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Selection of functional human antibodies from retroviral display libraries

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ABSTRACT

Antibody library technology represents a powerful tool for the discovery and design of antibodies with high affinity and specificity for their targets. To extend the technique to the expression and selection of antibody libraries in an eukaryotic environment, we provide here a proof of concept that retroviruses can be engineered for the display and selection of variable single-chain fragment (scFv) libraries. A retroviral library displaying the repertoire obtained after a single round of selection of a human synthetic scFv phage display library on laminin was generated. For selection, antigen-bound virus was efficiently recovered by an overlay with cells permissive for infection. This approach allowed more than 10^3-fold enrichment of antigen binders in a single selection cycle. After three selection cycles, several scFvs were recovered showing similar laminin-binding activities but improved expression levels in mammalian cells as compared with a laminin-specific scFv selected by the conventional phage display approach. Thus, translational problems that occur when phage-selected antibodies have to be transferred onto mammalian expression systems to exert their therapeutic potential can be avoided by the use of retroviral display libraries.

INTRODUCTION

The display of foreign polypeptides and proteins on the surface of viruses or cells provides an important tool for the engineering of biomolecules and the analysis of their interactions with binding partners (1,2). Display technology has made great progress over the last 10 years and covers applications ranging from basic research to diagnosis and therapy. One of the most successfully and extensively used display technology is the isolation of recombinant antibodies [variable single-chain fragments (scFvs)] from large combinatorial libraries displayed on the pIII coat protein of the filamentous bacteriophage (3). Such antibodies that recognize, for example, cell-surface markers, growth factors or extracellular matrix proteins were also proved to be effective for novel therapeutic strategies including cancer treatment. Recently, for example, it has been shown that an anti-laminin antibody (L36), isolated from a large synthetic scFv display library with a repertoire of $>5 \times 10^{10}$, was able to inhibit blood vessel formation and to prevent tumour growth (4,5).

Besides phage, other display platforms have been developed including yeast and bacteria cells, and also retroviruses (6–9). The envelope spike glycoprotein (Env) of the murine leukaemia virus (MLV) proved to be especially amenable to N-terminal extensions by foreign polypeptides (10). The Env protein is a homotrimeric complex (11,12) with each subunit of the trimer consisting of the SU (surface) and the TM component, which anchors the complex in the viral membrane (13). The SU glycoprotein mediates the attachment of the virion to its cellular receptor (14). Receptor choice determines the host range of MLV. Ecotropic viruses use the murine Rec-1 protein as receptor. As the human allele does not encode a functional receptor the tropism of these viruses is restricted to murine cells.

Growth factors, cytokines, extracellular parts of transmembrane proteins and also scFvs have been displayed on MLV by extending the N-terminus of the SU protein (15). These modifications usually result in the binding of the virus particles to the corresponding cell surface receptor or antigen. However, efficient functional cell entry via the targeted cell surface molecule resulting in an infectious cycle does not occur (15,16). The potential of retroviral display for the generation and screening of eukaryotic expression libraries has so far been demonstrated for small peptides of ~7–10 amino

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acids. These retroviral peptide display libraries were successfully selected for the identification of protease substrates (17,18) or antibody epitopes (19). In this study, we present the first retroviral scFv display library, which allowed as a proof of concept the selection of functional human anti-laminin antibodies.

MATERIALS AND METHODS

Generation of the plasmids

All the plasmids encoding scFv viruses in this study were derived from pE-Mo (18). First, the ecotropic *env* gene in pE-Mo was exchanged with the ecotropic *env* gene harbouring N-terminally a factor Xa cleavage site from the plasmid pN-XMo (kindly provided by M. Chadwick) via the NotI/ClaI restriction sites to give pE-XMo, in which a factor Xa cleavage site is encoded between the NotI site and the first codon of the SU protein. To construct the viral scFv library as well as the 7A5-XMo and L36-XMo viruses, the scFv-coding regions were amplified from the Griffin.1 library (5) or pHEN-2-L36 (20) or pHEN2-7A5 (21) by PCR using primer LMB3 (5′-CACAGGAAACAGCTATGAC-3′) and pHEN-Seq (5′-CTATGCGGCCCCATTC-3′). The PCR fragments were SfiI/NotI-digested and ligated into the SfiI/NotI-digested pE-XMo. RT–PCR fragments encoding the selected scFvs were cloned into the pGEM-T-Easy vector (Promega). To reconstitute the viruses L6-, L9- and L28-XMo, the scFv-coding sequences were subcloned from the corresponding pGEM-T-Easy plasmids into pE-XMo via SfiI/NotI. To generate the scFv expression plasmids, the L6 and L28 scFv-coding regions were amplified by PCR from the plasmids pscFv-L6-XMo and pscFv-L28-XMo using primer pairs L6XMoC (5′-CCATCGATGCAGGTGCAGCTGGTGC-3′) and scFvXN (5′-CCTCGATTGCGGCCGCACCTAGGA-3′) or L28XMoC (5′-CCATCGATGCAGGTGCAGCTGTTGC-3′) and scFvXN. The ClaI/NotI-digested PCR fragments were ligated into the ClaI/NotI-digested backbone of the plasmid pCR3.1-L36 (22), to obtain the plasmids pCR3.1-L6 and pCR3.1-L28. The identity of the sequence was verified using the primer BGHReverse (5′-TAGAAGGCACAGTCGAGG-3′).

Ligation and cloning conditions were basically the same as described previously with the exception that ElectroTen-Blue bacterial cells (Stratagene) were used in 1 cm cuvettes at 1.7 kV, 200 Ω and 25 μF (18). Electroporated bacteria were plated, pooled and subsequently grown in liquid medium for the purification of plasmid DNA. Restriction and sequence analyses of 45 randomly picked clones confirmed that >90% of the clones encoded complete scFvs.

Virus propagation, titration and detection

To generate virus stocks, 50–70% confluent HEK-293T cells [human kidney epithelia; originally referred to as 293tsA1609-neo (23)] grown in T175 flasks were transfected with 28 μg of plasmid DNA using Lipofectamine™ (Life Technologies). Two days after transfection, virus-containing supernatants were filtered through a 0.45 μm sterile filter and aliquots were stored at −80°C. Further harvests were possible after cultivation in serum-free DMEM overnight.

Infectious particles released from NIH-3T3 (mouse fibroblasts; ATCC CRL-1658) or HEK-293T cells were quantified by immunostaining using the goat anti RLV-p30 serum (Quality Biotech), as described previously (18). RT activity was determined using the C-type-RT Activity Assay (Cavidi Tech) in accordance with the manufacturer's instructions.

Binding-infection and particle binding assay

Murine EHS-derived laminin-1 (BD Bioscience), collagen IV (10 μg/ml; BD Bioscience) or milk powder [3% in phosphate-buffered saline (PBS), 0.45 μm sterile filtrated] were coated onto a 24-well cell culture plate overnight at 4°C. Next day, antigen-containing wells were blocked using 3% milk powder in PBS for 1 h at room temperature. After washing with PBS, infectious viral particles were added and incubated for 3 h at 4°C. After washing with ice-cold PBS, NIH-3T3 cells (4×10^5 per well) were added and incubated for 24 h at 37°C in an atmosphere of 5% CO_2. The number of infected cells was quantified by immunostaining using the goat anti RLV-p30 serum (Quality Biotech), as described previously (18).

For the virus particle binding assay, serial dilutions of scFv viruses were incubated with laminin-1 (10 μg/ml) coated 96-well cell culture plates or enzyme-linked immunosorbent assay (ELISA) plates for 3 h at 4°C. After washing with ice-cold PBS, bound viral particles were lysed and RT activity was determined using the C-type-RT Activity Assay (Cavidi Tech) in accordance with the manufacturer's instructions. Binding curves were calculated by plotting the input RT activity against the laminin-bound RT activity.

Selection of the virus library and fingerprint analysis

For selection of the scFv virus library, one well of a 6-well dish was coated with murine laminin-1 (10 μg/ml). On the next day, 6×10^6 i.u. (infectious units) of the scFv library (3.7×10^5 i.u./ml) was added for 3 h at 4°C. After washing with ice-cold PBS, 2.5×10^6 NIH-3T3 cells were added and cultivated overnight at 37°C in an atmosphere of 5% CO_2. Cells were then transferred onto a T25 cell culture flask and the cell culture supernatant was harvested 48 h later and stored at −80°C till further analysis or was directly used for a next round of selection. The stringency was increased by two and three washing steps at the second and the third round of the selection using ice-cold PBS followed by rigorous shaking for 10 min at 4°C. After selection, virus was harvested from the cell culture supernatant by ultracentrifugation in an SW41 rotor (Beckman) at 30 000 r.p.m. for 1 h at 4°C and RNA was purified using the QIAamp Viral RNA Mini kit (Qiagen) followed by treatment with DNase I (Promega).

For cloning and sequence analysis, the scFv-coding region of the viral RNA was reverse transcribed into cDNA (primer EMoSeq, 5′-CGTCTCCCGATCTCCATTGGTTAC-3′), which was then amplified by PCR using primers CB6 (5′-CCCCTAATCCCCTTAATTCTTC-3′) and EMoSeq. The PCR fragments were subcloned and plasmid from single bacterial clones was sequenced using the standard primers SP6 and T7. For the fingerprint analysis, the scFv-coding region was amplified by colony-PCR using primers CB6 and EMoSeq, and the PCR fragments were digested with BstNI (NEB).

Western-blot analysis

Virus particles were harvested into serum-free medium and then concentrated by ultracentrifugation in an SW41 rotor (Beckman) at 30 000 r.p.m. for 1 h at 4°C. Virus collected from 11 ml supernatant was then suspended in 100 µl of ice-cold PBS, separated by electrophoresis on a 10% SDS–polyacrylamide gel, and electrotransferred onto a nitrocellulose membrane (Hybond™ ECL; Amersham). The membranes were probed with the anti RLV-gp70 or anti RLV-p30 goat antisera (Quality Biotech) and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-goat antibodies (DAKO). Protein bands were visualized using ECL (Pierce).

Expression of scFv and specificity ELISA

HEK-293T cells were transiently transfected with pCR3.1, pCR3.1-L36, pCR3.1-L6 or pCR3.1-L28 plasmid using Lipofectamine (Life Technologies). Supernatant was collected at 48 h and 20 µl was analysed for scFv expression by western blotting using anti-myc monoclonal antibody (mAb) and ELISA as described previously (22). Briefly, 96-well Maxisorp® plates were coated with laminin-1 (10 µg/ml) in PBS overnight at 4°C. Cell-free supernatants from transfected cells were added for 1 h at room temperature and bound scFv was detected with the anti-myc-HRP antibody (Life Technologies).

RESULTS

Establishing the selection system

A key issue for setting up an effective selection system for retroviral scFv display libraries is the recovery of virions bound to the cognate antigen. We expected that virus particles displaying the scFv via a flexible linker peptide will be able to attach to and then enter into the permissive cells while being specifically bound to an antigen of choice. To verify this hypothesis and to set up a system for the selection of scFv from retroviral display libraries, we chose murine laminin-1 as an antigen and the scFv L36 as a model system (20). Three different types of viruses all based on the ecotropic Moloney MLV strain (MoMLV) were used in this study. Starting from the plasmid pE-XMo, which encodes the complete MoMLV genome modified with an epidermal growth factor (EGF) coding SfiI/NotI cassette inserted between the 3′ codon of the signal peptide and the 5′ codon of the SU domain, viruses displaying L36 (L36-XMo) or the unrelated scFv 7A5 (21) (7A5-XMo) were generated (Figure 1). All these viruses were then assayed for their ability to bind to laminin-1 and various control antigens and for the subsequent infection of NIH-3T3 cells being overlaid onto the virus–antigen complex ('binding-infection assay').

To allow binding of viral particles, cell culture plates were left either uncoated or coated with laminin-1, collagen or milk powder. Subsequently, equivalent amounts of particles of each virus type were added and allowed to bind for 3 h at 4°C. After stringent washing, NIH-3T3 cells were added. Infected cells were determined after 24 h by immunostaining of the viral capsid protein p30. In contrast to the 7A5-XMo and E-XMo viruses, the L36-XMo virus was able to bind to laminin-1 and

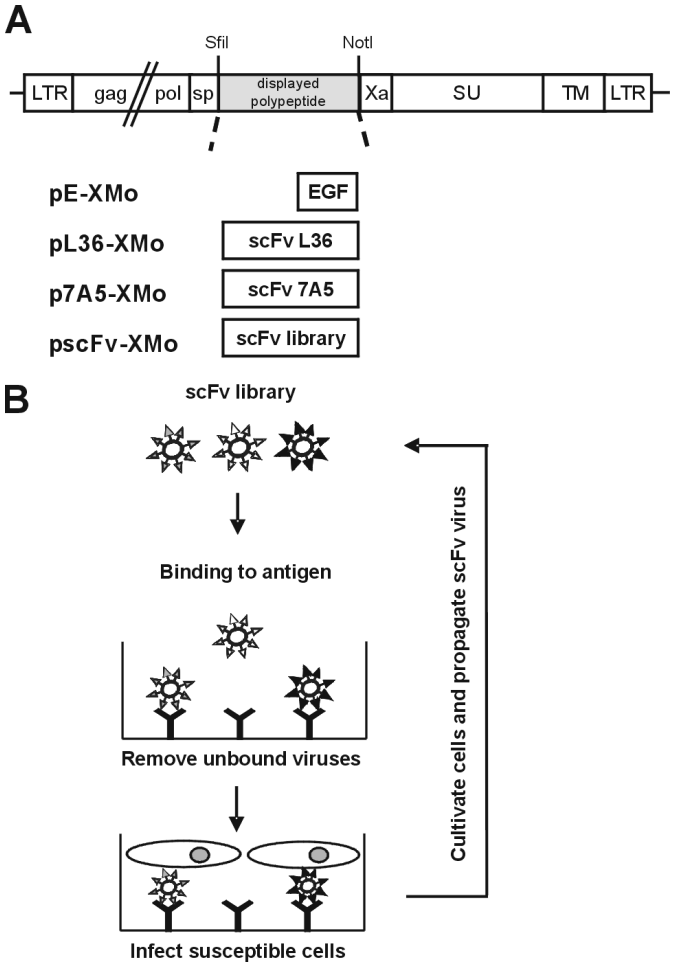

Figure 1. Schematic representations of the retrovirus constructs and the selection procedure. (**A**) All constructs contain the complete genome of the MoMLV being comprised of the *gag*, *pol* and *env* genes. A SfiI/NotI cassette is inserted in the *env* gene between the 3′ codon of the signal peptide (SP) and the 5′ codon of the SU domain. Polypeptides inserted into this cassette will be displayed on the viral Env protein via a factor Xa cleavage site (Xa). (**B**) Selection procedure for retroviral scFv libraries. The virus library is released from HEK-293T cells transfected with pscFv-XMo. The virus particles are then incubated with laminin-1 coated cell culture plates to allow binding. After binding and washing NIH-3T3 cells are added for infection. The infected cells are then cultivated to produce virus particles for the next round of selection.

Table 1. Percentage of virus[a] recovered upon binding to different antigens

Virus	Antigen Laminin	Collagen	Plastic	Milk powder
MoMLV (%)	0.1	0.28	0.3	0.52
E-XMo (%)	0.04	0	0	0
7A5-XMo (%)	0.02	0.04	0	0
L36-XMo (%)	>60	0.02	0.06	0

[a]5000 i.u. applied.

to infect NIH-3T3 cells (Table 1 and Figure 2A). Interestingly, >60% of the input infectious particles of L36-XMo had been retained on the antigen and infected the overlaid NIH-3T3 cells (Table 1). In addition, all the viruses including the L36 virus hardly bound to collagen, plastic and milk powder

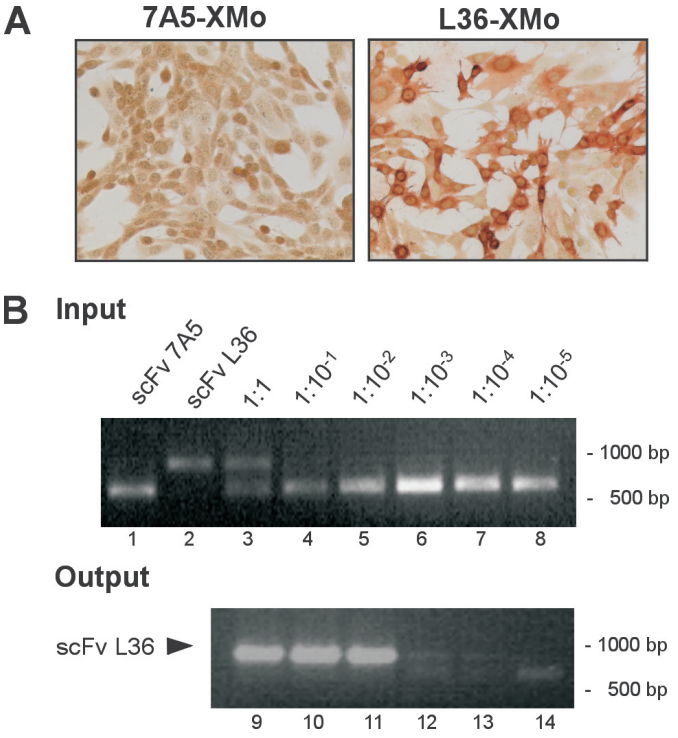

Figure 2. Laminin-bound retroviruses can be recovered. (**A**) NIH-3T3 cells were added to immobilized laminin-1 incubated with 2.5×10^6 particles of the 7A5-XMo (left panel) or the L36-XMo virus (right panel) after removal of unbound particles. Infected cells (appearing in red colour) were detected by immunostaining using specific antiserum for the viral capsid protein. (**B**) Recovery efficiency of the L36-XMo. Approximately 2.5×10^7 7A5-XMo virus particles were spiked with decreasing amounts (10-fold dilutions) of L36-XMo virus particles. The relative amounts of both viruses were determined upon ClaI digestion of RT–PCR fragments generated on viral genomic RNA before (Input, lanes 3–8) and after laminin binding and infection of NIH-3T3 cells (Output, lanes 9–14) to laminin-1. Each of the lanes 3–8 and 9–14 corresponds to 10-fold dilutions of L36-XMo. The ClaI-digested fragments derived from PCR using plasmids p7A5-XMo and pL36-XMo as template served as controls, respectively (lanes 1 and 2).

(Table 1). These results demonstrated that the rescue of antigen-bound virus by the addition of permissive cells is highly efficient and, therefore, suitable for the selection of retroviral scFv libraries.

Next, we determined the minimal amount of laminin-binding virus that can be recovered by this approach. For this purpose, L36-XMo and 7A5-XMo particles were mixed in different ratios from 1:1 to $1:10^{-5}$ starting with 5 mU RT activity, which corresponds to $\sim 2.5 \times 10^7$ virus particles each (24). After binding and infection, viral RNA from supernatants of the infected cells was isolated and amplified using RT–PCR. To distinguish between both virus types, fragments were digested with the restriction enzyme ClaI (Figure 2B, lanes 1–14). Detectable signals of the scFv L36 after binding and infection were found up to the 10^{-4} dilution (Figure 2B, lanes 9–13) corresponding to ~2500 laminin-specific viral particles among 2.5×10^7 non-specific particles. Given that in stocks of wild-type MoMLV only ~1% of the particles are infectious, a few single antigen-bound infectious virus particles can obviously be recovered by this approach.

Selection of a retroviral scFv library on laminin-1

To generate a retroviral scFv library, we used the phage population that was recovered after a first selection cycle of the human synthetic scFv phage display library Griffin.1 for binding to laminin-1 (5,20). In total, 4×10^3 bacterial clones derived from infection with the selected phage particles were pooled and used for preparation of the pHEN-2 plasmid library encoding the single round selected scFv repertoire. The retroviral plasmid library pscFv-XMo was generated by substituting the SfiI/NotI cassette of pE-XMo (Figure 1A) for the corresponding cassette removed from the pHEN-2 plasmid library. The resulting plasmid library pscFv-XMo was purified from a total of 2.3×10^4 bacterial clones. The virus library was then produced on HEK-293T cells, which are negative for the MoMLV receptor and thereby excluding any bias in the virus library through virus propagation. Supernatants harvested 48 h after transfection with pscFv-XMo contained 6×10^6 i.u.

For selection, laminin-1 was coated on 6-well cell culture plates. On the next day, the plate was blocked with milk powder and incubated with 1.5×10^5 i.u. of the scFv-XMo library at 4°C to allow binding. After binding, two washing steps were performed to remove unbound viral particles. Then, NIH-3T3 cells were added and incubated for 24 h to allow infection. The cells were then detached and transferred onto cell culture flasks to allow virus propagation for 48 h (Figure 1B). Two additional rounds of laminin binding, infection and virus propagation were performed. With each round the stringency of selection was increased by additional washing steps. The propagation of virus was monitored by determining infectious particles after each round of selection as well as by RT–PCR on genomic RNA purified from viral particles harvested from the cell culture supernatant. In addition, specific PCR fragments for the scFv-coding region were recovered after each selection round (data not shown).

To follow the selection on the molecular level, we performed fingerprint assays of the scFv-coding sequences isolated from the virus library before and after the third round selection (Figure 3). The scFv-coding sequences from 50 independent randomly picked bacterial clones were amplified by colony PCR and digested with the high-frequency cutting restriction enzyme BstNI. Before selection highly diverse restriction patterns were obtained, none of which occurred more than twice (Figure 3A). After selection in contrast, distinct patterns occurring at high frequency were observed (Figure 3B). The most prominent clones L9, L28 and L6 appeared with frequencies of ~32, 14 and 8%, respectively. Thus, the selection process resulted in a strong enrichment of individual viruses displaying specific antibody fragments.

To determine the laminin-binding activity of the virus population before and after selection, equal amounts of virus particles were incubated with immobilized laminin-1. After binding and washing, the number of bound virus particles was determined by RT activity. Figure 4A shows that low but significant laminin-binding activity was detectable in the virus library before selection. After the selection procedure, however, the laminin-binding activity of the virus population increased by almost 10-fold. Thus, particles displaying antibodies specific for laminin-1 must have been enriched during selection.

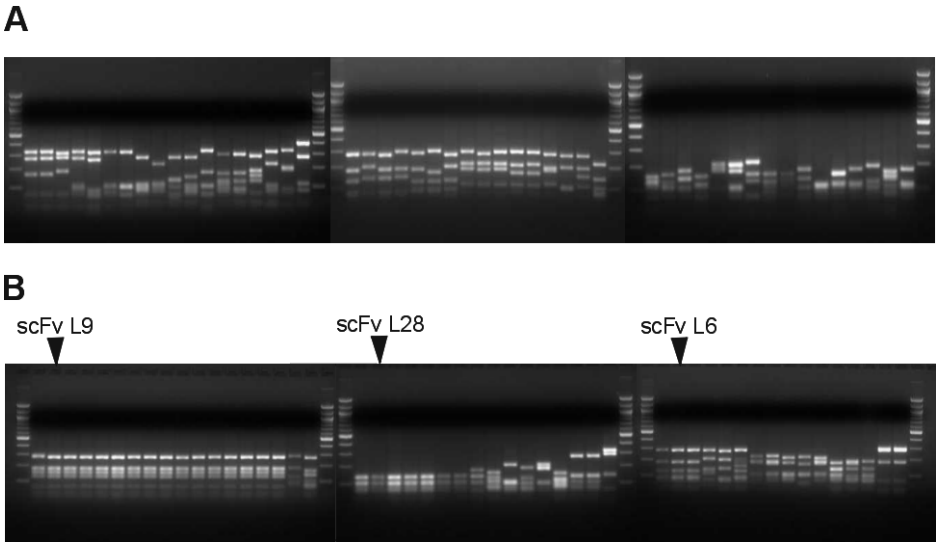

Figure 3. Fingerprint analysis of the selected scFvs. RT–PCR fragments derived from genomic viral RNA representing the scFv fragment coding sequences before (**A**) and after three rounds of selection (**B**) were cloned into bacteria. Colony-PCR fragments from 50 randomly picked bacterial clones were restricted with BstNI, respectively. Similar restriction patterns were grouped and the patterns of the most prominent clones L9, L28 and L6 are indicated.

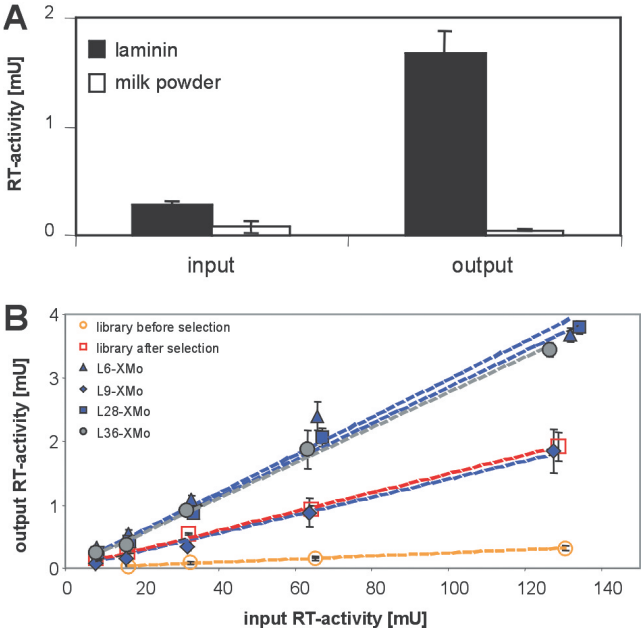

Figure 4. Laminin-1-binding activity of virus pools and selected scFv viruses. (**A**) An aliquot of 130 mU RT activity of the unselected (input) and the selected (output) virus populations were incubated with laminin-1 or milk powder coated ELISA plates. After washing, RT activities of the retained virus particles were quantified, respectively. (**B**) Different amounts of RT activity (input) of the unselected (orange line) and the selected (red line) virus populations as well as of the reconstituted L6-XMo (closed triangles, blue line), L9-XMo (closed diamonds, blue line), L28-XMo (closed squares, blue line) and L36-XMo viruses (closed circles, grey line) were incubated with laminin-1 coated ELISA plates. After washing, RT activities of the retained virus particles were quantified, respectively (output). Average values of three independent experiments are given.

Characterization of the selected viruses and scFvs

To characterize the selected scFvs, we cloned and sequenced the three most prominent scFvs, such as L9, L28 and L6. The CDR3 regions of all three scFvs differed completely from those of L36. Only the V_L CDR3 of L28 was related to that of L36 (Table 2). We then reconstituted virus particles displaying the three selected scFvs, and compared their laminin-binding activity to that of the L36-XMo virus, respectively. Serial dilutions of monoclonal virus populations were incubated with immobilized laminin-1. All the reconstituted viruses bound laminin at least as efficiently as the virus population after selection. Clones L28-XMo and L6-XMo were up to 2.5-fold more active than the pool of viruses after selection and also slightly more active than the L36-XMo virus (Figure 4B).

Next, we addressed the question if basic viral properties had changed due to the selection process. First, we determined the infectivity rates of the viruses L28-XMo, L6-XMo and L36-XMo when being bound to laminin-1. As all the three viruses were similar in their laminin-binding activities, subtle differences in this assay must be due to differences in the viral cell entry events. Equivalent particle numbers were added to the laminin-1 coated plates. After binding, NIH-3T3 cells were added and the number of infected cells was determined. As shown in Figure 5A, the L6- and L36-XMo viruses showed very similar infectivity rates, while the increase in the ratio of infectious among non-infectious particles was ~2-fold for the L28-XMo virus.

The increased infectivity rate of the L28-XMo virus could be due to increased amounts of Env protein being incorporated into the viral particles. To explore this, we performed western-blot analysis to determine the relative amounts of the scFv-SU proteins and the capsid protein p30. Both selected viruses showed very similar Env protein incorporation levels, which were slightly above that of the L36-XMo virus (Figure 5B).

Finally, we determined the expression and binding activities of the retrovirus selected anti-laminin scFvs when expressed as scFv alone in the absence of the viral context. For this purpose, the antibody genes (L6, L28 and L36) were cloned into the mammalian expression vector pCR3.1 (4). Transfection of HEK-293T cells with plasmids pCR3.1-L6, pCR3.1-L28 or

Table 2. CDR3 sequences of retrovirus and phage[a] selected scFvs

ScFv	V_H CDR3	V_L CDR3
L6 (retrovirus)	GDIRLRDP	NSRDSSGIQNV
L9 (retrovirus)	GEFSLMLEA	NSRDSSGNHV
L28 (retrovirus)	VELDSFDY	AAWDDSLGLI
L32 (phage)	SLFPLFD	AAWDDSLAFV
L34 (phage)	TALTAQL	AAWDDSLLSFV
L36 (phage)	YSAMADY	AAWDDSLPAIV

[a]Most efficient laminin-1-binding scFvs obtained after three cycles of selection of the Griffin-1 library (4).

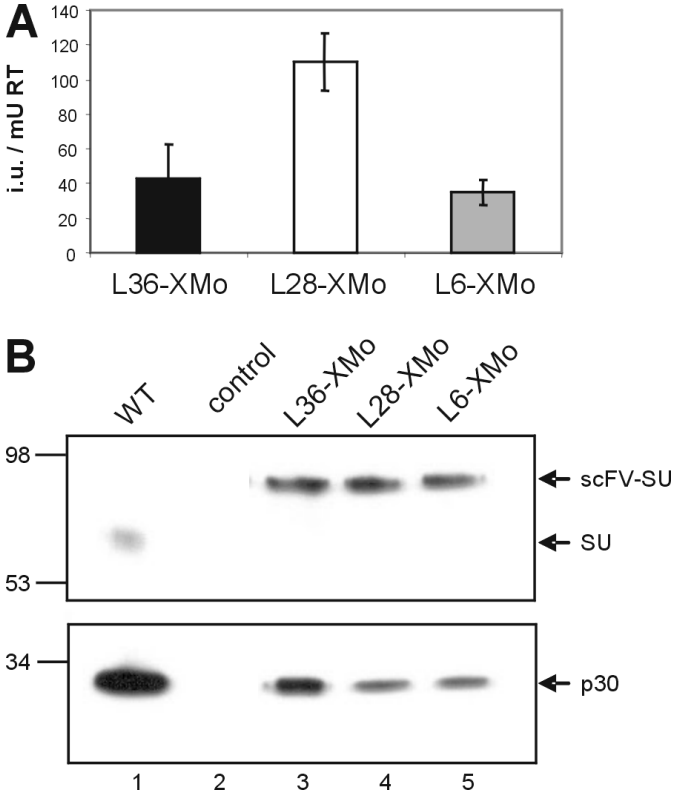

Figure 5. Characterization of the selected scFv-displaying viruses. (**A**) Infection rates of the selected viruses after binding to laminin-1. An aliquot of 1 mU of RT activity of the indicated viruses, respectively, was incubated with laminin-1 coated cell culture plates. After washing, NIH-3T3 cells were added and the number of infected cells was quantified 24 h post-infection by immunostaining using the MLV capsid-specific antiserum. The average ratio of the number of infected cells and the input RT activity, based on three independent experiments, is provided. (**B**) Env protein incorporation into viral particles. HEK-293T cells were transfected with plasmids, pL6-XMo (lane 5), pL28-XMo (lane 4) and pL36-XMo (lane 3), respectively, or left untransfected (lane 2). At day two after transfection, virus particles released from the cells were separated on a 10% SDS–polyacrylamide gel. MoMLV wild-type virus was loaded as a molecular weight marker control for the viral proteins. Immunoblotting was performed using SU and capsid protein-specific antisera.

pCR3.1-L36 resulted in the secretion of soluble active scFv at significant levels. Upon incubation of the cell culture supernatant with immobilized laminin, specific binding activity was detectable for both retrovirus selected scFv molecules (Figure 6A). In fact, the L28 and especially the L6 scFvs were significantly more efficient in laminin binding than the phage selected L36 scFv (Figure 6A). These binding differences could be due to either differences in the affinity of

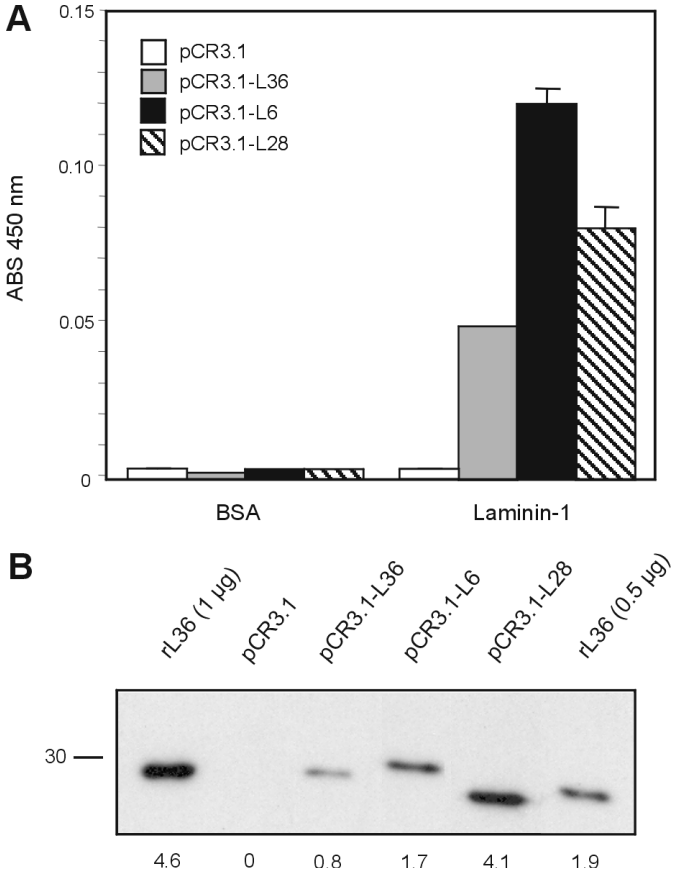

Figure 6. Functional characterization of scFv antibody molecules produced by human cells. (**A**) Secretion of scFvs into the cell culture supernatant by HEK-293T cells transiently transfected with plasmid pCR3.1, pCR3.1-L36, pCR3.1-L6 or pCR3.1-L28. Antibody functionality was demonstrated by ELISA against plastic immobilized BSA and laminin-1, respectively. Bound scFv was detected with an anti-myc mAb. (**B**) Western-blot analysis of scFv secreted into the cell culture supernatant by pCR3.1 (lane 2), pCR3.1-L36 (lane 3), pCR3.1-L6 (lane 4) or pCR3.1-L28 (lane 5) transfected HEK-293T cells, respectively. An aliquot of 20 µl of supernatant of each transfectant was loaded in each lane. An aliquot of 1 µg and 500 ng of recombinant L36 scFv antibody purified from bacterial culture supernatant were loaded in lanes 1 and 6, respectively. Immunoblotting was performed using an anti-myc mAb. Intensity of individual bands was evaluated by PCBas software (Raytest, Germany). The values were normalized to the intensity of control and expressed underneath the respective blot lane.

binding or differences in the antibody expression level. Although further experiments are necessary to determine antibody affinities, our data demonstrate that the retrovirus selected scFvs were expressed at higher levels than the phage selected scFv, especially the L28 clone, which was about four times more efficiently expressed than the phage-derived L36 clone (Figure 6B).

DISCUSSION

The data presented here demonstrate that scFv libraries displayed on the surface of the retrovirus MoMLV can be generated and selected to obtain functional scFvs for a given antigen. While other display platforms, such as phage and yeast (3,5,25), are well established for the selection of scFvs the use of animal viruses infectious for mammalian cells has not yet been described.

Retroviruses offer a number of beneficial properties for scFv display. While naked viruses, e.g. adenovirus, only tolerate insertions of small peptides into their capsid proteins, large polypeptides can be displayed on the surface of retroviruses without compromising viral infectivity, also including heavily glycosylated proteins, such as the prion protein (26). Therefore, it was possible to establish a replication competent library system which, in contrast to conventional phagemid-based scFv libraries, is independent from helper virus. Especially when replication competent display platforms are being used, the possibility that other parameters than antigen binding drive the selection process is a concern. However, basic viral properties, such as Env protein incorporation and infectivity, did not change considerably during our selection procedure.

Instead, laminin-1-binding activities and expression levels of the scFv molecules improved. Most importantly, this was also the case when the selected scFvs were analysed as soluble molecules in the absence of virus. Expression of the selected scFvs in HEK-293T resulted in considerably large amounts of ~25 mg/l that are even above the range obtained upon expression in yeast (1–10 mg/l) (25). We, therefore, conclude that antigen binding and expression were indeed the main driving forces of selection. The improved binding activity of the soluble scFvs also suggests that the multivalent nature of scFv expressed on the surface of retrovirus particles where ~50–100 Env molecules exist did not reduce the affinity of scFvs selected from retroviral libraries. Moreover, the application of a replication competent display system offers, due to its high mutation and recombination frequency, an additional level of scFv diversification.

An important observation while setting up the retrovirus-based selection system was the unimpaired infection efficiency of laminin-bound virus particles being virtually as high as that of free virus. This high degree of flexibility in the cell entry events involving receptor attachment and membrane fusion seems to be a unique property of retroviruses but is not limited to the use of laminin as antigen. The immobilization of retrovirus particles to other cell adhesion molecules, transmembrane receptors or antibodies similarly resulted in unimpaired or even increased infectivity if compared with free virus (19,27). For the library selection process, scFv displaying retroviral particles bound to their cognate antigen can, therefore, easily be rescued by adding permissive mammalian cells. Thus, there is no need for elution procedures so that high-affinity antibodies cannot get lost during selection. Moreover, this concept may also be easily applied to the use of living human cells as antigen source if ecotropic retroviruses as shown here are used as display platform. Then, scFvs directed against transmembrane cell surface proteins that are difficult to purify in a native conformation can be selected.

Although the repertoires that can be covered with retroviral libraries, at least in their current stage of development, are clearly below that of phage libraries, enrichment efficiencies as well as sensitivity were well within the range of phage systems. Our data show that a single selection round enriched at least 10^3-fold and that as few as 25 antigen bound infectious particles were sufficient to go into the next selection round. Retrovirus display libraries covering complexities of 10^6–10^8 can be generated (18,19). This level of complexity is sufficient for the screening of the scFv repertoire present in immunized donors. Alternatively, single round selected pools of phage particles as demonstrated here can serve as ideal starting point for the selection in retroviral systems. In this respect, it is remarkable that the scFvs selected from the retroviral library clearly differed in their CDR sequences from scFvs that were obtained after two further phage selection rounds (Table 2). Most probably, these differences reflect the adaptation of molecular parameters, such as codon usage, polypeptide folding and resistance to inactivation by mammalian cell proteases. Translational problems that occur when molecules selected in the context of phage have to be transferred onto mammalian expression systems to exert their therapeutic potential can be avoided. Thus, antibodies selected in the context of retroviruses will, for example, improve their expression from retroviral vectors or their use as anti-tumoral targeting domains in gene therapy applications.

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