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Generation and characterization of a chimeric rabbit/human Fab for co-crystallization of HIV-1 Rev

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Abstract

Rev is a key regulatory protein of HIV-1. Its function is to bind to viral transcripts and effect export from the nucleus of unspliced mRNA thereby allowing the synthesis of structural proteins. Despite its evident importance, the structure of Rev has remained unknown, primarily because Rev's proclivity for polymerization and aggregation is an impediment to crystallization. Monoclonal antibody antigen-binding domains (Fabs) have proven useful for the co-crystallization of other refractory proteins. In the present study, a chimeric rabbit/human anti-Rev Fab was selected by phage display, expressed in a bacterial secretion system, and purified from the media. The Fab readily solubilized polymeric Rev. The resulting Fab/Rev complex was purified by metal ion affinity chromatography and characterized by analytical ultracentrifugation which demonstrated monodispersity and indicated a 1:1 molar stoichiometry. The Fab binds with very high affinity, as determined by surface plasmon resonance, to a conformational epitope in the N-terminal half of Rev. The complex forms crystals suitable for structure determination. The ability to serve as a crystallization aid is a new application of broad utility for chimeric rabbit/human Fab. The corresponding single chain antibody (scFv) was also prepared, offering the potential of intracellular antibody therapeutics against HIV-1.

Keywords

rabbit antibody; phage display; humanized Fab; scFv; crystallization chaperone

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Introduction

The unique antibody repertoire development in rabbits (*Oryctolagus cuniculus*) has been exploited for the generation of polyclonal and monoclonal antibodies with exceptionally high avidity, affinity, and specificity¹. While rabbit polyclonal antibodies have had a long standing as research reagents, the more recent generation of rabbit monoclonal antibodies through both phage display^{2; 3; 4; 5} and hybridoma technology⁶ has provided access to a highly defined research reagent of unlimited supply. In addition to high affinity and specificity, rabbit monoclonal antibodies can recognize epitopes conserved between human, mouse, and rat antigens^{7; 8; 9}. This cross-reactivity along with the demonstration that rabbit monoclonal antibodies can be humanized has raised an interest in utilizing rabbit monoclonal antibodies for therapeutic applications¹⁰. Their ability to target epitopes that differ from those recognized by mouse monoclonal antibodies makes rabbit monoclonal antibodies attractive research reagents for functional and biophysical studies of antigen/antibody interactions.

A rabbit monoclonal antibody format of particular interest is the chimeric rabbit/human Fab which consist of rabbit variable domains V_H and V_K and human constant domains $C_{\gamma 1}$ and C_K ^{5; 11}. We have demonstrated that chimeric rabbit/human Fab libraries can be generated from spleen and bone marrow of immunized rabbits, in particular b9 allotype rabbits^{7; 8; 9}, and subsequently selected by phage display to yield chimeric rabbit/human Fab of high affinity, specificity, cross-reactivity, and convertibility to chimeric rabbit/human IgG1. The rabbit variable domains V_H and V_K of chimeric rabbit/human Fab can be humanized^{5; 12}.

While the 150-kDa IgG molecule is the most commonly used format of monoclonal antibodies in basic research as well as diagnostic, preventative, and therapeutic applications, the smaller 50-kDa Fab molecule, which can be expressed in *E. coli*, has facilitated the generation, affinity maturation, and humanization of monoclonal antibodies through *in vitro* evolution technologies, most prominently phage display^{10; 13; 14}. Thus, in most instances, the Fab molecule has been a facilitating format for generating and evolving IgG for particular purposes. Nonetheless, Fab have also been utilized in their own right for an increasing number of applications that exploit its smaller size and easier manufacturability compared to IgG¹⁵. An important application in basic research is the utilization of Fab for the co-crystallization of proteins in general and transmembrane, hydrophobic, and aggregating proteins in particular. In addition to providing crystal contacts through protruding hydrophilic surfaces, Fab can support crystallization by locking in conformations and blocking aggregation. For example, Fab have been used as crystal chaperones¹⁶ in the determination of the three-dimensional structure of transmembrane ion channels and G protein-coupled receptors^{17; 18; 19}. Notably, phage display has facilitated the generation and evolution of Fab with superior co-crystallization properties^{16; 19}.

Rev (13 kDa) is an essential regulatory protein of the HIV-1 virus which functions by binding to, and preventing splicing of, the viral mRNA, thereby facilitating transition to the late phase of the replication cycle (for review see²⁰). Despite its importance, and considerable efforts directed at its elucidation, the structure of Rev remains unknown, due largely to the protein's strong propensity to polymerize into long filaments^{21; 22}. Here we describe the preparation, characterization, and crystallization of a complex formed between Rev and a chimeric rabbit/human Fab selected by means of phage display. The preparation of the corresponding high-affinity single chain antibody fragment (scFv), which has anti-HIV therapeutic potential, is also described.

Results

Selection of Rev-specific antibody fragments using phage display

Following immunization with purified recombinant HIV-1 Rev, spleen and bone marrow from two rabbits were collected and processed for total RNA preparation, RT-PCR amplification of rabbit V_k , V_λ , and V_H encoding sequences, V_k - C_k - V_H cassette assembly, and asymmetric *Sfi*I ligation into phage display vector pC3C essentially as described¹⁵. The resulting library, which consisted of approximately 2×10^8 independently transformed chimeric rabbit/human Fab clones, was screened by phage display on recombinant Rev protein that had been selectively biotinylated at the C-terminus and immobilized on streptavidin-coated plates. After four to five rounds of panning, selected clones were subjected to an initial characterization by ELISA, DNA fingerprinting, and DNA sequencing. All selected clones were identical. The encoded chimeric rabbit/human Fab was termed SJS-R1. The deduced amino acid sequences of the variable domains of SJS-R1, which are shown in Fig. 1, revealed unique rabbit V_k and V_H sequences with the highest similarity (85% and 77% amino acid sequence identity, respectively) to b9-allotype-derived rabbit variable domains in GenBank⁸.

Expression and purification of antibody fragments

The Fab was expressed in *E. coli* using an expression cassette with two N-terminal signal sequences pelB and ompA which direct the separate secretion of the V_k - C_k and V_H - C_H1 chains into the periplasmic space where enzymic oxidation processes form two intramolecular disulfides per chain and one inter-chain disulfide²³. Although high expression was achieved, most of the Fab protein was retained in the periplasm with only a small amount being secreted into the media. It was found that freezing and thawing of the cells followed by a brief sonication dramatically increased the yield. The His tag on the V_H - C_H1 chain provided the basis for affinity purification and two cycles of Ni-Sephadex chromatography, one for batch-wise capture and the second using gradient elution, gave good results in terms of yield and purity. When further purification was required, this was carried out using Superdex S200 gel filtration in the presence of 1 M urea to increase Fab solubility (Fig. 2). Using the same expression and purification methods, the corresponding single chain Fab (scFv) was also produced.

The identities of the antibody fragments were confirmed by mass spectrometry. Reduced Fab gave mass values of 23,464 Da (23,468) and 24,472 Da (24,466) for the light and heavy chains, respectively; and oxidized scFv gave a mass of 26,429 Da (26,430). Values in parentheses are those predicted from the DNA sequences (Note: the Fab heavy chain fragment and scFv include C-terminal His tags). SDS-PAGE of non-reduced Fab (Fig. 2b) and scFv (Fig. 3a, lane scFv) also gave bands with mobilities consistent with the predicted masses. Molecular weights under native conditions were measured by sedimentation equilibrium and both Fab and scFv were found to be monomeric (Fig. 4c; data for Fab not shown). Although the antibody fragments in standard buffers and at neutral pH were well behaved below 1 mg/mL, at higher concentrations there was a tendency towards aggregation and, as mentioned above, the addition of 1 M urea helped maintain solubility without compromising conformational integrity.

Binding of Fab to Rev

The binding kinetics of the Fab to Rev were measured by surface plasmon resonance with Rev immobilized on the chip and Fab as the analyte (Fig. 5). The binding was characterized by a typical on-rate ($k_a = 2.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) but the off-rate ($k_d = 0.8 \times 10^{-5} \text{ s}^{-1}$) was very low: a low off-rate is characteristic of high affinity (see for example, Drake et al.,²⁴). With such a low off-rate, and with the technical limitations of the method, the subnanomolar affinity value determined (~40 pM) needs confirmation by another approach but regardless, a high affinity interaction is certainly suggested.

Rev in solution self-associates: monomers and dimers at low protein concentration associate to form high molecular weight filaments at higher concentrations²². The polymerization fits an isodesmic self-association model in which the association constant for the addition of a monomer to each aggregate is equal with a K_d of $\sim 1.0 \mu\text{M}$ ²⁵. The Rev filaments (Fig. 6a) are stable and are hollow with an outer diameter of $\sim 15 \text{ \AA}$ ²¹.

Addition of equimolar Fab to the filaments causes rapid depolymerization with the formation of small, uniformly sized complexes (Fig. 6b). In some views these complexes appear to have a central stain-penetrable hole (Fig. 6b, inset). In a similar manner the scFv also depolymerized Rev filaments (data not shown). In both cases, the facile disruption of the protein-protein interactions is consistent with a high affinity interaction.

To approximately locate the Rev epitope, the C-terminal deletion mutant Rev $\Delta 60-116$ was used. Electron microscopy showed that this mutant by itself cannot assemble into a filament, probably due to the absence of the carboxy-terminal half. When the scFv was added uniform complexes were formed that appeared similar, though smaller, to those observed with full-length Rev (data not shown). This suggested that the epitope is present, and located in the N-terminal 1–59 residues of Rev.

Preparation and characterization of Fab/Rev and scFv/Rev complexes

Immune complexes were prepared by mixing the antibody fragments with a several-fold molar excess of Rev and then purifying them by means of metal chelate chromatography on Ni-Sepharose taking advantage of the C-terminal His tags. SDS-PAGE of the non-reduced Fab/Rev complex gave two main bands of $\sim 45 \text{ kDa}$ and $\sim 16 \text{ kDa}$ corresponding to oxidized Fab and Rev (Fig. 3a, lane Fab/Rev). It should be noted that the predicted mass of Rev is 12,905 Da but that it appears to have an anomalously low mobility in gel electrophoresis. Reduction of the complex also produced two bands, in this case Rev and an $\sim 25 \text{ kDa}$ band corresponding to unresolved Fab heavy chain fragment ($\sim 24.5 \text{ kDa}$) and light chain ($\sim 23.5 \text{ kDa}$; Fig. 3b). Densitometry indicated a Fab/Rev 1:0.9 molar ratio, assuming equal Coomassie dye binding capacity. A similar analysis of the scFv/Rev complex under non-reducing (Fig. 3a, lane scFv/Rev) and reducing conditions (Fig. 3c) indicated a 1:0.87 molar ratio. Thus, both the Fab and scFv antibodies appear to form equimolar complexes with Rev.

Following affinity purification, it is common to use gel filtration to finalize purification (polish), and at the same time confirm physical homogeneity as evidenced by symmetrical elution peaks. Unfortunately, both Rev and Rev complexed with antibody fragments adsorb strongly to the commonly used gel filtration matrices ruling out this approach. As the primary aim of this work was to crystallize the immune complex it was important to ascertain monodispersity. For this purpose we used sedimentation velocity analysis after removing any protein aggregates by centrifugation at $100,000 \times g$ for 1–2 hours. Typical data (Fig. 7, insert) show a single moving boundary equivalent to a single species and characteristic of a monodisperse system. In a more detailed analysis, the sedimentation coefficient distribution plot (Fig. 7) also indicates a single species, analogous to a gel filtration peak²⁶. From fitting the data, the diffusion coefficient can be obtained and, hence, a molecular weight estimate made $\sim 63 \text{ kDa}$ (Fig. 7). This result is consistent with a complex of Rev and Fab in an equimolar ratio with a predicted mass of 60.81 kDa .

A more robust approach to mass and constituent stoichiometry determination is sedimentation equilibrium analysis. Protein gradients of Fab/Rev were analyzed assuming the system was ideal, for example, no reversible association, with a mass determination of 68 kDa (Fig. 4a). Small amounts of aggregate, evidenced as systematic error in residuals at the bottom of cell, accumulate during the course of the centrifugation. We have not examined in detail variations in buffer composition and pH that might stabilize the complex during analysis, although using

lower temperatures had no effect. Even when the aggregated protein absorbance is truncated prior to data fitting, mass estimates are ~65–68 kDa. If the gradient is analyzed as monomer-dimer system a better fit is obtained with a $K_d \sim 0.1$ mM, indicating a weak dimerization potential probably mediated by Rev (Fig. 4a). Native mass spectrometry also detects a Fab/Rev complex as the main component (with lesser amounts of oligomers of this 1:1 complex) but where none of the complexes contained dimeric Rev (Uetrecht, C. and Heck, A., personal communication). The mass and stoichiometry determinations with the single chain antibody complex are clearer, the scFv/Rev having a determined mass of 40.4 kDa (Fig. 4b), which is close to that predicted for an equimolar complex (39.34 kDa).

Crystallization of Fab/Rev complex and direct identification of epitope

Based on the physiochemical analysis, Fab/Rev appears homogeneous when freshly prepared, with the caveat that this assessment by analytical centrifugation was made using protein concentrations less than 1 mg/mL. In order to screen for homogeneity at higher protein concentrations, dynamic light scattering was used (Fig. 4d). This technique, which measures the translational diffusion coefficient, is widely used to assess the suitability of samples for crystallization²⁷. The Fab/Rev complex at concentrations between 1–9 mg/mL gave a main peak (~97% total protein) with mass estimations close to those obtained from centrifugation. (The mass determination by the DLS plate reader is not as rigorous as that by sedimentation equilibrium but in this case was consistent). Aggregates did accumulate slowly when the complex was incubated at room temperature for up to 24 hours but these were easily removed by filtration or low speed centrifugation.

Fab/Rev was crystallized (see Material and Methods) forming long rods (Fig. 8). The crystals were suitable for X-ray structure determination and the diffraction pattern (Fig. 9) indicated resolution to beyond 3.3 Å. The data were indexed in a primitive triclinic crystal system, with unit-cell parameters $a = 87.7$ Å, $b = 87.6$ Å, $c = 177.4$ Å, $\alpha = 95.3^\circ$, $\beta = 94.9^\circ$, $\gamma = 104.3^\circ$. The structural determination of the Fab/Rev complex will be described elsewhere (Dimattia et al., manuscript in submission).

HIV-1 Rev epitope and Fab paratope

The composition of the epitope was derived from the structure determination of the Fab/Rev complex at 3.2 Å resolution (Dimattia et al., manuscript in submission). Briefly, the epitope is conformational and located in the N-terminal region of Rev. Specific interactions with the Fab involve 16 residues encompassing amino acids within the N-terminal 63 residues that contact either one or both domains (V_K and V_H) of the Fab (Fig. 10; Table 1). The paratope (Fig. 10) reveals a Fab/Rev interface of ~ 720 Å². This large binding footprint is certainly consistent with high affinity binding and stabilization of the Fab/Rev 1:1 molar complex.

Discussion

Stabilization of HIV-1 Rev for structural studies

The selection of Fab SJS-R1 is remarkable in that, although we have so far only obtained this single clone, it has such advantageous properties. The antibody binds to monomeric Rev with very high affinity producing a stable immune complex. As we have previously mentioned, at concentrations >80 µg/mL Rev rapidly polymerizes to form filaments. The Rev used for the rabbit immunizations was below this critical concentration and was therefore probably a monomer-dimer mixture. For selection of the chimeric rabbit/human Fab library that was generated from Rev-immunized rabbits, solid phase Rev immobilized *via* a C-terminal biotinylated Avitag was used. This modified Rev does not form filaments and although confirmation of its association state was not done, the immobilized protein at high dilution was likely to be either monomeric or dimeric and binding to these non-polymeric species was

selected for. In future selections from this library, we will use Rev and mutant variants in other physical states in order to pan for clones binding to other epitopes. The high affinity of Fab SJS-R1, estimated to be ~40 pM, is primarily due to an exceptionally low dissociation rate (Fig. 5). Because our panning protocol¹¹ enriches clones with low dissociation rates it is conceivable that the Fab outcompeted other anti-Rev Fab early in the selection process. Mutant variants of Rev without the epitope of Fab SJS-R1 (Table 1) or epitope masking²⁸ with purified Fab will be used to identify additional chimeric rabbit/human Fab clones.

Antibody fragment-mediated crystallization with high affinity reagents directed at conformation-sensitive epitopes is commonly used to improve crystallization by reducing protein flexibility and providing different surface contacts. This approach has been particularly important for membrane proteins²⁹ but is also potentially useful for protein systems which exhibit physical heterogeneity due to self-association. Such systems may include proteins, as in the present study, that polymerize into filaments of indefinite length. Crystallization conditions may increase the propensity for self-association and antibody fragments which bind to an epitope located at or near protein-protein interaction interfaces may form stable, crystallizable complexes. The rapid depolymerization of Rev by the Fab or scFv clearly indicates binding to N-terminal protein oligomerization sites (Fig. 6) and indicates high affinity interactions. The Fab/Rev complex is monodisperse (Fig. 7) but during protracted sedimentation (>16 hours), some dimerization can occur. For this system, velocity sedimentation analysis (2 hours) appears to give a more reliable mass estimate. Nevertheless, the complex readily formed crystals (Fig. 8) which diffract to ~3 Å (Fig. 9) and have been suitable for structural determination which will be reported elsewhere (Dimattia et al., manuscript in submission).

Anti-HIV-1 therapeutic potential of antibody fragments

Although the primary motivation of the present study was the production of anti-Rev antibody fragments for structural studies, the high affinity and site of binding of Fab and scFv SJS-R1 predict that it may have anti-HIV-1 therapeutic potential. Although high affinity alone cannot be predictive of efficacy we suggest that binding to the Rev epitope, which so effectively blocks Rev oligomerization, is significant. The function of Rev appears to depend on its ability to assemble as a multiprotein complex on the viral RNA targeting sequence RRE (see for example, Jain and Belasco³⁰) Recent work suggests that following Rev binding to the RRE, it assembles into a tetramer and probably higher order complexes by oligomerization one Rev molecule at a time³¹. Direct visualization of the Rev-RRE by atomic force microscopy indicates a complex containing up to 13 Rev molecules³². The binding affinities of the initial Rev-RRE interaction are 0.26 nM with subsequent bindings to tetramer in the range of 0.79-0.48 nM³¹. The Rev-Rev interactions on the RRE complex appear to be of much higher affinity than the simple self-association of Rev of 10 µM per monomer²⁵. From this work, the affinity of both the initial Rev-RNA binding and the subsequent Rev oligomerization are weaker than the sub-nanomolar affinity of the Fab-Rev interaction (Fig. 5). The anti-HIV-1 potential of Fab and scFv SJS-R1 is, thus, based on its blocking the protein-protein interactions that are essential for Rev action. This could be achieved through anti-Rev intracellular immunization³³ with scFv SJS-R1 targeted to the cytoplasm or nucleus. In a previous study, two murine-based anti-Rev scFv, which were targeted to the cytoplasm, inhibited HIV-1 replication in human T-cells and peripheral blood mononuclear cells³⁴. These antibodies had relatively low affinities (0.1-0.01 µM) and bound to epitopes in the C-terminal region of Rev (residues 69-94). Interestingly, Fab SJS-R1 binds to the N-terminal domain of Rev and the C-terminal domain (residues 69-116) is not observed in the Fab/Rev crystal structure being either non-structured or highly mobile (Dimattia et al., manuscript in submission). Effective anti-HIV-1 agents based on anti-Rev intracellular antibodies may not be limited to any particular epitope but very high affinity binding would seem to be a prerequisite attribute. The anti-HIV-1 therapeutic potential of Fab

SJS-R1 is not restricted to intracellular antibodies. The crystal structure of epitope and paratope of the Fab/Rev complex facilitates the computational modeling of lead peptides, peptidomimetics, or other small synthetic molecules that subsequently can be optimized by high-throughput screening of corresponding chemical libraries³⁵. A small synthetic molecule that interferes with Rev action by mimicking Fab SJS-R1 would provide an anti-HIV-1 agent with potential oral bioavailability.

Conclusions

For the first time a Fab with rabbit variable domains and human constant domains derived by phage display has been produced against an HIV-1 protein. The antibody binds with very high affinity to a unique conformational epitope located in the N-terminal half of HIV-1 Rev. Both the Fab and its scFv derivative potently depolymerize Rev filaments indicating that their binding blocks a protein-protein interaction interface. The Fab/Rev complex is stable and readily forms crystals suitable for structural determination. This is also the first example of a chimeric/rabbit human Fab employed as a crystallization chaperone, promising broad utility for such agents in structural studies using X-ray diffraction. Although the anti-HIV-1 activities of the Fab and scFv have not yet been tested, based on their binding properties and the central role that Rev plays in viral infection, they also have therapeutic potential.

Materials and Methods

Preparation of HIV-1 Rev

Rev (clone BH10) was expressed in *E. coli* and purified as previously described²². Protein in 4 M urea was diluted with 6 M urea to a concentration of 69 $\mu\text{g/mL}$ (i.e. below the polymerization concentration of 80 $\mu\text{g/mL}$) and then folded by dialysis against 50 mM sodium phosphate (pH 7), 150 mM sodium chloride, 600 mM ammonium sulfate, 1 mM EDTA, 1 mM DTT at 4 °C. The Rev was then dialyzed extensively against 50 mM sodium phosphate, pH 7.0, 150 mM sodium chloride. The material was sterile-filtered (0.2 μm) and then snap frozen in liquid nitrogen in aliquots at 66 $\mu\text{g/mL}$. A C-terminally truncated form of the protein, Rev $\Delta\text{C60-116}$, was also expressed and purified in a similar manner.

Biotinylation of Rev

Rev with a 14-residue biotin ligase substrate domain (Avitag) appended to its C-terminus³⁶ was expressed in *E. coli* and purified by ion-exchange and gel filtration chromatographies in buffers supplemented with 2 M urea. Biotinylation with biotin ligase (Avidity, LLC) was done according to the manufacturer's protocol. Following the reaction, the protein was gel filtrated on Superdex S200 using 20 mM Tris (pH 7.4) containing 2 M urea. The integrities of the Rev-AviTag and biotinylated proteins were confirmed by mass spectrometry.

Inoculation of rabbits with Rev

All immunization protocols were reviewed and approved by the Animal Care and Use Committees of the NIAID (ASPLI-6) and Spring Valley Laboratories (Sykesville, MD) where the animals were housed and injected. Two rabbits (1QQ174-1 and 1QQ82-1) homozygous for immunoglobulin allotypes V_Ha1 and C _{κ} b9 were bled for serum prior to immunization and seven days after each immunization and each rabbit was immunized with 0.5 mL of 66 $\mu\text{g/mL}$ Rev that was stored frozen as 1.2 mL aliquots for injection of two rabbits. Precautions were taken to not warm the protein when it was mixed with an equal volume of Ribi adjuvant and the mixture was placed on ice. One mL was injected per rabbit. Boosts were then given at 3-week intervals using the same amounts of antigen and Ribi adjuvant. After four boosts, when serum titers had stabilized in both rabbits, a final boost was given and 5 or 6 days later the

rabbits (1QQ174-1; 1QQ82-1) were euthanized and bone marrow and spleens collected and immediately stored in TRIzol (Invitrogen).

Rabbit anti-Rev antibody titers

Antibody titers were monitored by dot blot. Rev was immobilized directly on a 0.45 μ m PVDF membrane at a density of 500 ng per dot. Sera from both animals were diluted serially (10-folds) and antibody levels were determined with an anti-rabbit antibody kit (WesternBreeze, Invitrogen).

Selection of anti-Rev chimeric rabbit/human Fab by phage display

Spleen and bone marrow from both rabbits were processed for total RNA preparation and RT-PCR amplification of rabbit V_K , V_L , and V_H encoding sequences using established primer combinations and protocols¹¹. For each rabbit, rabbit V_L /human C_K /rabbit V_H segments were assembled in one PCR amplification step, digested with *Sfi*I, and cloned into phage display vector pC3C⁹. Electroporation of *E. coli* strain XL-1 Blue (Stratagene) yielded approximately 2×10^8 independent transformants. Based on established protocols¹¹, the library was selected against biotinylated HIV-1 Rev immobilized on streptavidin-coated microtiter plates (Sigma) at 100 ng/well. After four rounds of panning, 11 out of 12 clones that were analyzed by ELISA¹¹ revealed strong binding to biotinylated Rev. One clone was negative. *Alu*I restriction analysis showed that all 11 anti-Rev ELISA-positive clones had the same restriction pattern. The negative clone had a different pattern. *Alu*I restriction analysis was done on 19 additional clones. Thirteen had the same fingerprint as the 11 original positive clones. The six clones with a different fingerprint were all negative. A fifth round of panning did not yield additional positive clones. Six positive clones (three from the fourth and three from the fifth round of panning) were sequenced as described¹¹. The sequences were all identical. Surprisingly, all clones had a stop codon (TAG) at the position of the first codon of the V_H - C_H1 chain, which immediately follows the pelB signal sequence in pC3C (as the Fab was obviously being expressed, the host, *E. coli* strain XL1 Blue, must be suppressing this amber codon through its supE44 genotype).

Expression and purification of anti-Rev Fab and scFv

In order to maximize the expression of the selected anti-Rev Fab, the stop codon was changed, using PCR and the appropriate primers, from TAG (stop) to CAG (glutamine), the customary first codon of the V_H variable domain. A modified ompA- V_K - C_K -pelB- V_H - C_H1 -polyHis cassette (without HA tag) was transferred from pC3C into *E. coli* expression vector pET11a (Novagen) between the *Nde*I-*Bam*HI restriction sites. The anti-Rev Fab V_K and V_H sequences were also cloned into pET11a such that scFv versions of them would be expressed joined by the 18-residue linker GGSSRSSSSGGGGSGGGG, i.e. ompA- V_K -linker- V_H -polyHis. Similar to anti-Rev Fab, these scFv also had a C-terminal polyHis to facilitate purification. The expression plasmids for Fab or scFv production were transfected into *E. coli* strain BL21CodonPlusRIL (Stratagene) and the resulting transfectants fermented in a Biostat B 2-L bench-top fermentor (Braun Biotech) using a glycerol carbon source. Cells were grown at 37 °C and induced with IPTG with a typical cell yield of 50–60 g wet weight/L. The fermentation broth was frozen at –80 °C, thawed, briefly sonicated and then clarified by centrifugation in a JA-10 rotor at 14,000 $\times$ g for 30 minutes. The supernatant was added to ~100 mL of Chelating Sepharose Big Beads resin (GE Healthcare Life Sciences) charged with NiSO₄ and equilibrated with 20 mM sodium phosphate buffer (pH 7.2). The resin was gently mixed for ~30 minutes, filtered through a Buchner funnel, washed with column buffer containing 1 M urea and then packed into a Ni-Sepharose Fast Flow column (3.5 cm diameter $\times$ 10 cm length). After washing the column, the protein was eluted using a 10–500 mM imidazole gradient. Pooled fractions were further purified by gel filtration on a Superdex S200 column (2.6 cm diameter $\times$ 60 cm

length) equilibrated in 20 mM sodium phosphate (pH 7.2), 30 mM sodium chloride and 1 M urea. Column fractions were assayed by reducing (plus DTT) and non-reducing (minus DTT) SDS-PAGE. Note: the anti-Rev Fab is formally identified as Fab SJS-R1 (for simplicity hereafter 'Fab'), and the derived anti-Rev scFv as scFv SJS-R1 (hereafter 'scFv').

Preparation of Fab/Rev immune complexes

The Fab/Rev complex was produced by combining Fab and a 5-fold molar excess of Rev in 1 M Urea. The urea was necessary to prevent the precipitation of Rev. The mixture was dialyzed against PBS, any precipitated protein (usually excess Rev) was removed by centrifugation, and then applied to a Ni-Sepharose Fast Flow column equilibrated in dialysis buffer. The Fab/Rev complex was eluted with imidazole, dialyzed against 20 mM HEPES (pH 8.0) and concentrated to 8–9 mg/mL using an Amicon Ultra-15 10K NMWL centrifugal filter (Millipore). The scFv/Rev complex was produced and purified in a similar manner except that the final dialysis was performed against PBS.

SDS-PAGE and densitometry

Gel electrophoresis was performed using NuPAGE Novex 4–12% polyacrylamide gels (Invitrogen). Gels were scanned and TIF image files analyzed using Kodak Molecular Imaging software V4.0 (Carestream Health, Rochester, NY).

Analytical ultracentrifugation

A Beckman Optima XL-I analytical ultracentrifuge, absorption optics, an An-60 Ti rotor and standard double-sector centerpiece cells were used. Equilibrium measurements were taken at 20 °C and concentration profiles recorded after 16–20 hours at either 12,000–14,500 (Fab/Rev) or 18,000–21,500 (scFv/Rev or scFv) rpm. Baselines were established by over-speeding at 45,000 rpm for 3 hours. Data (the average of five scans collected using a radial step size of 0.001 cm) were analyzed using the standard Optima XL-I data analysis software. Protein partial specific volumes were calculated from the amino acid compositions³⁷ and solvent densities were estimated using the public domain software program SEDNTERP (<http://www.rasmb.bbri.org/>). Sedimentation velocity measurements at 20 °C were taken at 45,000 rpm for 3 hours with data collection at 5–10 minute intervals. Data (radial step size 0.003 cm) was analyzed using the program DCDT+ version 2.2.1²⁶.

Electron microscopy

Fab and Rev filaments prepared as described previously²¹ in cold 50 mM HEPES, 150 mM sodium chloride, 25 mM sodium citrate (pH 7.0) were mixed in equimolar ratio. The mixtures were incubated overnight at 4 °C (conditions under which Rev filament controls remained polymerized). Specimens were applied to 400-mesh carbon-coated copper grids made hydrophilic by air glow discharge and negatively stained with 1% uranyl acetate. Images were recorded with a Philips CM120 electron microscope by CCD at 45,000× magnification.

Surface plasmon resonance

The kinetics of the Fab binding to immobilized Rev was studied by surface plasmon resonance using a Biacore X (GE Healthcare). Rev was immobilized on the surface of a CM5 sensor chip at 1500 RU (1000 RU ~1 ng bound protein/mm²³⁸) by EDC-NHS coupling chemistry according to the manufacturer's protocol. HBS-EP buffer (GE Healthcare) was used as the running buffer and the analyte Fab (90 µl) in running buffer was passed over immobilized Rev at a flow rate of 30 µL/min. Analyte was injected at concentrations between 0.5–500 nM. For all assays, at least two replicate injections at the same concentrations were employed to calculate the kinetic data. Analysis was done using BIAevaluation software, version 3.1. The interaction was globally fitted to a 1:1 interaction Langmuir model with the association and

dissociation phases of the interaction fitted simultaneously. The goodness of fit between the fitted curves and the experimental curves was assessed by visual comparison. The rate constants (k_a and k_d) and the equilibrium constant (K_d) were calculated from the best-fit kinetic parameters (BIAevaluation handbook BR 1002-29, Biacore AB).

Liquid chromatography and mass spectrometry

An HP1100 LC-MS electrospray mass spectrometer (Agilent) coupled to a Zorbax C-3, 2.1 mm diameter $\times$ 15 cm length column was used. Protein (0.1–0.5 mg/mL) was diluted between 1:20 to 1:50 with either H₂O or 1% formic acid and samples (5 μ L) were applied to the column equilibrated in 0.1% formic acid, 5% acetonitrile. The column was washed for 15 minutes with column solvent and then a 35-minute gradient of 5% to 100% acetonitrile in 0.1% formic acid was applied. The flow rate was 0.2 mL/min and gradient eluate was analyzed by mass spectrometry.

Dynamic light scattering

Proteins (1–9 mg/mL) were either centrifuged or filtered prior to analysis and then 10 μ L samples were manually pipetted into a 384 well plate (Greiner Bio-One). Measurements were made at 20 °C using a DynaPro Plate Reader Plus and the data analyzed using Dynamics V6 software (Wyatt Technology Corporation).

Crystallization and data collection

Purified Fab/Rev in 20 mM HEPES (pH 8.0) at a concentration of 12.9 mg/mL was used. Crystallization trials were at 21°C in hanging drops containing 200 nL protein and 200 nL precipitant solution equilibrated against 50 μ L reservoirs in 96-well plates. Over 1600 reservoir conditions were assayed and only one resulted in crystalline nucleations. Microcrystals of Fab/Rev initially grew in 12% PEG 6000, 100 mM di-ammonium phosphate (DAP), and 100 mM Tris-HCl (pH 8.5) and were optimized with a screen varying concentration and pH of the initial reservoir components as well as screening of the initial crystallization condition against the 96-well Hampton Research (Laguna Niguel, CA, USA) additive screen kit. Optimized crystals were grown with Fab/Rev sample at 7.1 mg/mL in 20 mM HEPES, pH 8.0 in 1–2 weeks with 50 mM spermidine added to the drop and in 9–14% PEG 600, 100–200 mM DAP, 100 mM Tris (pH 8.5) and were cryoprotected by a quick pass through reservoir solution supplemented with 25% (v/v) ethylene glycol before flash cryo-cooling in a cold (100 K) stream of nitrogen gas. Diffraction data were recorded from a single Fab/Rev crystal at $\lambda = 0.97960$ Å at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The crystal used for diffraction was grown in 9.5% PEG 6000, 150 mM DAP, 100 mM Tris-HCl (pH 8.5), and 50 mM spermidine. Diffraction data were integrated and scaled using HKL2000 software (HKL Research, Inc.).

Sequence accession numbers

The sequences for the Fab SJS-R1 L- and H-chains have been deposited in GenBank with accession numbers GU223201 and GU223202, respectively.

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Abbreviations used

CDR	complementary determining region
RRE	Rev Response Element
VH	antibody heavy chain variable region
Vκ	antibody kappa light chain variable region

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Figure 1.
Amino acid sequences of the variable domains of Fab SJS-R1. Shown are framework regions and complementary determining regions (CDR) of V_K and V_H .

Figure 2.

Purification of the Fab by gel filtration chromatography. Fab from two cycles of Ni-Sepharose chromatography was applied to a Superdex S200 column (2.6 cm diameter $\times$ 60 cm length) equilibrated with PBS containing 1 M urea. The main protein peak eluted at ~250 mL. The smaller (conductivity) peak eluting at ~290 mL is the salt peak (included volume). Inset (a) a Coomassie stained SDS-PAGE of non-reduced (Oxd) fractions taken across the main peak. The bar at the top corresponds to peak fractions also indicated with a bar in the chromatogram. The main band at ~48 kDa corresponds to oxidized Fab. Inset (b), a Coomassie stained SDS-PAGE of purified Fab either not reduced (Oxd) or reduced (Red) with DTT. In the reduced sample, at the high protein loading employed, the light chain and the heavy chain fragment are not resolved and appear as a single band at ~24 kDa.

Figure 3.

SDS-PAGE of purified Fab, scFv, and immune complexes with Rev. Proteins were either not reduced (a) or reduced (b and c), and then analyzed on 4–12% acrylamide gels and stained with Coomassie dye. Standards (Std) are labeled in units of kDa. Though the Fab in panel (a) is underloaded, it can be seen to be pure by examining the Fab/Rev lanes in panels (a) and (b). The ~30 kDa band marked with an asterisk in panel (a) was identified as an oxidized dimer of Rev (data not shown).

Figure 4.

Molecular weights of the Fab/Rev and scFv/Rev complexes. The molecular weight determinations by sedimentation equilibrium ultracentrifugation analysis are indicated in panels (a) to (c). The protein concentration profiles, represented by UV absorbance gradients at 280 nm, versus the radial distance, are indicated. The solid lines indicate data fitted using a single species model, and open circles represent the experimental data. The upper panels of each graph show the residuals (difference between the fitted and experimental values) as a function of radial position. Panel (d) shows the hydrodynamic radius (R) distribution for the Fab/Rev complex as determined by dynamic light scattering. Monodispersity is indicated by the main species which constitutes 97.9% of the protein. The molecular mass is estimated from the intensity of the scattered light.

Figure 5.

Surface plasmon resonance analysis of Fab binding to immobilized Rev. Duplicate, and in some cases triplicate, injections were made over the concentration range 8–23 nM Fab (right hand ordinate). The binding curves show the very low off-rates. The kinetic parameters k_a and k_d and the dissociation constant determined from these data ($K_d = k_d/k_a \sim 40 \text{ pM}$) are given.

Figure 6.

The Fab depolymerizes Rev filaments. Panel (a) shows an electron micrograph of negatively stained Rev filaments. The filaments have a stain penetrable lumen and are quite long, often extending over entire grid squares. Panel (b) shows that when the Fab and Rev are mixed in equimolar ratio the high affinity Fab binding rapidly depolymerizes the Rev filaments and results in the formation of small, uniformly sized complexes. Bar = 100 nm; 50 nm in the inset.

Figure 7.

Sedimentation velocity centrifugation analysis of the Fab/Rev complex. The Fab/Rev immune complex was centrifuged at 45,000 rpm, 20 °C, with data collection every 10 minutes up to 3 hours. The insert shows protein absorbance at 280 nm as a function of radial distance. The movement of the single boundary toward the bottom of the cell is indicated by the arrow. The main panel shows the sedimentation coefficient distribution derived from data in the insert where the abscissa s^* is the apparent sedimentation coefficient and has units of Svedbergs (S) (1 Svedberg = 1×10^{-13} sec.) The ordinate $g(s^*)$, is the distribution function of apparent sedimentation values, and has units of absorbance units per Svedberg (AU S^{-1}). The solid line is the data fitted to a single species and the points are the actual data. The distribution function, $g(s^*)$, is used to derive the concentration, sedimentation coefficient, and diffusion coefficient²⁶. In the distribution plot the area under each peak gives the total amount of that species.

Figure 8. Crystallization of the Fab/Rev complex. Shown are several Fab/Rev crystals with an enlargement on the right. Crystals usually grew as long rods.

Figure 9.

X-ray diffraction pattern for a crystal of the Fab/Rev complex. Shown is a portion of a 1.0° oscillation diffraction pattern collected at the IO2 beamline at the Diamond Light Source (Didcot, UK). Concentric rings depict the 40.0, 20.0, 8.0, 4.0 and 3.2 Å resolution shells. In the final dataset data are measurable to 3.2 Å, with 6-fold non-crystallographic symmetry redundancy.

Figure 10.

The paratope of Fab SJS-R1. Shown is an isosurface rendering of a Rev monomer, as viewed onto the epitope, with the Rev C α -trace shown as dark grey ribbon and the paratope depicted in gold. Hydrogen bonding is in blue. The area of the Fab/Rev interface is $\sim 720 \text{ \AA}^2$, this large area accounts partially for the exceptionally high affinity of the interaction.

Table 1

Rev epitope residues engaged by the Fab

Rev residue	Buried Surface Area (%)	
	Into Heavy Chain	Into Light Chain
D11	20	30
K14	50	
A15	80	30
L18	90	20
F21	60	
L22	80	20
S25	10	
N26	10	
R48	40	
Q51	40	
I55	50	60
R58	10	80
L59	10	100
S61		10
T62		80
Y63	20	60