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Generation of affinity matured scFv antibodies against mouse neural cell adhesion molecule L1 by phage display

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Abstract

The recognition molecule L1 plays important functional roles in the nervous system and in non-neural tissues. Since antibodies to L1 are of prime importance to study its functional properties, we have generated affinity matured human single chain variable fragment (scFv) antibodies against mouse L1 by introducing random mutations in the complementarity determining regions (CDRs) of a previously isolated scFv antibody heavy chain (CDR1 and CDR2) and light chain (CDR3). After biopanning the mutant library, a clone (5F7) that gave the strongest ELISA signal was expressed, purified, and characterized. The dissociation constant of 5F7 (2.86×10^{-8} M) was decreased 60-fold compared to the wild type clone G6 (1.72×10^{-6} M). 5F7 detected L1 by Western blot analysis in mouse brain homogenates and recognized L1 in L1 transfected cells and cryosections from mouse retina and optic nerve by immunofluorescence. Bivalent 5F7 scFv antibody (5F7-Cys) was also generated and showed a dissociation constant of 5.22×10^{-9} M that is 5.5-fold lower than that of monomeric 5F7 antibody. The bivalent affinity matured L1 scFv antibody thus showed stronger binding by a factor of 310 compared to the wild type clone. This antibody should be useful in various biological assays.

Keywords: Affinity maturation; Bivalent scFv; Neural cell adhesion molecule L1; Phage display; scFv; Site-directed mutagenesis

The neural cell adhesion molecule L1 [1] has been shown to mediate cell adhesion, neuronal migration, neurite outgrowth, axon fasciculation [2], neuronal survival [3,4], and synaptic plasticity [5] in vitro. The phenotype of L1-deficient mice and humans carrying mutations in the L1 gene confirms a critical role of L1 for the normal development of the nervous system ([6,7], for reviews, see [8–10]). L1 interacts homophilically and heterophilically with several ligands including cell surface and extracellular matrix glycoproteins (for reviews, see [11–13]).

Many functions of L1 were characterized by using antibodies in vitro and in vivo. To be useful reagents for diagnostic purposes and investigations of the molecule's biological functions, antibodies need to be obtained that satisfy several requirements: (1) they should show high binding activity for L1; (2) they should be efficient reagents in different assay systems, such as ELISA, Western blot, and immunocytochemistry; (3) they should be functionally active in that they can either block or enhance the functions of L1; (4) they should be reproducibly obtained; and (5) they should be cheaply available in large amounts. To achieve these aims, we have made use of the phage display technology [14] and isolated single chain variable fragment (scFv) antibodies that bind to L1 [15]. The antibodies obtained by this method bind to mouse L1 in a specific and highly reproducible manner, but did not recognize L1 in Western blots prepared under reducing conditions and did not give specific signals by indirect immunofluorescence in cryosections of adult mouse brain. To obtain antibodies with high binding affinity, we used the method of affinity maturation with phage display technology to increase the affinity of the antibody by mutation of the complementarity determining regions, a process that the immune system uses naturally when exposed repeatedly for long times to a particular antigen. We not only mutated one complementarity determining region (CDR), but a

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total of three CDRs in the hope that affinity maturation in these three regions would increase the affinity of the antibody considerably. Further, we adopted the principle of making an antibody more avid by introducing bivalency, as naturally occurring in the immunoglobulin class G by homodimerization of the scFv antibody.

Here we report the affinity maturation by random mutagenesis and homodimerization of the affinity matured scFv antibody to yield an overall increase in affinity by a factor of approximately 300, from a dissociation constant in the micromolar range to a dissociation constant in the nanomolar range. This antibody was able to detect L1 in homogenates of adult mouse brain under reducing conditions by Western blot analysis and reacted specifically by indirect immunofluorescence in cryosections of mouse retina and optic nerve. This antibody will be useful in a broad range of future experiments on mouse L1.

Materials and methods

Design and construction of a mutant library for L1 scFv antibody affinity maturation and cloning. For the construction of a L1 scFv affinity matured library, the gene of L1 scFv clone G6 selected from a human synthetic phage display scFv library was used as template for PCR amplification. Oligonucleotides used for PCR amplification of heavy and light chain V-regions genes are described in Table 1. The amino acid residues of the antibody are numbered according to Kabat and colleagues [16]. The mutated scFv gene fragments were generated by PCR amplification with primers VHNcoback and VHCDR1for (Table 1) to introduce random mutations at positions 31–33 in the CDR1 region of the V_H gene [17,18], with primers VHCDR1back and VHCDR2for to randomly mutate positions 50, 52, and 54 in CDR2 of the V_H gene [17] and with primers VHCDR2 back and VLCDR3for to introduce random mutations at positions 91–95b of CDR3 of the V_L gene. PCR was performed in a volume of 50 μl using *Taq* DNA polymerase (Life Technologies, Eggenstein, Germany) and 500 pM of each primer for 25 cycles (1 min at 94 °C, 1 min at 65 °C, and 1 min at 72 °C). The three resulting PCR products were agarose gel-purified with the Gel Extract Rapid Kit (Life Technologies) and spliced together with overlap extension PCR to generate a scFv gene mutant library [19]. The mutated V_H and V_L gene fragments were spliced together in 100-μl PCRs containing 100 ng of the fragments and *Taq* DNA polymerase. The reactions were cycled eight times (95 °C, 2 min; 55 °C, 1 min; and 72 °C, 3 min) to join the fragments. The VHNcoback and VLNotfor primers were then added and the reaction was cycled another 25 times (94 °C, 1 min; 65 °C, 1 min; and 72 °C, 2 min) to amplify the assembled full-length scFv genes for making the mutant library. The overlap extension PCR was repeated three times.

Each individual PCR product was purified from the PCR, cut with restriction enzymes *Nco*I and *Not*I, and ligated into the phagemid of pHEN2 digested with *Nco*I and *Not*I. The pHEN2 phagemid has a myc tag for detection and a 6× His tag for purification using Ni–NTA agarose (Qiagen, Hilden, Germany). Approximately 9 μg vector and 3 μg insert were used in the ligation mix, which was purified by the PCR Rapid Purification Kit (Roche Molecular Biochemicals, Mannheim, Germany), eluted in 50 μl of sterile water, and electroporated into electrocompetent TG1 *Escherichia coli* cells. A mutant library containing 4.82×10^8 members was generated from the products of three ligations and seven electroporations. The library size was calculated by counting the number of ampicillin-resistant colonies. Library quality was verified by determining the percentage of clones with inserts of appropriate size for a scFv gene. The diversity of the library was confirmed by sequencing randomly picked clones. The resulting library was mutated in the V_H-CDR1, V_H-CDR2, and V_L-CDR3 regions of the G6 gene.

Purification of mouse L1Fc. The mouse L1Fc fusion protein (mL1Fc) which contains the whole extracellular domain of mouse L1 (amino acids 1–1123, DataBank Accession No. X12875) in fusion with the Fc portion of human IgG1 was expressed in CHO cells and purified as described [4].

Selection of L1 scFv antibodies from the mutant library. The selection of scFv binders by a biopanning procedure was performed essentially as described [20]. Binders were selected on mL1Fc-coated NUNC immunotubes (NUNC, Wiesbaden, Germany). Tubes were coated at concentrations of 100, 25, 10, 1, and 1 nM for 1–5 rounds of selection in phosphate buffered saline (PBS), pH 7.5, overnight at 4 °C. On the next day, tubes were blocked with 2% MPBS (2% skim milk in PBS) and incubated at room temperature (RT) for 2 h. After washing the tubes three times with PBS, phagemid particles (approximately 10^{12}–10^{13} cfu) were added in 4 ml of 2% MPBS and incubated for 2 h at RT. Tubes were washed 20 times with PBS containing 0.1% (v/v) Tween 20 and 20 times with PBS. Bound phages were eluted with 1 ml of 100 mM triethylamine and quickly neutralized with 0.5 ml of 1 M Tris–HCl, pH 7.4. The eluted phages were used to infect exponentially growing *E. coli* TG1 cells and grown overnight at 30 °C on TYE (10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 8 g NaCl in 1000 ml distilled water, pH 7.4) plates containing 100 μg/ml ampicillin and 1% glucose. After five rounds of selection, individual clones from the fifth round were analyzed by phage ELISA.

Indirect ELISA using scFv antibodies. Ninety-six-well microtiter plates were coated with 100 μl of either 1 μg/ml mL1Fc, 10 μg/ml human IgG or 10 μg/ml BSA in PBS overnight at 4 °C and blocked with 2% MPBS. Hundred microliter of bacterial supernatant was added to each well including 2% skim milk and incubated for 2 h at RT. After washing three times with 0.05% Tween 20 in PBS and three times with PBS, anti-myc polyclonal antibody (diluted 1:2000, Santa Cruz Biotechnology, Heidelberg, Germany) was added and incubated for 2 h at RT. The wells were washed again and incubated with HRP-conjugated anti-rabbit IgG (Dianova, Hamburg, Germany) for 1 h at RT. Binding was detected using TMB (3,3′,5,5′-tetramethylbenzidine) as substrate. For specificity analysis of scFv antibodies, human L1Fc (1 μg/ml) and P0Fc (10 μg/ml) were used as additional controls.

Table 1
Oligonucleotide primers used for construction of the mutant library for L1 scFv antibody affinity maturation

VHNcoback	5′-GCG GCC CAG CCG G*CC ATG G*CC GAG GTG CAG CTG GTG GAG TCT GG-3′
VHCDR1back	5′-ATG AGC TGG GTC CGC CAG GCT CC-3′
VHCDR1for	5′-GAG CCT GGC GGA CCC AGC TCA TMN NMN NMN NAC TAA AGG TGA ATC CAG AGG CTG-3′
VHCDR2back	5′-GAG AAA TAC TAT GTG GAC TCT GTG-3′
VHCDR2for	5′-GTC CAC ATA GTA TTT CTC ACT MNN ATC TTG MNN TAT MNN GGC CAC CCA CTC CAG CCC CTT C-3′
VLCDR3for	5′-CTT GGT CCC TCC GCC GAA TAC CAC $(MNN)_7$ GGA GTT ACA GTA ATA GTC AGC CTC-3′
VLNotfor	5′-GAG TTT TTG TTC T*GC GGC CGC* ACC TAG GAC GGT CAG CTT GGT CCC TCC GCC GAA TAC-3′

Sequence symbols are: A (adenine), C (cytosine), G (guanosine), T (thymidine). Wobble symbols: M (A or C), N (G or A or T or C).

Sequencing of clones. The sequences of selected clones were determined with the primers LMB3 (5′-CAG AAA CAG CTA TGA C-3′) and fdSEQ1 (5′-CAG AAA CAG CTA TGA C-3′) [20] by the dideoxy chain terminating method. Sequencing was repeated three times for verification. Automated sequencing was performed using a model 377 DNA sequencer from Applied Biosystems according to manufacturer's instructions. The sequence data were analyzed using the DNA Star program (DNASTAR, WI, USA).

Expression and purification of scFv antibodies. The scFv genes were transformed into the non-suppressor strain of *E. coli* (HB2151) for producing soluble scFv antibodies induced by IPTG since the pHEN2 carries a amber stop codon (TAG) between scFv gene and M13 minor coat protein gene3. The HB2151 is not a supE bearing strain which does not read TAG as glutamic acid. *E. coli* HB2151 cells transformed with selected scFv genes were maintained in LB medium containing 0.1% glucose and 100 μg/ml ampicillin at 37 °C on a shaker until they had reached an OD600 of approximately 0.8–0.9. Production of scFv antibodies was induced by the addition of IPTG to a final concentration of 1 mM into the culture medium and continued by shaking overnight at 30 °C. Antibodies from culture supernatants were purified via Ni–NTA agarose chromatography. The column was equilibrated with 50 mM phosphate buffer (pH 7.5), 500 mM NaCl, and 20 mM imidazole and washed with equilibration buffer once after loading of bacterial supernatant which had been dialyzed against equilibration buffer overnight at 4 °C. The scFv antibodies were eluted with 50 mM phosphate buffer (pH 7.5), 500 mM NaCl, and 100 mM imidazole and 2 ml fractions were collected. The eluted scFv antibodies were pooled, dialyzed against PBS, and concentrated by centrifugation in Centricon-10 tubes (Millipore-Amicon, Eschborn, Germany).

Western blot analysis. Brain homogenates of L1-deficient mice (50 μg) [6], crude membrane fractions (20 μg), and brain homogenates (20 μg) from wild type mice, and mL1Fc (2 μg) were subjected to SDS–PAGE (6% gel) under non-reducing and reducing conditions, and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The membranes were blocked for 2 h at RT with 5% MTBS (5% skim milk in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl). The scFv antibody (at a 1:2 dilution of 10× concentrated supernatant) was added and incubated overnight at 4 °C. Binding of scFv antibodies was detected with anti-myc antibodies and HRP-conjugated secondary antibodies as described [21].

Epitope mapping. For mapping of the L1 epitope recognized by the L1 scFv antibodies, recombinant mouse L1 fragments were expressed in *E. coli* using the pET expression vector system [22] and detected with L1 scFv antibodies by Western blot analysis. In brief, DNA fragments coding for immunoglobulin domains I–II (Ig1–2, amino acids 22–240), III–IV (Ig3–4, amino acids 239–427), and V–VI (Ig5–6, amino acids 426–661) and fibronectin type III repeats 1–2 (FN1–2, amino acids 631–826), and 3–5 (FN3–5, amino acids 828–1093) were derived from the full-length mouse L1 cDNA and cloned into the pET expression vector. Inclusion bodies containing the L1 fragments were resuspended in solubilization buffer (8 M urea, 0.1 M NaH_2PO_4, 1 mM dithioerythritol, and 20 mM Tris–HCl, pH 8.0) and incubated with agitation for 1 h at RT. The lysates were centrifuged at 10,000*g* for 20–30 min at RT to pellet the cellular debris. Supernatants were collected, dialyzed against 20 mM Tris–HCl, pH 7.5, separated by SDS–PAGE (12% gels), and transferred to nitrocellulose membranes. The membranes were blocked for 2 h at RT with 5% MTBS. The scFv antibodies (at a 1:2 dilution of 10× concentrated supernatant) were added and incubated overnight at 4 °C. Binding of scFv antibodies was detected as described for Western blot analysis.

Determination of antibody affinity by using competition ELISA. For determination of antibody affinity, a competitive ELISA was used [23, 24]. In brief, scFv antibodies at 5 nM (which was within the range of values that gave a linear relationship between antibody concentration and ELISA signal in titration experiments) were equilibrated with increasing concentrations of mL1Fc (ranging from 1 nM to 10 μM) in 100 μl volume MPBST (PBS containing 2% skim milk and 0.05% Tween 20) for 1 h at RT. The pre-equilibrated scFv antibodies were then transferred to a microtiter plate coated with mL1Fc and processed by standard ELISA assay as described above. The concentration of antigen at which the half-maximal ELISA signal is detected corresponds to the dissociation constant (K_d) [25].

Construction of bivalent scFv to improve binding avidity of the scFv antibody. For construction of bivalent scFv antibodies, we generated a new vector pLD-Cys in which a cysteine residue was introduced into the pLD1 vector between the myc tag and the 6 × His tag by PCR using primers pLDcysBgl2back (5′-AAG ATC TAG GTG GCT GCC ATC ATC ACC ATC ATC ATT AA TC-3′) and pLDcysDra3for (5′-TGG GTG ATG GTT CAC GTA GTG GGC CAT-3′). The PCR products were digested with *Bgl*II and *Dra*III, and ligated into pLD1 to obtain pLD-Cys. The pLD-Cys allows the scFv antibody to form homodimers by a disulfide bridge (Fig. 3A). The vector of pLD1 was derived from pHEN1 [26] by replacing the phage minor coat protein gene3 sequence between the *Not*I and *Eco*RI (filled in) sites with the fragment of *Not*I–*Nhe*I (filled in) of pOPE101-215(Yol) [27]. The resulting pLD1 contains a myc tag for detection and a 6 × His tag for purification of scFv antibody via Ni–NTA agarose chromatography. The genes of the scFv antibodies G6 and 5F7 were subcloned into the pLD-Cys vector via *Nco*I and *Not*I sites for production of the corresponding homodimers. Expression and purification of bivalent scFv antibodies were performed as described above for monovalent scFv antibodies.

Immunofluorescence staining. Indirect immunofluorescence of L929 cells stably transfected with mouse L1 was performed as described with minor modifications [28]. Briefly, cells grown on poly-L-lysine- (pLL) coated glass coverslips were washed with PBS. For live-cell-staining, cells were blocked with 5% fetal calf serum in PBS for 15 min at RT. ScFv antibodies were then applied at a concentration of 50 μg/ml and incubated for 1 h at RT. After washing, cells were fixed for 5 min in 4% paraformaldehyde and washed again with PBS, and anti-myc polyclonal antibodies (diluted 1:200) were added for another 1 h at RT. Antibody binding was detected with Cy3-conjugated secondary antibodies (Dianova). As a negative control, cells were treated in the same manner except that incubation with scFv antibodies was omitted.

Immunohistochemistry. Cryosections (16 μm thick) from retina and optic nerve of adult mice were mounted on pLL-coated glass coverslips, dried overnight, blocked with PBS containing 1% BSA for 30 min at RT, and incubated for 2 h at RT with scFv antibodies (10 times concentrated supernatant of bacterial cultures). Bound scFv antibodies were detected using anti-myc polyclonal antibodies (diluted 1:200, RT for 1 h) and Cy3-conjugated goat-anti-rabbit IgG (diluted 1:200, RT for 1 h). For control, cryosections were treated in the same manner except for the omission of the scFv antibodies.

Results

Construction of mutant phage display scFv library for affinity maturation of L1 scFv antibody

In a previous study [15], we have isolated a scFv antibody (G6, GenBank Accession No.: AF 394236) using the human synthetic phage display scFv library [29]. This antibody bears moderate affinity (K_d 2×10^{-6} M) and reacted specifically with mouse L1 by ELISA and Western blot analysis under non-reducing conditions. However, the antibody did not react with L1 by Western blot analysis under reducing conditions, or by indirect immunohistology on fresh frozen sections of adult mouse brain.

We therefore decided to isolate more tightly binding and more broadly applicable antibodies by affinity maturation by introducing random mutations in the complementarity determining regions (CDRs) of the scFv antibody heavy chains (CDR1 and CDR2) and light chain (CDR3) by site-directed random mutagenesis. The strategy for the design and construction of the affinity matured antibody is shown in Fig. 1. Wild type scFv clone G6 gene was modified by introducing random mutations in the V_H-CDR1, V_H-CDR2, and V_L-CDR3 regions using PCR (Fig. 1B). PCR products with random mutations in the V_H-CDR1 region (Fig. 1B,

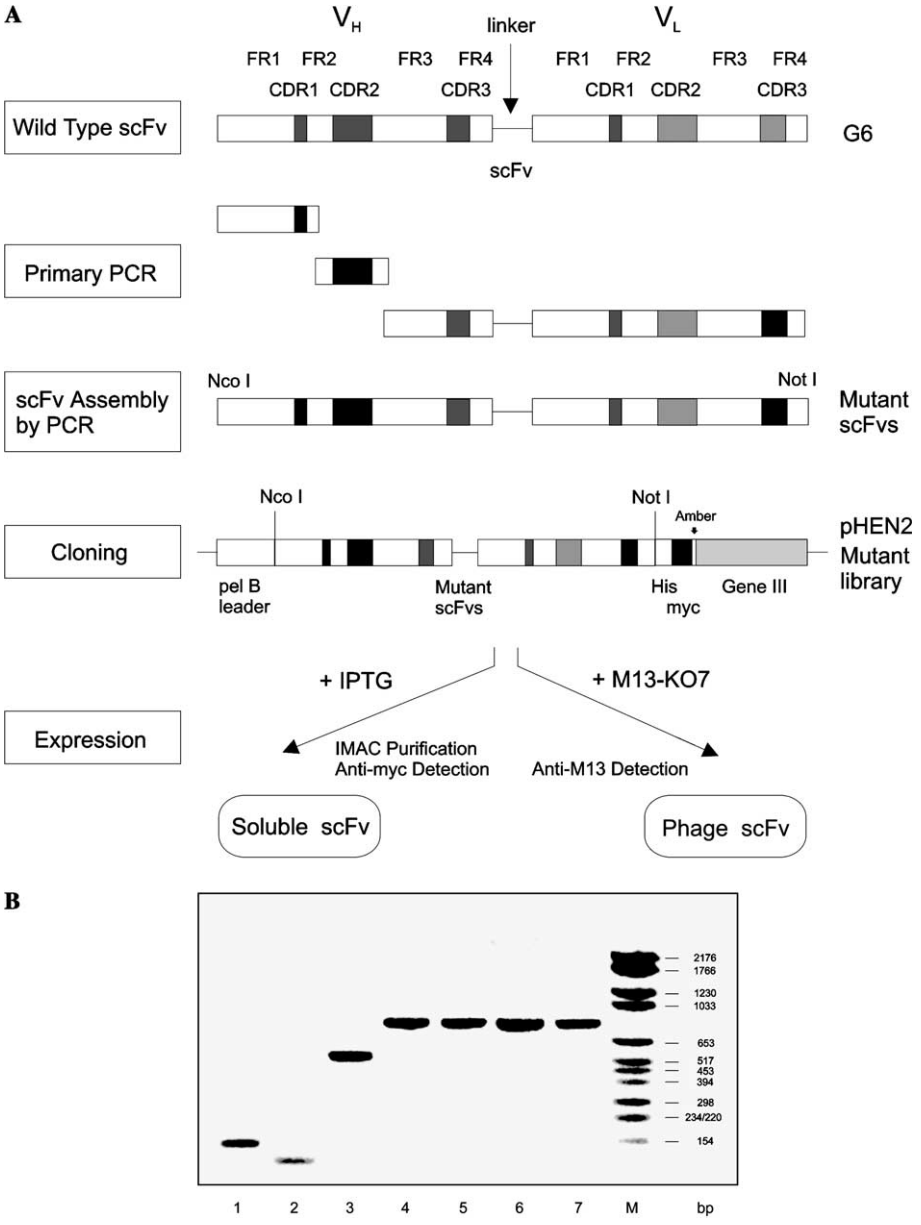

Fig. 1. Design and construction of the mutant phage display library for affinity maturation of L1 scFv antibody. (A) Schematic strategy to construct the mutant library for scFv affinity maturation. Wild type scFv clone G6 gene was modified by introducing random mutations in the V_H-CDR1, V_H-CDR2, and V_L-CDR3 regions (black rectangles) using PCR. Three successive PCR products were assembled to generate a full-length mutated scFv gene which was cloned into the phagemid pHEN2 via *Nco*I and *Not*I sites. The amber codon between the tags and the phage minor coat protein III gene allows expression of soluble scFv antibody in a non-suppressor strain of *E. coli* induced by IPTG and production of phage displayed scFv antibodies in a suppressor strain of *E. coli* using M13KO7 helper phage. Phagemid-expressed scFvs carry a 6× His tag for IMAC purification by Ni–NTA agarose and a myc tag enabling detection with the anti-myc antibody. (B) Gel profile of PCR products. PCR products with random mutations were run on 1.5% agarose gels and visualized with ethidium bromide. Lane 1, PCR products mutated in the V_H-CDR1 region; lane 2, PCR products mutated in the V_H-CDR2 region; lane 3, PCR products mutated in the V_L-CDR3 region; lanes 4–6, assembled full-length mutated scFv genes from the above three PCR products; lanes 4 to 6 indicate the three individually assembled PCR products; and lane 7, wild type scFv clone G6 gene. DNA molecular weight standards (lane M) are indicated at the right in bp.

lane 1), V_H-CDR2 region (Fig. 1B, lane 2), and V_L-CDR3 region (Fig. 1B, lane 3) were generated with the oligonucleotide primers indicated in Table 1. Full-length mutated scFv genes (Fig. 1B, lanes 4–6) were assembled using the three PCR products. The mutant library was then generated by cloning the full-length mutated scFv genes into phagemid pHEN2. The potential diversity of the mutant library is 20^{13} (8.192×10^{16}) members. However, our library contained only 4.82×10^8 clones, indicating that only a small fraction of the potential diversity was tapped, most likely because of the limitation in electroporation efficiency [30]. Restriction enzyme digestion of 18 randomly selected clones revealed an expected insert with an average size of about 750 bp in each clone. Sequence analysis of these clones showed unique sequences and relatively random mutations in the V_H-CDR1, V_H-CDR2, and V_L-CDR3 regions as expected. In the pHEN2 phagemid, the amber codon between the tags and the phage minor protein III gene allowed the expression of soluble scFv antibodies in the non-suppressor strain of *E. coli* after induction by IPTG. Production of phage displayed scFv antibodies was possible by using a suppressor strain of *E. coli* with the M13KO7 helper phage. Phagemids expressing scFv antibodies were constructed such that they carried a 6× His tag for purification by IMAC (immobilized metal affinity chromatography) using Ni–NTA agarose and a myc tag that allowed detection with myc antibody.

Selection of affinity matured scFv antibody against mouse L1 from a mutant phage display scFv library

To select for scFv antibodies with higher affinity, the mutant library was tested using decreasing concentrations of immobilized mouse L1Fc for each consequent selection round. After 5 rounds of panning, 24 clones were picked randomly after infection with eluted phages and individually tested for binding to L1Fc by the phage ELISA. Out of these clones, one clone (5F7) which showed the highest immunoreactivity to L1Fc by ELISA was chosen for further characterization. The resultant antibody reacted with mouse L1Fc, but not with human L1Fc, rat P0Fc, human immunoglobulin G or BSA (data not shown).

Sequence analysis revealed that mutations had been successfully introduced into positions 31–33 in the V_H-CDR1 region, positions 50, 52, and 54 in the V_H-CDR2 region and positions 91–95b in the V_L-CDR3 region of affinity matured scFv clone 5F7 compared to wild type clone G6 (Table 2). These data suggested that the PCR based site-directed random mutagenesis had generated mutations in the designed regions. The conservation of amino acids at position 32 (Y) of V_H-CDR1 and positions 91 (R), 94 (S), and 95 (G) of V_L-CDR3 indicated the importance of these residues for binding to L1.

Generation of homodimers and production and purification of recombinant scFv antibodies

Homodimers of scFv antibodies have been shown to have bivalent avidity in antigen binding [31]. To additionally improve the affinity of the L1 scFv antibodies, homodimers of wild type and affinity matured L1 scFv antibodies were generated. To this purpose, a vector (pLD-Cys) that could be used for one step subcloning of scFv antibodies to form homodimers in *E. coli* was generated. A schematic overview for the generation of pLD-Cys is shown in Fig. 2A The pLD-Cys allows the convenient construction, rapid expression, and easy detection of scFv homodimers linked by a disulfide bridge. The genes of scFv antibodies G6 and 5F7 were subcloned into the pLD-Cys vector via the *Nco*I and *Not*I sites for production of homodimers. Western blot analysis of the subclones expressed in bacteria showed that homodimers had indeed formed (Fig. 2B). When bacterial culture supernatants containing homodimers of G6 (Fig. 2B, lanes 1 and 2) and 5F7 (Fig. 3B, lanes 3 and 4) were separated by SDS–PAGE under non-reducing conditions proteins of approximately 60 kDa were detectable (Fig. 2B, lanes 2 and 4). The 60 kDa proteins were reduced to approximately 30 kDa when the same samples were run under reducing conditions (Fig. 2B, lanes 1 and 3). This indicates that the 60 kDa protein is a disulfide-linked homodimer of the scFv monomer.

For expression and purification of 5F7 scFv antibody, the construct that contains the DNA coding se-

Table 2
Sequences of CDR regions of wild type (G6) and affinity matured (5F7) L1 scFv antibodies

Cone	Heavy chain						Light chain						
	CDR1			CDR2			CDR3						
	31	32	33	50	52	54	91	92	93	94	95	95a	95b
G6	S	Y	W	N	K	G	R	D	S	S	G	N	H
5F7	**D**	***Y***	**A**	**D**	**S**	**S**	***R***	**T**	**P**	***S***	***G***	**S**	**A**

The numbers in parentheses denote the positions of the amino acids. The residues were numbered according to Kabat and colleagues [16]. The boldface letters indicate the mutated amino acids and differences between the wild type and affinity matured clones. The italic letters indicate silent mutations.

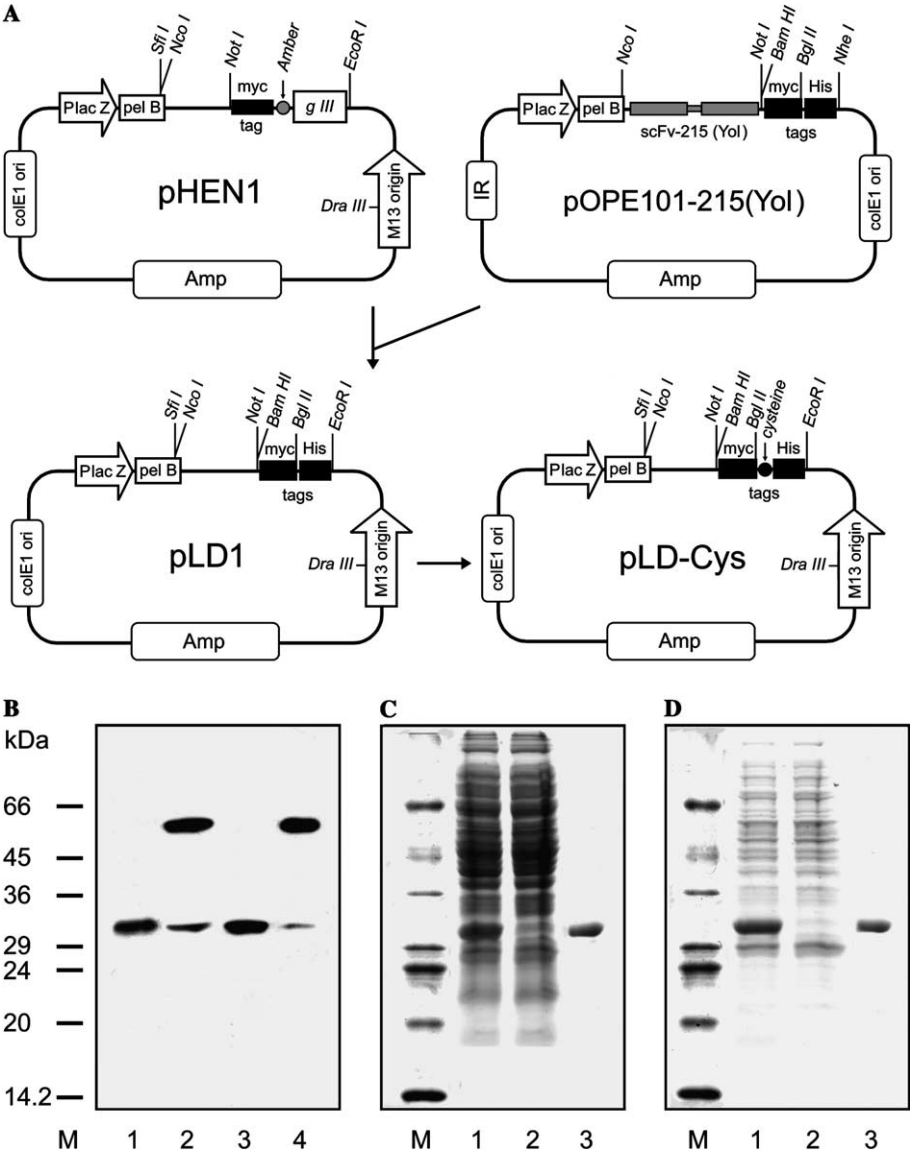

Fig. 2. Construction of a vector for expressing scFv homodimer and production and expression of L1 scFv antibodies and homodimers in *E. coli*. (A) For expression and formation of scFv homodimer, the vector pLD-Cys was generated from pHEN1 and pOPE101-215(Yol). First, vector pLD1 was generated by replacing the phage minor coat protein gene3 sequence between the *Not*I and *Eco*RI (filled in) sites in pHEN1 with the *Not*I–*Nhe*I (filled in) fragment of pOPE101-215(Yol). Second, a cysteine residue was introduced into the pLD1 vector between the myc tag and the 6× His tag by PCR. The respective PCR product was recloned into pLD1 via *Bgl*II and *Dra*III sites to generate vector pLD-Cys which allows easy subcloning of the scFv gene via restriction sites *Sfi*I/*Nco*I and *Not*I, and scFv antibody to form homodimer by a disulfide bridge in *E. coli*. (B) Formation of homodimers from L1 scFv antibodies. Bacterial culture supernatants containing homodimers of G6 (lanes 1 and 2) and 5F7 (lanes 3 and 4) were separated by SDS–PAGE (12% gel) under reducing (lanes 1 and 3) and non-reducing conditions (lanes 2 and 4) and detected with anti-myc antibody. (C,D) Expression and purification of affinity matured scFv antibody 5F7 (C) and its homodimer (D). 5F7 and its homodimer (5F7-Cys) were expressed in *E. coli*, purified by Ni–NTA agarose chromatography, and separated by SDS–PAGE (12% gel) under reducing conditions. Lanes M shows the molecular weight standards. Lanes 1, bacterial culture supernatant containing scFv antibodies; lanes 2, column flow-through; lanes 3, purified scFv antibody 5F7 or 5F7-Cys eluted with 100 mM imidazole. Abbreviations: P lacZ, lac Z promotor; pel B, bacterial peptide leader; Amp, ampicillin resistance gene; M13 origin, origin of phage M13 replication; IR, intergenic region of phage f1; colE1 ori, origin of *E. coli* replication.

quence of 5F7 was expressed in *E. coli* HB2151 and purified from the bacterial culture supernatant of large scale (100 ml) by Ni–NTA agarose chromatography. The bacterial culture supernatant contained a prominent protein with the molecular weight of approximately 30 kDa that was retained on the Ni–NTA agarose column and could be eluted with 100 mM imidazole (Fig. 2C). For expression and purification of the 5F7 homodimer (5F7-Cys), the 5F7 scFv DNA coding sequence was subcloned into the pLD-Cys vector to allow production in *E. coli* TG1. The 5F7-Cys was purified by Ni–NTA agarose chromatography from bacterial culture supernatants as described for the 5F7 monomer (Fig. 2D). Wild type scFv antibody G6 and its

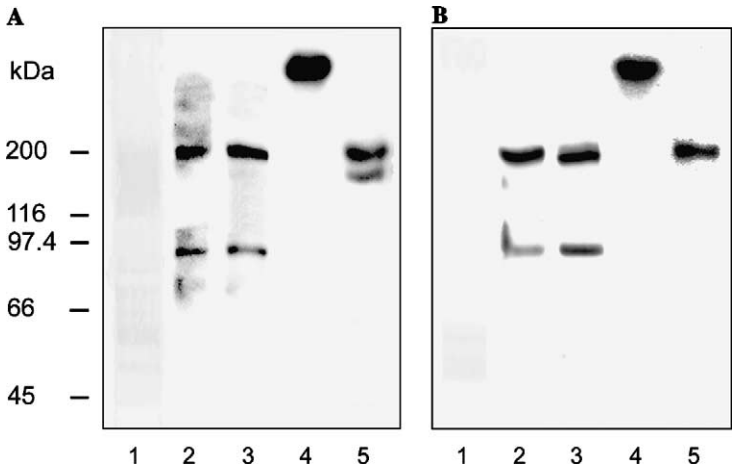

Fig. 3. Detection of L1 protein by Western blot analysis using affinity matured scFv antibody from the mutant library and homodimer. Brain homogenates of L1-deficient mice (50 μg, lanes 1), crude membrane fractions (20 μg, lanes 2), and brain homogenates (20 μg, lanes 3) from wild type mice were separated by SDS–PAGE (6% gel) under reducing conditions, and L1Fc (2 μg) were separated by SDS–PAGE (6% gel) under non-reducing (lanes 4) and reducing (lanes 5) conditions. The gels were blotted onto nitrocellulose membranes and probed with affinity matured L1 scFv antibody clone 5F7 (A) and its homodimer 5F7-Cys (B) and detected with anti-myc antibody. Molecular weight standards are indicated at the left in kDa.

homodimer (G6-Cys) were produced and purified in a similar manner (data not shown).

Characterization of the affinity matured L1 scFv antibody

Western blot analysis was carried out after separation of proteins under reducing and non-reducing conditions (Fig. 3). Antibody 5F7 (Fig. 3A) and its homodimer 5F7-Cys (Fig. 3B) recognized mouse L1 from crude membrane fractions of mouse brain (Fig. 4, lanes 2), total mouse brain homogenates (Fig. 3, lanes 3) under reducing conditions, and recombinant mouse L1Fc fusion protein under non-reducing (Fig. 3, lanes 4) and reducing conditions (Fig. 3, lanes 5), while wild type scFv antibody G6 did not detect L1 under reducing conditions [15]. The results suggest that the antigen binding affinity of L1 scFv antibody was increased upon affinity maturation and engineering. In addition, brain homogenates from L1-deficient mice [6] did not show reactivity with the antibodies (Fig. 3, lanes 1), indicating that the antibodies recognize L1 specifically in Western blotting.

Mapping of the epitope for the affinity matured L1 scFv antibody binding to L1

As for the wild type G6 clone, we investigated which domain of mouse L1 was recognized by the scFv 5F7 antibody. To this aim, different fragments of mouse L1 were subcloned into the pET vector and expressed in

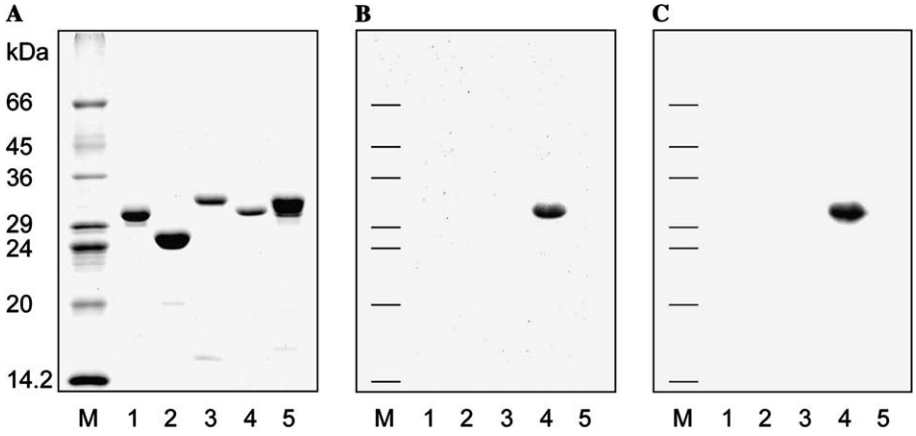

Fig. 4. Epitope mapping of L1 scFv antibodies using different fragments of L1 by Western blot analysis. Extracts of bacteria expressing different fragments of L1 were separated by SDS–PAGE (12% gel), blotted onto a nitrocellulose membrane which was probed with soluble L1 scFv G6 and 5F7 antibodies, and detected with anti-myc antibody. Lanes 1, Ig1–2; lanes 2, Ig3–4; lanes 3, Ig5–6; lanes 4, FN1–2; lanes 5, FN3–5. Molecular weight standards (lane M) are indicated at the left in kDa. (A) Fragments of L1 stained with Coomassie blue. (B) Fragments of L1 detected with wild type L1 scFv antibody clone G6. (C) Fragments of L1 detected with affinity matured L1 scFv antibody clone 5F7.

E. coli [22]. The inclusion bodies of bacteria were boiled in SDS sample buffer, subjected to SDS–PAGE, and visualized by Coomassie brilliant blue (Fig. 4A). All fragments gave the expected molecular masses as calculated from the protein sequences (Fig. 4A). Proteins from the gels were transferred to nitrocellulose membranes and probed with wild type L1 scFv antibody G6 (Fig. 4B) and affinity matured L1 scFv antibody 5F7 (Fig. 4C). Both antibodies, the wild type and the affinity matured antibodies reacted with the fibronectin type III repeats 1–2 (FN1–2) of mouse L1.

Determination of dissociation constants of wild type and affinity matured scFv antibodies

To determine whether the affinity matured scFv antibodies and the homodimers derived thereof showed increased affinity to L1, the dissociation constant (K_d) of the individual antibodies was determined (Table 3) by competition ELISA as described previously [23,24]. Clone G6 had a K_d of 1.72×10^{-6} M. The homodimer of G6 had a K_d of 4.74×10^{-7} M, thus showing an increase in affinity of a factor of 3.5. Affinity matured clone 5F7 had a K_d of 2.86×10^{-8} M, showing an increase of approximately 60-fold over the wild type clone G6. The homodimer of 5F7 showed a K_d of 5.22×10^{-9} M, resulting in an increase in binding affinity of approximately 5.5-fold. Comparison of the K_d values for G6 and the 5F7 homodimer thus demonstrates an increase in the affinity of the antibody by a factor of 310.

Characterization of the scFv antibodies by indirect immunofluorescence

Indirect immunofluorescence staining was performed to determine the ability of the antibodies to bind to live cells and to cryosections of retina and optic nerve from adult mice. Fig. 5 shows binding of scFv antibodies to L1 transfected L929 cells by indirect immunofluorescence. Staining with 5F7 antibody showed a strong signal of immunoreactivity (Fig. 5B) when compared to the G6 antibody (Fig. 5A). No staining of L1 transfected cells was observed when incubation with primary scFv antibodies was omitted (Fig. 5C). Furthermore, indirect immunofluorescence of non-transfected, L1 negative L929 cells did not show any non-specific binding of the 5F7 antibody (Fig. 5D).

Table 3
Dissociation constants of scFv antibodies compared between wild type, affinity matured, and homodimerized mouse L1 scFv antibodies

Clone of scFv	K_d (M)
G6	1.72×10^{-6}
G6-Cys	4.74×10^{-7}
5F7	2.86×10^{-8}
5F7-Cys	5.22×10^{-9}

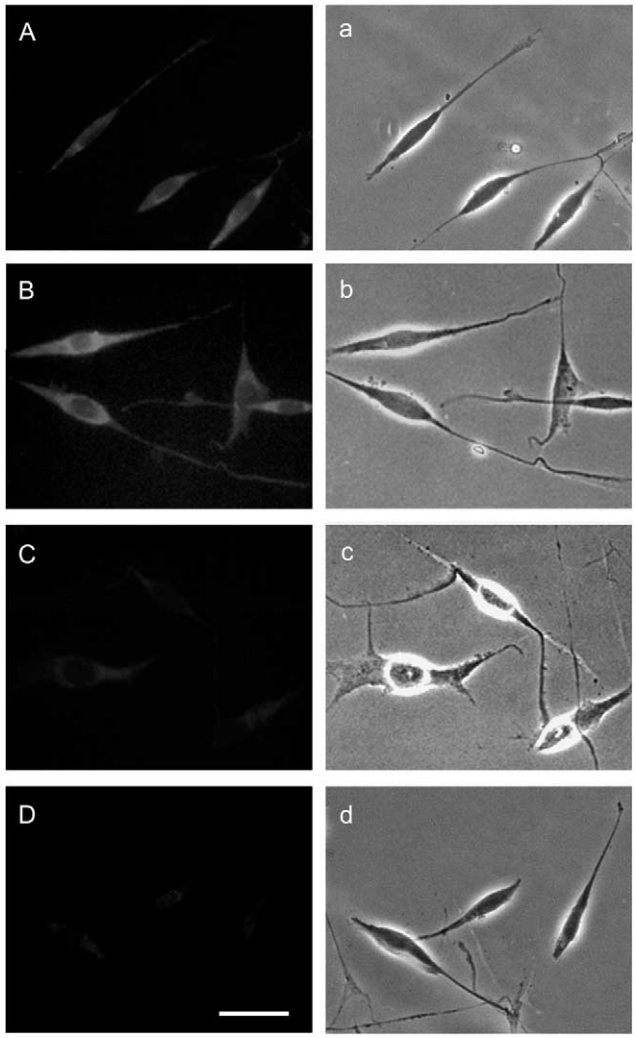

Fig. 5. Detection of L1 on L1 transfected L929 cells by immunofluorescence using affinity matured scFv antibody. Live-cell-staining of L929 cells stably transfected with L1 was performed by incubation with scFv antibodies. Binding of scFv antibody was visualized using anti-myc antibody followed by Cy3-conjugated anti-rabbit IgG. Panel A represents the cells incubated with scFv clone G6. Panel B represents the cells incubated with mutated scFv clone 5F7. Panel C represents a negative control without scFv antibody incubation. Panel D represents non-transfected parental cells incubated with scFv clone 5F7. The right panels (a–d) represent the corresponding phase contrast micrographs of the left panels. Bar in D: 70 μm for all panels.

Antibodies were also applied to fresh frozen sections from retina and optic nerves of adult mice as shown in Fig. 6. In the retina, L1 is expressed in the nerve fiber layer and inner and outer plexiform layer. L1 is additionally detectable in the unmyelinated retinal end of the optic nerve and is expressed on unmyelinated retinal ganglion cell axons in the myelinated segment of the nerve [32]. The G6 antibody did not label any of these L1-immunoreactive structures in the primary visual pathway (Fig. 6A). However, an L1-specific pattern of immunoreactivity was observed when sections were incubated with the homodimer of G6 (G6-Cys, Fig. 6B). Compared

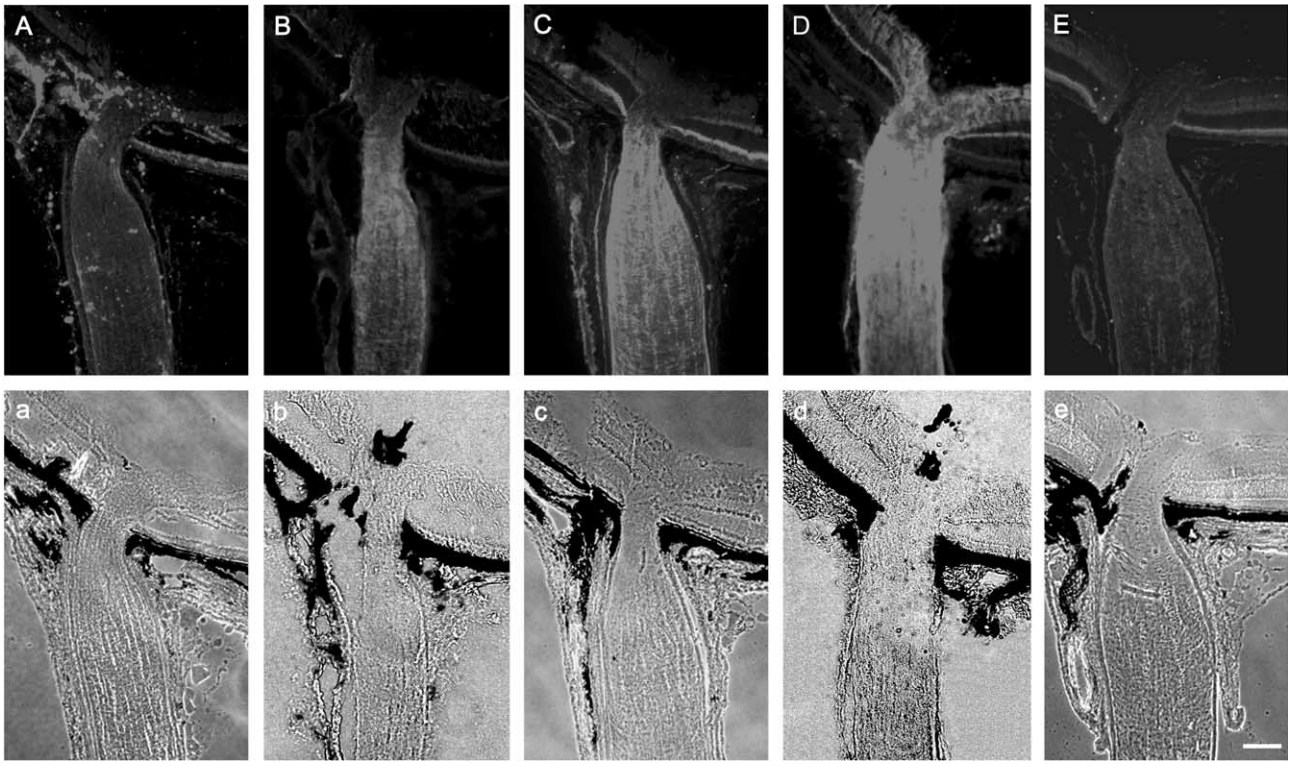

Fig. 6. Staining of cryosections from retina and optic nerve with wild type, affinity matured and bivalent L1 scFv antibodies. Cryosections of retina and optic nerves from adult mice were incubated with L1 scFv clone G6 (A) and its homodimer G6-Cys (B), 5F7 (C), and its homodimer 5F7-Cys (D), or without scFv antibody (E). Bound scFv antibodies were visualized using anti-myc antibodies followed by Cy3-conjugated anti-rabbit IgG. Clone 5F7 and 5F7-Cys detect L1 in the retina and optic nerve, while clone G6 and G6-Cys are not or less immunoreactive, respectively. The lower panels (a–e) represent the corresponding phase contrast micrographs of the upper panels. Bar in e: 100 μm for all panels.

to the G6-Cys, intensity of the immunofluorescence was increased when affinity matured 5F7 scFv antibody was used for staining (Fig. 6C). An even stronger L1-specific labeling was obtained with the homodimer of 5F7 (5F7-Cys) antibody (compare Figs. 6C and D). Unspecific labeling of the inner segments of photoreceptor cells and weak and diffuse background labeling of nervous tissue was observed when incubation of sections with scFv antibodies was omitted (Fig. 6E). The combined immunocytological and immunohistochemical data clearly show a correlation between the increase in labeling intensities (Figs. 5 and 6) and the decrease in dissociation constants (Table 3) of the various antibodies.

Discussion

We have been successful in producing a second generation of single chain variable fragment (scFv) antibodies against the murine neural cell adhesion molecule L1 by site-directed random mutagenesis. We generated a library that contains mutations in the three complementarity determining regions (CDRs) of the scFv heavy chains (CDR1 and CDR2) and the light chain (CDR3) of a previously isolated low affinity binding scFv antibody against mouse L1 [15]. Introduction of mutations in the CDRs is a general principle on the way to affinity mature scFv antibodies [33,34]. In our approach, we have introduced mutations not only into one CDR, but have inserted mutations into three CDRs to be comprised in one antibody. We have chosen the heavy chain for introduction of mutations into CDR1 (residues 31–33) and CDR2 (residues 50, 52, and 54) and the light chain for introduction of mutations into CDR3 (residues 91–95b). We chose these domains to introduce mutations because of the importance of these regions for the formation of antigen–antibody complexes [17,18,35]. Analysis of the mutated amino acids revealed silent mutations, but also mutations that result in alteration of the primary structure of the antigen combining sites. Interestingly, as shown in Table 2, affinity matured 5F7 clone contained wild type amino acids in position (32, Y) in V_H-CDR1, and in positions (91, R; 94, S; and 95, G) in V_L-CDR3. These amino acid residues are therefore probably essential for the binding to L1 or for efficient folding of the scFv antibody [36]. The domain of V_H-CDR3 of the scFv antibodies isolated from the human synthetic scFv phage display library already contained random mutations specific for antigen binding [5,29].

In addition to affinity maturation of the wild type scFv L1 antibody, we generated a homodimer of this

antibody with the aim to increase the avidity over that given by the monomeric antibody. For homodimerization, we have introduced a cysteine residue between the myc tag and the 6× His tag to allow the scFv antibody to form homodimers by a disulfide bridge when expressed in *E. coli*. Both, affinity maturation by site-directed random mutagenesis and homodimerization yielded significant increases in affinity of the antibodies as measured by competition ELISA. The homodimer of the original wild type scFv led to an approximately 3.5-fold increase in affinity. Affinity maturation resulted in an approximately 60-fold increase in affinity to mouse L1. Dimerization of the affinity matured scFv antibody gave an increase of approximately 5.5-fold. The overall increase in affinity from the wild type antibody to the homodimer of the affinity matured scFv thus resulted in an approximately 300-fold increase in antibody binding affinity. This value compares well to others in the literature which report increases between 100- and 1000-fold for various antibodies [37–39].

The increased avidity of the L1 antibody resulted in two major improvements in the properties of the antibody: the affinity matured homodimer reacted by Western blot analysis under reducing conditions with bands that are characteristic of L1 from mouse brain, namely a major band at 200 kDa and a proteolytic degradation product described for the membrane associated form containing part of the fibronectin type III homologous repeats with the molecular weight of approximately 80 kDa [40]. Detection of L1 in homogenates of brain is conventionally best achieved with selected polyclonal antibodies which require milligram quantities of purified L1 molecule for successful production by immunization of rabbits. Detection of L1 is less successful with some monoclonal antibodies prepared by the hybridoma technology [41]. Thus, affinity maturation has led to a significant improvement in the generation of a valuable reagent. Another important improvement in the range of antibody application was the successful use in immunocytochemistry and immunohistochemistry. This technique requires antibodies with high affinity which again was conventionally mostly attained by polyclonal antibodies and less successfully with conventional monoclonal antibodies produced by hybridoma technology. Affinity maturation was one step leading to an improvement, but homodimerization in addition proved to be a crucial next step. We thus generated an antibody that can be used successfully by ELISA, Western blot analysis under reducing and non-reducing conditions, and immunocytochemistry of cultured cells and tissue sections.

We have generated high affinity human antibody against L1 that is not only useful for research purposes, but has the potential to be a therapeutic reagent in vivo, since it is reactive at low concentrations and its size is well below that of immunoglobulins G and M which provides the basis for good penetration into complex tissues. The fact that the antibodies are of human origin would make applications to the human directly feasible.

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