

Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display

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Here we applied ribosome display to *in vitro* selection and evolution of single-chain antibody fragments (scFvs) from a large synthetic library (Human Combinatorial Antibody Library; HuCAL) against bovine insulin. In three independent ribosome display experiments different clusters of closely related scFvs were selected, all of which bound the antigen with high affinity and specificity. All selected scFvs had affinity-matured up to 40-fold compared to their HuCAL progenitors, by accumulating point mutations during the ribosome display cycles. The dissociation constants of the isolated scFvs were as low as 82 pM, which validates the design of the naive library and the power of this evolutionary method. We have thus mimicked the process of antibody generation and affinity maturation with a synthetic library in a cell-free system in just a few days, obtaining molecules with higher affinities than most natural antibodies.

Keywords: ribosome display, *in vitro* selection, single-chain antibody fragments, Human Combinatorial Antibody Library, affinity maturation

Ribosome display^{1,2} is a technology for the *in vitro* selection and evolution of very large protein libraries. The main feature distinguishing this technique from other selection techniques, such as phage display^{3–5}, is that the entire procedure is performed *in vitro*, without using cells at any step. Ribosome display was developed and applied first for peptide libraries⁶, and was then systematically improved to be suitable for screening and selection of folded proteins⁷. The principle of ribosome display is depicted in Figure 1. In ribosome display genotype and phenotype are linked through ribosomal complexes, consisting of messenger RNA (mRNA), ribosome, and encoded protein, that are used for selection. Using this technology, a scFv fragment of an antibody with picomolar affinity for a GCN4-variant peptide was isolated from a library prepared from immunized mice⁸. Alternative *in vitro* selection methods such as “RNA-peptide fusion”^{9,10}, in which an *in vitro*-synthesized polypeptide is covalently attached to its encoded message, have been demonstrated to work for peptide libraries.

If a high-fidelity proofreading DNA polymerase is used during the PCR amplification steps (Fig. 1), the repertoire of the library employed is virtually maintained¹¹. However, a particularly interesting feature of the ribosome display technology is that it can also be used for the directed evolution and affinity maturation that occurs during the selection process, if a low-fidelity DNA polymerase is used that introduces mutations during amplification (Fig. 1). A directed evolution can also be achieved when *in vivo* selection technologies are used, for example by transforming into particular mutator strains¹² when using phage display. However, this entails the risk of simultaneously introducing unwanted and possibly detrimental mutations in the plasmid or the host genome. For more efficient and controlled mutagenesis, it is usually necessary to switch between the diversification steps *in vitro* and the selection steps *in vivo*, which includes additional laborious cloning and transforma-

tion after each cycle^{13–15}. Thus, an important advantage of ribosome display is that selection and evolution can easily be performed entirely *in vitro* and thus these impediments can be avoided.

In this study we applied the ribosome display technology for the selection and evolution of scFvs from the synthetic HuCAL, which contains 2×10^9 independent members and has been designed to cover most of the antibody structure space¹⁶. The HuCAL is several orders of magnitude more diverse than a typical library from immunized mice and is not enriched for specific binders before selection.

We show here that by ribosome display it is possible to select a range of different scFvs from a naive library, which have affinities up to 82 pM. All the selected antibodies accumulated mutations through amplification with low-fidelity DNA polymerase, and thereby improved their affinities for antigen up to 40-fold when compared to the progenitor sequences originally present in the HuCAL and thus evolved during ribosome display selection.

Results and discussion

To validate ribosome display as an *in vitro* selection and evolution system, we decided to use the HuCAL (ref. 16). The HuCAL is a naive, completely synthetic antibody library, and thus the sequences of all frameworks and the randomized regions are exactly known. Mutations in the antibodies selected by ribosome display are likely to have been introduced during ribosome display selection, because mutations are rare in antibodies isolated from the HuCAL by phage display¹⁶. The HuCAL consists of 7 consensus human heavy-chain frameworks representing the human antibody subclasses according to Kabat¹⁷ (VH1A, VH1B, VH2-6), and 7 consensus human light-chain frameworks (V κ 1–V κ 4, V λ 1–V λ 3) in all 49 different possible combinations, thereby capturing most of the structural diversity of CDR1 and CDR2 (complementarity-determining regions) in both domains. Most of the diversity is generated by the CDR3s, where

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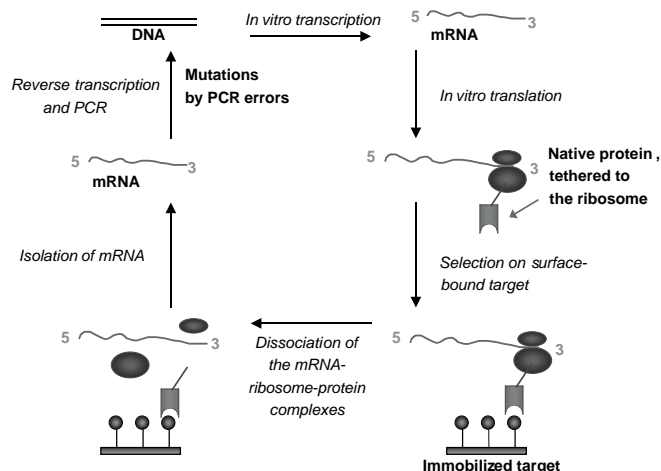


Figure 1. Principle of ribosome display. A library of scFvs is transcribed and translated in vitro. The resulting mRNA lacks a stop codon, giving rise to linked mRNA-ribosome-scFv complexes. These are directly used for selection on the immobilized target. The mRNA incorporated in bound complexes is eluted and purified. Reverse transcription-PCR can introduce mutations and yields a DNA pool enriched for binders that can be used for the next iteration.

both light and heavy chains carry fully randomized cassettes, with respect to both sequence diversity and length. In the case of CDR3 of the heavy chain, a sub-stoichiometric coupling of mixtures of trinucleotide codons¹⁸ was used to ensure a length distribution similar to human antibodies¹⁶.

Preparation of the ribosome display construct. We converted the HuCAL, which is in a phage display format¹⁶, into a construct suitable for ribosome display. The ribosome display construct of the scFv does not have a signal sequence, and the C terminus is fused to a spacer⁷ that tethers the synthesized protein to the ribosome by maintaining the covalent bond to the transfer RNA (tRNA). Furthermore, because the ribosomal tunnel can cover at least 20–30 amino acid residues^{19,20}, the spacer needs to provide an unstructured portion at the C terminus for allowing the remainder of the protein to be folded correctly.

The ribosome display version of HuCAL was prepared by in vitro ligation, without transforming cells. The pool of genes encoding the scFv library was ligated to the DNA fragment encoding the spacer. A twofold molar excess of spacer was used for this ligation, resulting in >50% of the scFv library-encoding DNA fragments ligating correctly to the DNA encoding the spacer. Thus, every member of the library was statistically 50 times overrepresented as a template for the subsequent two-step PCR, where all features necessary for efficient ribosome display were introduced: on the DNA level, the T7 promoter; and on the RNA level, the 5' and 3' stem-loops that stabilize mRNA against ribonucleases, and the ribosome-binding site necessary for efficient in vitro translation⁷. During the 40 PCR cycles using low-fidelity *Taq* DNA polymerase, the diversity of the original library was further increased by PCR errors, and we assume the actual size of the library to be even larger than 2×10^9 .

Selection for insulin binders. Three ribosome display experiments were carried out, each consisting of six selection cycles, using bovine insulin as an antigen. For all experiments, the affinity selections were performed using biotinylated bovine insulin and streptavidin magnetobeads in the first three cycles, followed by another three cycles with the same antigen but avidin-agarose as a capture reagent. This was done in order to prevent the enrichment of either streptavidin or avidin binders.

The last step of each ribosome display cycle is the PCR amplification of the reverse transcript of the enriched mRNA, and thus the

amount of PCR product reflects the amount of mRNA of enriched binders. Upon comparison of band intensities after the fourth and fifth cycles of ribosome display, we observed increasing amounts of PCR product, indicating that a specific enrichment of binders from the HuCAL pool was taking place. To achieve a higher enrichment of specific binders in the respective pools a sixth cycle was performed. The enriched pools were analyzed by radioimmunoassay (RIA) at room temperature (Fig. 2) for binding and inhibition by insulin. All three pools (A, B, and C), obtained after the fifth and sixth cycle showed binding to immobilized insulin and could be inhibited with soluble insulin, albeit to different extents (Fig. 2). Neutravidin and BSA were used as a negative control. Although higher enrichment for specific binders was achieved in the sixth round in all pools, the amount of noninhibitable binders (unspecific or possibly binding only biotin-insulin) increased proportionally in pools B and C (Fig. 2).

Sequences of selected scFvs: framework usage. After the sixth cycle of ribosome display from all three experiments (A, B, and C), the enriched pools were cloned and plasmids isolated and transcribed to mRNA. The mRNAs of single clones were used for in vitro translation, followed by RIA analysis for binding and inhibition with 10 nM and 100 nM insulin. Twenty-two binding scFvs from experiment A, 13 binders from experiment B, and 11 binders from experiment C were identified. A few clones contained stop codons or deletions (see below). The sequences of all isolated scFvs having no stop codon or deletions are shown schematically in Figure 3.

Among the scFvs obtained in each particular experiment (A, B, or C), a family of closely related scFvs was identified, which differed only in a few amino acid residues (Fig. 3). An overview of all identified scFv framework combinations is shown in Figure 4. In experiment A, 9 scFvs out of 15 were closely related. All 15 scFvs contained the same heavy-chain framework, VH3, but two different CDR-H3 sequences (12 and 3 scFvs, respectively). These scFvs were rather diverse in their light-chain usage: 10 scFvs contained V κ 2 (two different CDR3s), one V κ 4, two V λ 1 (different CDR3s), and two V λ 2 (different CDR3s) (Fig. 4). In experiment B, seven scFvs contained VH6 and V λ 1, all having the same CDR-H3 sequences. Two other antibodies isolated in experiment B were very closely related to the predominant group of scFvs from experiment A (VH3 and V κ 2, same CDR3s). In experiment C two different groups of antibodies were found: nine of them possessed VH1A and V κ 1 and two had VH1A and V λ 2 (different CDR-H3) (Fig. 4).

Compared to the original HuCAL framework sequences, all selected scFvs had mutations (two–nine mutations per scFv within the V_H and V_L domains, mean 4.3) (Fig. 3), which were introduced during PCR.

In the three experiments, a variety of scFvs with different frameworks were selected, demonstrating that there are many solutions to developing a high-affinity binder to a protein target. It also indicates that under the conditions used complete sampling of the library did not occur. The introduction of diversity by PCR errors in each cycle results in overlapping processes: Binders are enriched during the selection cycles and after several rounds of selections start to compete for insulin binding. Simultaneously, the binders can be mutated and thereby gain or lose affinity to insulin. Thus, new binders can be generated or weak binders can affinity-mature. In contrast to phage display or other selection systems where mutations occur rarely, the sequence space is not strictly limited by the size of the initial library in ribosome display. As a consequence of this dynamic system, every selection might give a different and new result. The selection of ligand families for a protein target has been observed before using SELEX (ref. 21), and is an indication that the selection is nearing its final round, because these families contain the binders with the highest affinity. We would expect that earlier-round pools are more complex and contain ligands with a wider range of affinities.

Table 1. Dissociation constants of selected scFvs^a

scFv	K _d (nM)	scFv	K _d (nM)
Awt	31 ± 1	Cwt	3.4 ± 0.32
A1	7.7 ± 0.3	C33	0.88 ± 0.04
A3	23 ± 2	C46	0.73 ± 0.04
A21	2.0 ± 0.3	C49	0.30 ± 0.03
A23	3.2 ± 0.6	C59	0.082 ± 0.019
B17	19.5 ± 0.9	C67	0.14 ± 0.01

^aDissociation constants of antibody scFv fragments binding bovine insulin selected by ribosome display estimated by competition BIAcore at 20°C.

The occurrence of somatic mutations. Among the mutations of the selected scFvs in the HuCAL framework sequence, several prevalent mutations were identified.

In the VH1A framework selected in experiment C, Thr56 mutated to either Ala or Lys. This mutation is apparently of particular significance because all picomolar binders (C49, C59, and C67 in Table 1) share this CDR-H2 mutation. All antibodies with the framework combination VH1A-Vκ1 use Val85, but not Thr85 in V_L. The oligonucleotide used to encode CDR3 in Vκ1 and Vκ3 was designed to encode an equimolar mixture of valine and threonine at position 85. This region at the variable/constant (V/C) domain interface is exposed in scFvs, but may still contribute to stability by lateral interactions. Alterations at the V/C interface have been implicated in affecting the folding yield of scFvs (ref. 22).

In many of the VH3 sequences selected in experiment A and B, Glu46 mutated to Lys. Molecular modeling studies (A. Honegger, personal communication) show that this negatively charged glutamate at position 46 is positioned to form a salt bridge with the positively charged Arg38 of V_H, and it is also close to Lys43. A mutation giving rise to a positively charged residue (lysine) at position 46 may affect the interactions present in this domain and thus potentially its stability. In VH3-CDR2 of these scFvs, Gly52A changed to Asp, and in the corresponding Vκ2-CDR1 Ser27A mutated to Gly; both mutations probably have no significant effect on insulin binding (Table 1). In some of the scFvs from selection experiment A, Leu108 in V_H mutated to proline (Fig. 3). This leucine residue in the wild-type scFv is located at the surface of the scFv in a β-sheet, and a change to proline could have some effect on the geometry of this β-sheet segment.

In experiment B, all selected scFvs with framework VH6 showed a mutation from the conserved original Trp103 to Leu. Trp103 in the wild-type scFv is located beneath the binding pocket and is involved in extensive hydrophobic interactions to the light-chain domain. This Trp → Leu change may affect the V_H to V_L orientation, which may in turn alter the pocket geometry and thus indirectly contribute to insulin binding. Several related scFvs selected in the experiment B lost the highly conserved Cys23 in Vλ1 by mutation to Arg. The concomitant loss of a disulfide bond, however, apparently does not influence the ability of the scFv to bind the antigen. This is reminiscent of a natural antibody with unexpected replacements for a cysteine, which also retained its binding ability^{23,24}. In general, to afford the loss of a disulfide bond, antibodies have to be rather stable²⁴.

All isolated scFvs contained an intact 20-amino acid linker. Most mutations found in the original A(G₃S)(G₄S)₃ linker were Gly → Ser (five times), and Gly → Asp, Gly → Cys, and Ser → Pro substitutions (once each).

Characterization of selected scFvs. We compared these mutated scFvs to their respective HuCAL progenitors. Because the scFvs with HuCAL consensus sequences have not been selected themselves, we constructed two consensus sequence scFvs, one for the VH3-Vκ2 family isolated in experiment A and the second for the VH1A-Vκ1 family of experiment C (Fig. 4). These two groups were chosen because they were expected to bind the antigen with the highest

affinity, based on RIA inhibition analysis. The original HuCAL framework sequences were therefore fitted with the CDR3s in V_L and V_H isolated in the ribosome display experiments. We thus obtained the consensus sequence scFv Awt (VH3-Vκ2; CDR3 of V_H: FFDADMDS, CDR3 of V_L: QQYGGVPY) and the consensus sequence scFv Cwt (VH1A-Vκ1, with a Thr85 in V_L; CDR3 of V_H: RMYFDS, CDR3 of V_L: QQWSSFPF).

The related antibodies A1, A3, A21, A23, B17, and their consensus scFv Awt were subcloned into the secretion vector pJB33 (see Experimental Protocol), expressed in the periplasm of *Escherichia coli*, purified, and used for K_d determination at 20°C by competition BIAcore analysis^{25,26}. The competition analysis allows the determination of the correct dissociation constants in solution, independent of any BIAcore rebinding error²⁵⁻²⁷. From the sensorgrams K_d values were calculated as described⁸. In agreement with our results from the RIA analysis and as summarized in Table 1, all scFvs analyzed from experiment A and the related scFv from experiment B had improved their affinities to insulin. The best improvement achieved amounted to 15-fold when compared to the “wild-type” HuCAL scFv.

A similar analysis was performed with five related scFvs isolated in experiment C and their consensus sequence scFv Cwt. The selected scFvs were produced as inclusion bodies in *E. coli*, refolded, purified, and used for K_d determination by competition BIAcore analysis, as shown in Table 1. Again, all analyzed scFvs from experiment C improved their affinities to insulin, the best of them by 40-fold to 82 pM, compared to the original HuCAL scFv.

Suppression of stop codons and frameshifting. The sequence analysis revealed that among the 46 binders there were also scFv genes present that contained either a stop codon (four scFvs) or +1 / -1 frameshifts in their gene sequences (seven scFvs) (data not shown). However, all these “incorrect” antibody sequences gave rise to protein molecules that bound to insulin and could be inhibited specifically in RIA. All of these alterations were found in sequence clusters where most sequences were “correct” and thus indicate alterations occurring at a later stage in the selection experiment, as they were clearly derived from a functional progenitor.

Previously, we had established that for successful ribosome display enrichment the absence of a stop codon is absolutely essential^{1,7}. Thus, we wished to understand how these scFvs could have been selected despite containing a stop codon. We therefore analyzed the in vitro-translated protein products of all “incorrect” scFvs, synthesized in the presence of [³⁵S]methionine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography revealed that in every case investigated, in addition to the translation product corresponding to the shortened scFv

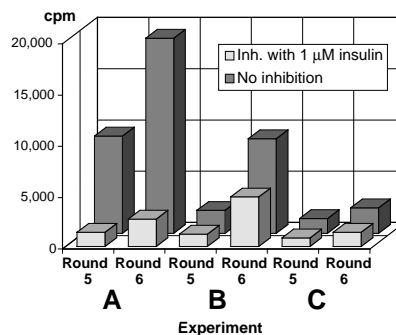
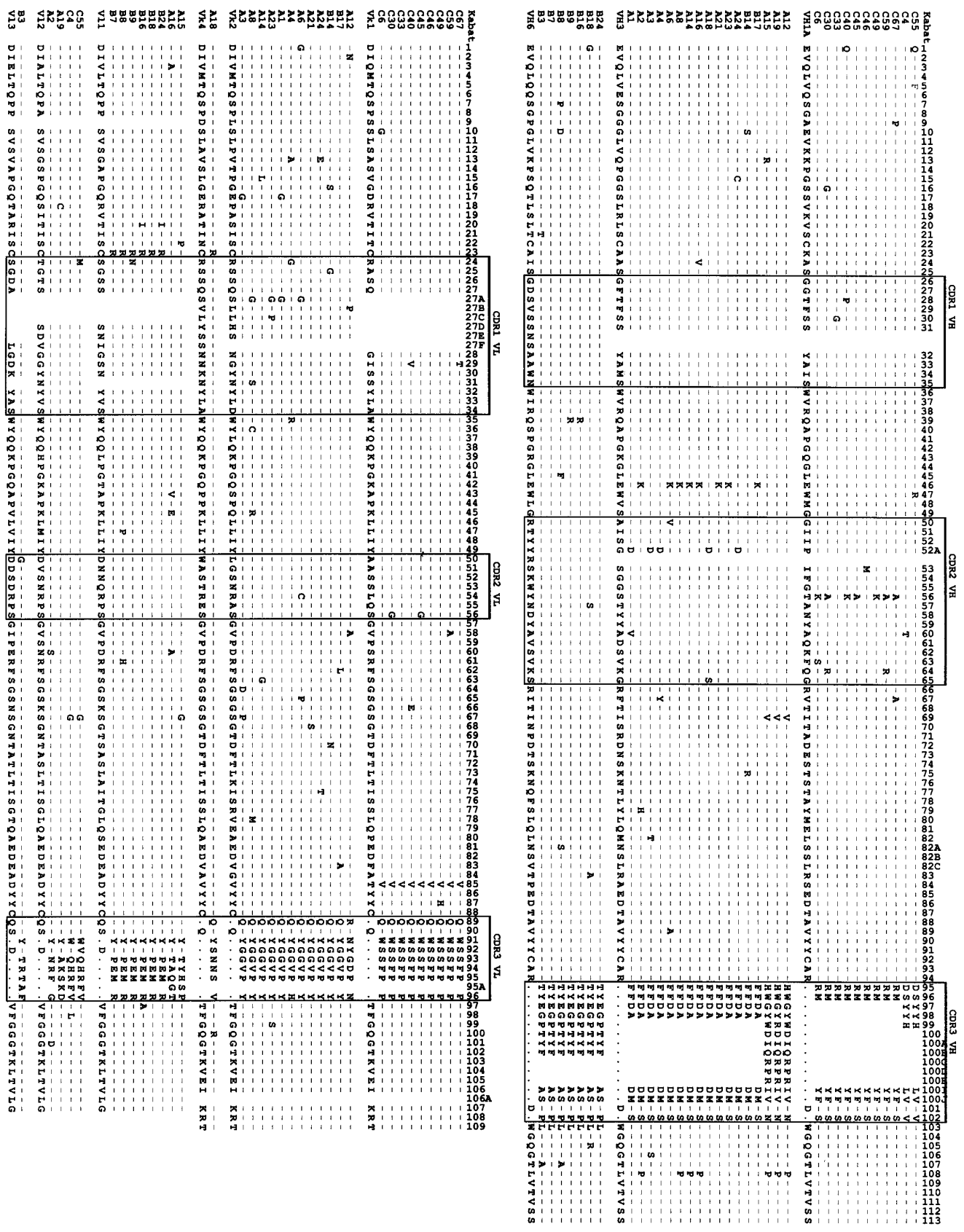


Figure 2. Radioimmunoassays of the pools after the fifth and sixth rounds of ribosome display. Pooled RNAs from three independent ribosome display experiments A, B, and C were translated in vitro in the presence of [³⁵S]methionine, and translation mixtures containing 0 and 1 μM bovine insulin were analyzed by RIA at room temperature as described in the Experimental Protocol section. Each bar represents the average of two samples.

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Figure 3. Alignment of the amino acid sequences of V_H and V_L of the scFvs. Residues identical to the original HuCAL light- and heavy-chain domains are represented by dashes. Dots in the original HuCAL sequences represent the randomized sequence of CDR3s with different length (for details, see text). ScFvs included in experiments A, B, or C are denoted accordingly. Numbering of amino acid residues in V_H and V_L and the labeling of CDRs is according to Kabat¹⁷.



VH1A	C(9)						C(2)
VH1B							
VH2							
VH3		A(9+7*) A(1) B(2)		A(1)	A(1) A(1)	A(1) A(1)	
VH4							
VH5							
VH6					B(6+4*)		B(1)
	Vκ1	Vκ2	Vκ3	Vκ4	Vλ1	Vλ2	Vλ3

Figure 4. Framework usage of the insulin-binding HuCAL scFvs. The vertical and horizontal axes denote the HuCAL heavy-chain and light-chain variable domains (for details, see text). ScFvs isolated in experiments A, B, or C are denoted accordingly. Numbers in parentheses represent the number of closely related scFvs with the same CDRs, but different point mutations. Numbers with an asterisk represent the numbers of closely related scFvs with the same CDRs, but having either a stop codon or frameshift (see text).

version, the full-length protein was also synthesized. In all cases the upper band on the SDS-PAGE gel corresponded to that of the correct full-length scFv protein, which on average amounted to ~10% of the truncated version. This large amount of correct, full-length scFv indicates that during the in vitro translation step of ribosome display, under the conditions used, the suppression of the stop codon and of +1 or -1 translational frameshifts must be possible. These results clearly explain why these genetically incorrect scFvs could nevertheless be isolated by ribosome display. A fully intact protein molecule was affinity-selected, and the enrichment did not result from unspecific binding.

Conclusions

In this report we show that it is possible to isolate specific high-affinity antibodies completely in vitro, without involving any natural sources or in vivo steps, starting from a naive library of synthetic, designed genes. By applying the ribosome display technology for the screening of the HuCAL, a number of different scFvs have been isolated that specifically bind insulin, with monomeric dissociation constants as low as 82 pM measured in solution. This process of antibody generation closely mimics the generation of antibodies by the immune system, by first selecting from a preexisting variety and then by further optimizing binders introducing somatic mutations. The resulting molecules with monomeric affinities in solution as low as 8×10^{-11} M validate both the library and the evolutionary technology.

The affinity maturation process expands the actual sequence space sampled to beyond that of the initial library of 2×10^9 members. Because each of the 2×10^9 sequences is initially theoretically represented by ~100 functional ribosomal complexes⁷, rapid diversification is possible. A "single-pot" library, containing the same coverage of sequence space in the absence of point mutations occurring during selection, would have to be several orders of magnitude larger in size. To improve the resulting molecules even further, the ribosome display format is ideally set up to be combined with DNA shuffling²⁸, error-prone PCR²⁹, or the staggered extension process³⁰, because no transformation is needed for subsequent library generation. We believe that ribosome display, especially in conjunction with designed protein libraries such as HuCAL, will be a very powerful technology for the generation and evolutionary fine-tuning of custom proteins for a wide range of applications.

Experimental protocol

Construction of ribosome display library. Because the HuCAL was in phagemid format¹⁶, it was necessary to first prepare the ribosome display construct. The *E. coli* culture containing the HuCAL on a phagemid was grown overnight at 37°C in Luria-Bertani medium containing 30 µg/ml chloramphenicol. A plasmid pool was isolated using the Plasmid Midi Kit (Qiagen, Hilden, Germany), cut with *Xba*I/*Eco*RI, and separated by agarose gel electrophoresis. The scFv library fragment, encoded on a 750 bp fragment, was extracted from the gel with the QIAEX Gel Extraction Kit (Qiagen). The C-terminal spacer was isolated from fd phage³¹ (f17/9) in a similar way, by using an *Eco*RI/*Hind*III digestion. The purified library *Xba*I/*Eco*RI fragment (350 fmol) was ligated in a 30 µl reaction mixture with the *Eco*RI/*Hind*III spacer fragment (175 fmol) overnight at 16°C.

In one of the selections (experiment C) a spacer with a length of 182 amino acids and a sequence derived from the periplasmic part of TonB from *E. coli* was used. The spacer was amplified from *E. coli* genomic DNA by PCR using the TonBfor primer (5'-TATATGGCCTCGGGGCC-GAATTCAGCCGCCACCGAG-3') and the TonBtrev primer (5'-CCG-CACACCAGTAAGGTGTGCGGTCAGATATTCACCACAATCCC-3'). The PCR product was digested with *Eco*RI, purified by agarose gel electrophoresis, and ligated to the *Xba*I/*Eco*RI fragment of the HuCAL as described above. The ligation efficiency was 50% as judged by analytical gel electrophoresis. In order to introduce the features necessary for ribosome display, the ligation mixtures were directly amplified in two steps by PCR, using in the first step the primers SDA (5'-AGACCACAACGGTTCCCTCTAGAAAT-AATTTTGTTAACCTTAAGAAGGAATATATCCATGGACTACAAAGA-3') and T5Te (5'-CCGCACACCAGTAAGGTGTGCGGTATCACCAGTAG-CACC-3') or TonBtrev, respectively, and in the second step primers T7B (5'-ATACGAAATTAATACGACTCACTATAGGGAGACCACACCGG-3') and T5Te or TonBtrev. The oligonucleotides T7B and SDA introduced the 5'-untranslated region of the mRNA, which is derived from gene 10 of phage T7 (ref. 32), and includes the T7 promoter, the 5' stem-loop, and the ribosome binding site. The oligonucleotides T5Te and TonBtrev introduced a 3' stem-loop of mRNA, which is derived from the early terminator of phage T3 (ref. 33) and which has been slightly modified to continue the open reading frame. PCR products were directly used for in vitro transcription with T7 RNA polymerase³⁴, and RNA was purified by lithium chloride precipitation.

Affinity selection. In vitro translations were performed in an *E. coli* S-30 system as described⁷. After 7 min at 37°C the translation reaction was stopped by diluting it fourfold with ice-cold washing buffer (50 mM Tris acetate pH 7.5, 150 mM NaCl, 50 mM magnesium acetate, 2.5 mg/ml heparin, and 0.1% Tween-20) and centrifuged for 5 min at 4°C at 10,000 *g* to remove insoluble components. After mixing 1 ml of the ice-cold diluted and centrifuged translation mixture with 250 µl of ice-cold 10% biotin-free sterilized milk powder, it was supplemented with biotinylated bovine insulin (Sigma, Buchs, Switzerland) to 10 nM concentration, and the mixture was transferred to the ice-cold milk-blocked panning tubes (5 ml volume). The tubes were placed inside of an appropriate larger tube filled with ice and rotated end-over-end for 1 h in a cold room. After affinity selection, the solutions from the panning tubes were added to 200 µl of Streptavidin Magnetic Particles (Roche Diagnostics, Rotkreuz, Switzerland) (ice-cold; washed with washing buffer before use) prepared in a 1.5 ml tube and rotated end-over-end on ice for 10–15 min in the cold room. After five washes with ice-cold washing buffer using a magnet, the retained ribosomal complexes were dissociated with 200 µl ice-cold elution buffer (50 mM Tris acetate pH 7.5, 150 mM NaCl, 20 mM EDTA, 50 µg/ml *Saccharomyces cerevisiae* RNA) for 5 min on ice by gentle shaking, and released mRNA was recovered using the High Pure RNA Isolation Kit (Roche Diagnostics). Intermittently, affinity capturing was carried out with avidin immobilized on agarose (Sigma) with the following modifications: 50 µl of avidin-agarose was used for capturing, and beads were separated from the washing solution with a centrifuge. Purified RNA was subsequently used for reverse transcription PCR⁷. After in vitro transcription of PCR products, RNA was purified by lithium chloride precipitation and used either for RIA analysis or for the next round of ribosome display.

RIA analysis. Microtiter plate wells were coated overnight at 4°C with 100 µl per well of 4 mg/ml neutravidin in PBS (10 mM sodium phosphate buffer pH 7.4, 140 mM NaCl, 15 mM KCl). The following day 50 pmol of biotinylated bovine insulin in 100 µl PBS per well was incubated for 30 min at 25°C and, after washing with PBST (PBS with 0.05% Tween 20), the wells were blocked with 4% milk powder in PBS. The RNA of the pool or of sin-

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gle clones was translated *in vitro* for 30 min at 37°C in an *E. coli* S-30 system^{7,8}. The reaction mixture contained 0.3 µM of [³⁵S]methionine (50 µCi/ml) but no cold methionine. After translation, the reaction mixture was diluted fourfold with PBST and centrifuged. The supernatant was diluted with the same volume of 4% milk in PBST containing 0, 20, or 200 nM bovine insulin and preincubated for 1 h at room temperature. Binding to insulin, immobilized to the microtiter wells, was carried out for 30 min at 25°C with gentle shaking. After five washes with PBST, bound radioactive protein was eluted with 4% SDS in PBS and quantified in a scintillation counter.

Periplasmic expression and protein purification. Selected scFv sequences (C46, Cwt, A1, A3, A21, A23, Awt, B17) were cloned in the secretion vector pJB33, a pAK400 (ref. 35) derivative encoding the periplasmic folding factor Skp (ref. 36), which introduces a C-terminal histidine tag to the expressed protein. The scFvs were expressed as described⁸. Cells were collected by centrifugation (8,000 g, 10 min, 4°C) and resuspended in PBS containing DNaseI (Roche Diagnostics). Cell disruption was achieved by French Press lysis, the resulting crude extracts were centrifuged (20,000 g, 25 min, 4°C) and filtered through a 0.22 µm filter. Histidine-tagged scFv fragments were purified by immobilized-metal ion affinity chromatography (IMAC) using Ni-NTA Superflow (Qiagen) according to the manufacturer's recommendations. The eluate was dialyzed against HBS buffer (20 mM HEPES, pH 7.2, 150 mM NaCl, 3 mM EDTA), and the monomeric scFv was isolated by gel filtration using a HiLoad Superdex75 column (Pharmacia, Freiburg, Germany).

Cytoplasmic expression and protein purification. The scFvs were cloned in the vector pTFT74 (ref. 37), expressed and refolded as described⁸. Refolded scFvs were purified by IMAC using Ni-NTA Superflow (Qiagen) and gel filtration chromatography (Superdex75) as described above.

Competition BIAcore. Competition BIAcore analysis was performed under conditions of mass-transport limitation as described⁸, using a CM5 chip (BIAcore, Freiburg, Germany) coated with 2,000 relative response units of bovine insulin, which was coupled by classical amine chemistry. An uncoated chip was used as a control. Each binding/regeneration cycle was performed at 20°C with a constant flow rate of 25 µl/min using 20 mM HEPES, pH 7.2, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20 (HBST). Samples of 250 µl of antibody (about 1 nM) in HBST, containing various amounts of antigen, were injected through the sample loop of the system, followed by regeneration of the surface by injection of 20 µl of 6 M guanidinium chloride in HBST. The antibodies were preincubated with free bovine insulin at different concentrations for at least 1 h at 4°C before injection. Data were evaluated using the BIAevaluation software (Pharmacia) and Kaleidagraph (Synergy Software, Reading, PA). Slopes of the association phase of linear sensorgrams were plotted against the corresponding total antigen concentrations, and the dissociation constant was calculated as described⁸.

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