) with a specific mAb and FITC-conjugated secondary antibody (green channel in b). Note: Co-localization of both markers in (b) results in yellow color. The fluorescence signal in CK knockout mice (f, lower half) is due to reaction with mouse IgG. For further details see Materials and methods. Abbreviations: BA, Bergmann glia axon; BC, Bergmann glia cell; D, dermis; E, epidermis; G, granular layer; HF, hair follicle; M, molecular layer; P, Purkinje cell layer; PCB, Purkinje cell body; PC, panniculus carnosus, PD, Purkinje dendrites; SC, subcutis. Bars = 10 μm.

the dermis in k.o. mice is also present when using only secondary and tertiary antibodies (results not shown). It is most probably due to higher levels of endogenous IgG in k.o. mice (Schlattner, unpublished data), reacting with the tertiary anti-mouse mAb. Staining with *i5-2* and *p5-8* was weaker, but more specific for BB-CK, while *i5-3* and *p5-7* revealed the lowest signals (data not shown).

4. Discussion

We have shown here that scFv antibodies binding to different and even poorly immunogenic epitopes of cytosolic human B-CK can be isolated by in vitro screening from a human synthetic phage display library. Both applied panning strategies were successful: (i) competition screening, using integral, native human BB-CK and competition with excess native rabbit MM-CK; and (ii) peptide screening, using huBpep, a 17-mer corresponding to the so-called isoenzyme-specific box of human B-CK [17]. Various techniques including ELISA, immunoblot, immunohistochemistry, and SPR spectroscopy proved that the scFv antibodies recognize native as well as denatured B-CK, and to some extent also M-CK. Due to high sequence homology among CK isoenzymes and the poor immunogenicity of some differing epitopes, only few mAb have been developed so far that react with native as well as denatured cytosolic BB-CK [21–23].

ScFv selected for binding to integral human BB-CK (*i* clones) show affinities that are only slightly below those of mouse mAb. Competition with rabbit MM-CK successfully minimized recognition of this isoenzyme (e.g. *i5-2* and *i5-3*), although some reactivity towards human MM-CK persisted for most scFv clones, especially in human muscle extracts containing high amounts of this isoenzyme. This cross-reactivity is seen with many available anti-BB-CK antibodies and is due to the extremely high similarity among cytosolic CK isoenzymes (about 80% sequence identity) as compared to mitochondrial CKs [17]. Interestingly, all scFv obtained with this first screening strategy recognized surface epitopes near the N terminus, which was possibly more accessible.

Besides the N terminus, the so-called isoenzyme-specific box (residues 254–270) is the only sequence motif showing considerable difference between BB-CK and MM-CK or mitochondrial CK isoenzymes (only 41% identity; see Fig. 1b and Ref. [17]). This motif corresponds to a surface-exposed, amphiphatic α-helix in the C-terminal CK domain [18] and is of special interest because it represents a putative interaction site with hitherto unknown binding partners [32]. We have used this motif for peptide screening, since no specific mAb have been available so far. The obtained scFv (*p* clones) show some cross-reactivity with MM-CK, but similar affinities as the *i* clones, except the higher affinity clone *i4-4*. However, all *i*-clones perform better with denatured protein in immuno-stainings, possibly because dena-

turation makes the N-terminal epitopes more accessible. To improve binding properties, scFv could potentially be affinity maturated by random mutation of specific residues and panned with a more stringent competition by MM-CK.

The scFv characterized in this study will be a useful tool for a variety of applications. Their main advantages are (i) the presence of multiple tags (His-and FLAG-tags), (ii) the specificity for different epitopes (the isoenzyme-specific box [*p5-7*, *p5-8*], the N terminus [*i5-2*, *i5-3*] or other epitopes in the N-terminal domain [*i4-1*, *i4-4*, *i5-5*]), and (iii) the possibility of intracellular expression. Through multiple tags, one can potentially avoid the use of anti-mouse IgG in immunohistochemical detection. These Ab often give false positive signals in mouse tissue due to reaction with endogenous IgG. The variety of recognized epitopes further facilitates detection of BB-CK in histological sections, since certain epitopes may not be accessible as reported with mouse mAb directed against the N-terminal peptide [21]. The specificity of scFv for different epitopes can also be used to directly probe CK interactions with partner molecules. For example, cytosolic expression of these scFv in cell culture [7] may be used to block such interactions and to study their physiological significance.

The work presented here already demonstrates that (i) isoenzyme-specific scFv antibodies can be readily generated from a human synthetic phage display library by different selection strategies, and that (ii) scFv antibodies compare favorably with mouse mAb in regard to their affinity as well as their suitability for a variety of typical applications.

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