Published in final edited form as:

Methods Mol Biol. 2014 ; 1131: 549–561. doi:10.1007/978-1-62703-992-5_35.

Generation and Use of Antibody Fragments for Structural Studies of Proteins Refractory to Crystallization

Stephen J. Stahl, Norman R. Watts, and Paul T. Wingfield

Abstract

With the rapid technological advances in all aspects of macromolecular X-ray crystallography the preparation of diffraction quality crystals has become the rate-limiting step. Crystallization chaperones have proven effective for overcoming this barrier. Here we describe the usage of a Fab chaperone for the crystallization of HIV-1 Rev, a protein that has long resisted all attempts at elucidating its complete atomic structure.

Keywords

Fab; scFv; Phage-display; Crystallization; Chaperone; HIV; Rev

1 Introduction

The method of X-ray crystallography remains the gold standard for determining the structure of macromolecules. The number of protein atomic structures is increasing rapidly, but certain types of proteins remain resistant to this approach due to their inability to form diffraction quality crystals, notably membrane proteins, proteins with a strong propensity to polymerize, and intrinsically disordered proteins. Proteins resistant to the usual approach of simply screening many solution conditions can sometimes be crystallized by more informed means, including site-specific mutations, surface entropy reduction (SER), trimming of flexible regions, addition or insertion of chaperones (fusion with proteins with high crystallization potential), and use of non-covalent chaperones. Several such auxiliary chaperones have been developed, including monoclonal Fab, scFv, camelid single-chain antibodies, DARPINS, and others. Excellent reviews are provided in [1 – 4]. Here we describe methods which result in the crystallization of a protein that has both a strong tendency to polymerize and aggregate and has an intrinsically disordered domain.

Rev is an HIV-1 regulatory protein that mediates the nuclear export of unspliced and partially spliced viral mRNA, thereby initiating the transition to the late phase of viral replication. The 13-kDa protein has two domains: an N-terminal domain, comprising half

© Springer Science+Business Media New York 2014

¹³The protein complex is crystallized without removal of the His-tag. The presence of the tag does not interfere with crystallization. For studies involving the binding of RNA to the complex the tag is removed as polyhistidine can bind nonspecifically to nucleic acid.

¹⁴A single-chain (scFv) version of the anti-Rev Fab is constructed where the anti-Rev V_L and V_H chains are linked with an 18-residue linker. This construct is expressed and purified analogous to the Fab. The anti-Rev scFv also acts as an efficient crystallization chaperone with the advantage that the crystals diffract to higher resolution (DiMattia et al., unpublished observations).

the protein, with a helix-turn-helix structure, and a C-terminal domain that is predicted to be intrinsically disordered (Fig. 1a, b). In vivo, Rev initially interacts with viral mRNAs by binding, via an arginine-rich motif (ARM) in its N-terminal domain, to a highly structured region in the RNA named the Rev response element (RRE) and then oligomerizing in a highly cooperative manner to form a 200–300 kDa ribonucleoprotein complex containing 8–12 Rev monomers (Fig. 2a). Rev binds, via a nuclear export sequence (NES) in its C-terminal domain, to the host protein Crm1 (exportin-1) to direct the mRNA to the cytoplasm in a manner that avoids default splicing. After dissociating from the RNA, Rev binds to importin- β via the nuclear localization sequence (NLS) in its N-terminal domain and returns to the nucleus for further rounds of mRNA export [5 – 7]. More recently it has been recognized that Rev also performs several other functions, including the prevention of multiple viral integration events in the early phase of infection and enhancement of viral translation and genome encapsidation of genomic RNA into virions in the late phase [8 , 9].

In vitro, in the absence of RNA, Rev monomers form dimers at a concentration of $\sim 80 \mu\text{g/ml}$ and then polymerize into long hollow filaments. These filaments have a diameter of $\sim 15 \text{ nm}$ and are of indefinite length [10 , 11]. Such filaments have a strong tendency to associate laterally and form aggregates (Fig. 2b). This association can be readily prevented by the presence of 50 mM citrate ion. However, the formation of the filaments effectively prevents the formation of crystals. Agents such as the divalent cation Mg^{+2} ; poly-anions such as RNA (both cognate and heterologous), DNA, and oligo-glutamate ($n = 8$ or 10); and detergents such as CHAPS cause disassembly to variously sized oligomers, their heterogeneity precluding crystallization. Rev filaments also react rapidly and stoichiometrically with unpolymerized tubulin (or microtubules) to form heterogeneous ring complexes [12] and with tubulin pre-formed into monodisperse rings with the macrolide cryptophycin into homogeneous ring complexes. Both types of Rev–tubulin rings associate laterally in an antiparallel manner. The latter double rings have eightfold symmetry, are exceedingly homogeneous, and readily form square arrays, but they do not stack, and they do not form crystals (our unpublished observations). However, a mutated and truncated form of Rev has been crystallized and the structure solved [13].

Full-length Rev can be crystallized with the aid of a Fab chaperone (Figs. 2c and 3). The rationale and method [14] are summarized as follows. By immunizing rabbits with Rev at concentrations below that at which it polymerizes one can induce antibody production against epitopes otherwise buried in the polymer. Following affinity maturation in the animal one can then screen and further increase the affinity of the corresponding Fab by means of phage display. High-affinity Fab are then rescreened for the ability to form well-behaved soluble complexes with Rev, the complexes are purified and characterized, and then screened for crystallization. In retrospect, with the atomic structure in hand [15], the Fab functions as follows: Rev monomers associate in an antiparallel manner via the A-faces of their helix-turn-helix N-terminal domains and then further associate via their B-faces [13 , 15]. The Fab binds to and blocks the B-face of the Rev dimer, preventing helical polymerization and allowing crystallization (Fig. 1c, d).

It should be noted that whilst the method described here produces material that can yield diffraction quality crystals from the full-length Rev protein, only the N-terminal domain is

ordered—the C-terminal domain remains disordered. We are investigating other approaches to resolve this issue. These include rescreening the above libraries for Fab that can bind and potentially order the C-terminal domain, forming complexes with Rev's normal binding partner proteins, and further exploiting by NMR and electron microscopy the order present in the Rev polymers themselves.

2 Materials

2.1 Rev Folding

1. Folding buffer 1: 50 mM sodium phosphate, 150 mM sodium chloride, 1 mM DTT, 1 mM EDTA, 600 mM ammonium sulfate, pH 6.8.
2. Folding buffer 2: 50 mM sodium phosphate, 150 mM sodium chloride, 1 mM DTT, 1 mM EDTA, 10 mM ammonium sulfate, pH 6.8.
3. Millex-GS 0.2 μ m filter units (Millipore).

2.2 Rabbit Immunization

1. Ribi Adjuvant System (RAS) is a stable oil-in-water emulsion that may be used as an alternative to the water-in-oil emulsions (Sigma Aldrich).
2. TRIzol Reagent (Invitrogen).

2.3 RNA Isolation

1. 1-Bromo-3-chloro-propane (BCP; Molecular Research Center).
2. RNA storage buffer: RNase-free 1 mM sodium citrate (pH 6.4) (Life Technologies/Ambion).

2.4 RT-PCR Antibody V_L and V_H Gene Library Preparation

1. SuperScriptII PCR Kit: RT enzyme mix. This includes SuperScriptIII RT and reaction mixture; oligo(dT)₂₀ random hexamers; dNTPs and *E. coli* RNase H (Invitrogen).
2. Qiagen MinElute Gel Extraction Kit (Qiagen).

2.5 Assembly and Cloning of the Fab Gene Library

1. Qiagen PCR Purification Kit (Qiagen).
2. XL1-Blue cells (Stratagene).
3. PEG 8000: Polyethylene glycol average molecular weight 8 kDa (Sigma-Aldrich).

2.6 Selection Anti-Rev Fab Clones by Phage Display

1. Streptavidin magnetic beads (Dyna).
2. TBS: Tris buffered saline (Quality Biologics Inc.).
3. PBS: Phosphate buffered saline (Life Technologies).

4. LB Broth (Quality Biologics Inc.).
5. IPTG: Isopropylthio- β -galactoside (Life Technologies).
6. Peroxidase-conjugated goat anti-human IgG (Jackson Laboratories).

2.7 Production of Anti-Rev Fab

1. Ni-Sepharose 6 Fast Flow (GE Healthcare).
2. Amicon stirred ultrafiltration cell (Millipore): Model 8200, 200 ml (63.5 mm membrane), or Model 8400, 400 ml (76 mm membrane). Ultrafiltration Disc (membranes), PM30, Amicon, 30 kDa NMWL (Millipore).

2.8 Crystallization and Structural Determination

Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane (Millipore).

3 Methods

3.1 Expression and Purification of HIV-1 Rev (See Note 1)

1. If Rev has been stored frozen, thaw the tube rapidly under warm water.
2. Add additional solid urea to a final concentration of 6 M and DTT to a final concentration of 5 mM. Adjust the protein concentration to 1 mg/ml.
3. Fold the protein by diluting with an equal volume of cold folding buffer 1, and then dialyzing overnight at 4 °C versus a large volume (1:1,000) of folding buffer 1, and then remove the ammonium sulfate by two sequential dialyses against folding buffer 2.
4. Confirm successful folding of Rev by monitoring α -helical content by far-UV circular dichroism [10] and the formation of long, regular filaments by negative-stain electron microscopy [11].
5. Sterile-filter with a 0.2 μ m Millex-GS filter unit, dilute the Rev to below 80 μ g/ml, and snap freeze 0.5 ml aliquots in liquid nitrogen (see Note 2).

3.2 Rabbit Immunization

1. Inoculate rabbits with 0.5 ml of 66 μ g/ml purified Rev protein mixed with an equal volume of Ribi Adjuvant.
2. Give boosts at 3-week intervals with the same amount of antigen and adjuvant.
3. After four boosts and when serum titers have stabilized in the rabbits, a final boost is given.

¹The expression in *E. coli* and purification of HIV-1 Rev are described in [10]. Rev has 115 residues and a mass of 12,905 Da.

²Rev folds and polymerizes well at 0.5 mg/ml, forming long filaments. As the protein concentration is increased nucleation will increase and the filaments will become short, making visual assessment of successful folding by microscopy more difficult. The filaments are very prone to lateral aggregation and massive precipitation, but this can be prevented by the inclusion of 25–50 mM sodium citrate in all buffers and by keeping the protein cold at all times. The extinction coefficient is 0.7 at 280 nm for a 1 mg/ml solution.

4. Euthanize the rabbits 5 or 6 days later, and collect the bone marrow and spleens. Store in TRIzol at -80°C .

3.3 RNA Isolation

1. Homogenize the spleens and bone marrow separately in 30 ml of TRIzol and then centrifuge at $2,500 \times g$ at 4°C .
2. Mix the supernatants with 3 ml of BCP and, after standing 15 min at room temperature, centrifuge at $17,500 \times g$ for 15 min at 4°C .
3. Transfer the upper aqueous phase to a fresh centrifuge tube and add 15 ml isopropanol.
4. After 10 min at room temperature, centrifuge at $17,500 \times g$ for 10 min at 4°C .
5. Discard the supernatant and add 30 ml of 70 % ethanol. Centrifuge at $17,500 \times g$ for 10 min at 4°C .
6. Discard the supernatant, and air-dry the pellets for ~5 min. Dissolve the pellets in 250 μl RNA storage buffer.
7. Determine the RNA concentration by measuring $A_{260\text{ nm}}$ ($1\text{ OD}_{260} = 40\text{ }\mu\text{g/ml}$).

3.4 RT-PCR Antibody V_L and V_H Gene Library Preparation

1. Dilute the RNA to 20 μg in a final volume of 32 μl .
2. Add 4 μl of a solution at 2.5 mM of each of the four dNTPs and 4 μl 50 μM Oligo (dT)₂₀.
3. Add 40 μl of SuperScriptIII 2 $\times$ RT reaction. Mix (8 μl 10 $\times$ reaction buffer, 16 μl 25 mM MgCl_2 , 8 μl 0.1 M DTT, 4 μl 40 units/ μl RNaseOUT, 4 μl 200 units/ml SuperScriptIII RT).
4. Incubate for 50 min at 50°C and then 5 min at 85°C .
5. Cool on ice, then add 4 μl of 2 units/ μl RNase H, and incubate for 20 min at 37°C .
6. The rabbit immunoglobulin V_L and V_H genes are PCR amplified from this SS DNA library using 11 primer pairs (see **Note 3**).
7. The PCR products are purified with 1 % agarose gel electrophoresis and eluted using the Qiagen MinElute Kit.

3.5 Assembly and Cloning of the Fab Gene Library

1. Combine the collection of V_L and V_H gene fragments with the C_K containing stuffer fragment. The primers used to amplify the 3' end of the V_L gene fragments and the 5' end of the V_H gene fragments in Subheading 3.4 are designed to overlap with the ends of the C_K coding region. PCR primers that contain an *Sfi*I restriction site and overlap with the 5' end of the V_L gene fragments or the 3' V_H gene

³See Table 1 : described in detail [17]: see Chapter 9, Protocol 9.6.

fragments are used to generate large *SfiI* restriction fragments comprising the V_L – C_K – V_H coding sequences (*see Note 4*).

2. Purify the V_L – C_K – V_H PCR products using the Qiagen PCR Purification Kit, then digest with *SfiI*, and then separate as above with 1 % agarose gel electrophoresis.
3. Ligate these purified *SfiI* DNA fragments into phage display vector pC3C. (This vector provides the coding sequence for C_H , resulting in a complete V_L – C_K – V_H – C_H coding sequence as well as the regions needed for expression and transport of the Fabs.) Transfect the ligation mixture into XL-1-Blue cells using electroporation.
4. Use these transfected cells to prepare 200 ml phagemid prep, using VCSM helper phage (*see Note 4*).
5. Harvest the phagemid from the culture supernatant with PEG 8000 precipitation (*see Note 4*).

3.6 Selection of Anti-Rev Fab Clones by Phage Display

1. Mix 1 ml of the phagemid prep with 20 μ l of 0.25 mg/ml biotinylated Rev (*see Note 5*). Incubate for 1 h on a roller at 37 °C.
2. Wash streptavidin magnetic beads (100 μ l/1 mg) 3 $\times$ with 100 μ l 1 % BSA in TBS.
3. Add the phagemid/Rev mixture to the beads and continue rolling at 37 °C for 1 h.
4. Isolate the beads with a magnet and wash 5–10 $\times$ at 5 min per wash on the roller at 37 °C with TBS + 0.05 % Tween-20.
5. Isolate the phage bound to the beads by incubating with 50 μ l of 10 mg/ml trypsin in PBS for 30 min at 37 °C.
6. Use this “panned” preparation of phagemid to transfect 2 ml of log-phase XL1-Blue cells in SB. Grow this culture, and continue with another four more rounds of panning as described (*see Note 4*).
7. Isolate individual colonies, inoculate 2 ml of LB broth, and shake at 37 °C until the culture is slightly turbid. Add 20 μ l of 0.1 M IPTG, and continue shaking overnight. Freeze and thaw the cultures, and then remove the bacteria with centrifugation for 10 min at 4,000 $\times g$.
8. To identify anti-Rev Fab-producing clones, assay the culture supernatants with ELISA. Microtiter plate wells are coated with Rev, washed with H_2O , and then incubated with blocking solution (5 % milk powder in PBS). Dilute the culture supernatants 1:1 with blocking solution and add to the Rev-coated wells. After 3 h

⁴Described in detail by [18].

⁵Biotinylation of Rev: A 14-residue peptide biotin ligase substrate domain (Avitag: GGGLNDIFEAQKIEWHE) is appended to the C-terminus [19], 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) is done according to the manufacturer's protocol. Following the reaction, the protein is gel filtrated on Superdex S200 using 20 mM Tris–HCl, pH 7.4, containing 2 M urea. The proteins are characterized by mass spectrometry.

at 37 °C, wash the wells with H₂O and add peroxidase- conjugated goat anti-human IgG (anti-human IgG is used as the constant regions in these Fabs are derived from human IgG). After another 1 h of incubation, wash the plates and develop with a peroxidase substrate.

3.7 Production of Anti-Rev Fab

1. Subclone the bi-cistronic coding sequence (OmpA leader-V_L - C_L and pelB leader-V_H -C_H -polyHis) of the identified anti-Rev Fabs into *E. coli* expression vector pET11a [16].
2. Grow cells transfected with the pET11-RevFab plasmid (see Fig. 4) in 1 L of LB broth, and induce Fab production with the addition of IPTG to 1 mM (see **Note 6**).
3. After 3–5 h of IPTG induction, freeze the cultures at –80 °C.
4. Thaw the culture, centrifuge at 11,000 × *g* for 20 min, and add the supernatant to ~75 ml Ni-Sepharose 6 Fast Flow equilibrated with PBS. Gently shake for 30 min at 4 °C.
5. Collect the resin with a sintered glass funnel and wash with 250 ml PBS and then with 300 ml 50 mM imidazole in PBS.
6. Elute the Fab with 250 ml of 0.5 M imidazole in PBS. Concentrate to about 25 ml using an Amicon stirred ultrafiltration cell and dialyze against PBS. The yield is typically about 10 mg (see **Notes 7** and **8**).

3.8 Rev:RevFab Complex Formation and Purification

1. Combine 10 mg of RevFab (in 25 ml PBS) with 6 mg Rev (in 10 ml 50 mM Tris, 20 mM ammonium sulfate, 5 mM DTT, 2 M urea, pH 7.5) (see **Note 9**).
2. Incubate for 30 min at 4 °C, and then dialyze against PBS at 4 °C.
3. Remove any precipitate (mostly excess Rev) by centrifugation at 18,000 × *g* for 15 min, and then load the supernatant on a Ni-Sepharose 6 Fast Flow column (1.6 × 0.8 cm). Wash with 25 ml of PBS and then with 50 ml of 50 mM imidazole in PBS.
4. Elute the Rev:RevFab complexes with 0.5 M imidazole in PBS, collecting 10 ml fractions.
5. Monitor the fractions with SDS-PAGE, and combine the fractions containing the Rev:RevFab complex (Fig. 5).
6. Dialyze the pooled fractions against 20 mM Hepes, pH 8.0.

⁶Cells are grown using a 1-L shake flask or as described [14] using a Biostat B 2-L bench top fermentor (Braun Biotech) using minimal media and a glycerol carbon source. Cells were grown in the fermentor at 37 °C and induced with IPTG with a typical cell yield of 50–60 g/L.

⁷Further purification, if required, can be performed by gel filtration using Superdex S200. Urea (1 M) was included in the PBS column buffer to increase the solubility of the Fab [14].

⁸The sequences for the Fab L- and H-chains have been deposited in GenBank with accession numbers GU223201 and GU223202, respectively.

⁹This is a molar ratio of ~2 Rev:RevFab.

3.9 Crystallization and Structural Determination

1. Characterize the protein complex using some of the biochemical and biophysical methods described in [14] (*see Note 10*).
2. Concentrate the Rev:RevFab complex to ~10 mg/ml using a Millipore Ultra-15 centrifugal concentrator (*see Note 11*).
3. Remove any precipitate by centrifugation at $12,000 \times g$ for 10 min.
4. Crystallize the protein complex as described by [15] (*see Notes 12 – 15*).

Acknowledgments

This work was supported by the Intramural Research Program of NIAMS, National Institutes of Health.

References

1. Derewenda ZS. Application of protein engineering to enhance crystallizability and improve crystal properties. *Acta Crystallogr D Biol Crystallogr*. 2010; 66(Pt 5):604–615. doi: 10.1107/S090744491000644X. [PubMed: 20445236]
2. Koide S. Engineering of recombinant crystallization chaperones. *Curr Opin Struct Biol*. 2009; 19(4): 449–457. doi: 10.1016/j.sbi.2009.04.008. [PubMed: 19477632]
3. Kim J, Stroud RM, Craik CS. Rapid identification of recombinant Fabs that bind to membrane proteins. *Methods*. 2011; 55:303–309. [PubMed: 21958987]
4. Lieberman RL, Culver JA, Entzminger KC, Pai JC, Maynard JA. Crystallization chaperone strategies for membrane proteins. *Methods*. 2011; 55(4):293–302. doi: 10.1016/j.ymeth.2011.08.004. [PubMed: 21854852]
5. Pollard VW, Malim MH. The HIV-1 Rev protein. *Annu Rev Microbiol*. 1998; 52:491–532. [PubMed: 9891806]
6. Cullen BR. Nuclear mRNA export: insights from virology. *Trends Biochem Sci*. 2003; 28(8):419–424. doi: 10.1016/S0968-0004(03)00142-7. [PubMed: 12932730]
7. Strebel K. Virus-host interactions: role of HIV proteins Vif, Tat, and Rev. *AIDS*. 2003; 17(Suppl 4):S25–S34. [PubMed: 15080177]
8. Groom HC, Anderson EC, Lever AM. Rev: beyond nuclear export. *J Gen Virol*. 2009; 90(Pt 6): 1303–1318. doi: 10.1099/vir.0.011460-0. [PubMed: 19321757]
9. Grewe B, Uberla K. The human immunodeficiency virus type 1 Rev protein: menage à trois during the early phase of the lentiviral replication cycle. *J Gen Virol*. 2010; 91(Pt 8):1893–1897. doi: 10.1099/vir.0.022509-0. [PubMed: 20538907]
10. Wingfield PT, Stahl SJ, Payton MA, Venkatesan S, Misra M, Steven AC. HIV-1 Rev expressed in recombinant *Escherichia coli*: purification, polymerization, and conformational properties. *Biochemistry*. 1991; 30:7527–7534. [PubMed: 1854752]
11. Watts NR, Misra M, Wingfield PT, Stahl SJ, Cheng N, Trus BL, Steven AC, Williams RW. Three-dimensional structure of HIV-1 Rev protein filaments. *J Struct Biol*. 1998; 121(1):41–52. [PubMed: 9573619]
12. Watts NR, Sackett DL, Ward RD, Miller MW, Wingfield PT, Stahl SS, Steven AC. HIV-1 rev depolymerizes microtubules to form stable bilayered rings. *J Cell Biol*. 2000; 150(2):349–360. [PubMed: 10908577]

¹⁰Rev binds to the Fab with a 1:1 molar stoichiometry and with a very high affinity ($K_d < 10^{-10}$ M).

¹¹The complex in this buffer can be concentrated to about 10 mg/ml, whereas in PBS the complex starts to precipitate above concentrations of ~1 mg/ml.

¹²The methods used for protein crystallization and structure determination are beyond the scope of this chapter. Some of the research tools and methods used can be found at <http://www.hamptonresearch.com>.

¹⁵The structure of the Rev–Fab complex has the PDB accession code 2X7L.

13. Daugherty MD, Liu B, Frankel AD. Structural basis for cooperative RNA binding and export complex assembly by HIV Rev. *Nat Struct Mol Biol.* 2010; 17(11):1337–1342. doi: 10.1038/nmsb.1902. [PubMed: 20953181]
14. Stahl SJ, Watts NR, Rader C, DiMattia MA, Mage RG, Palmer I, Kaufman JD, Grimes JM, Stuart DI, Steven AC, Wingfield PT. Generation and characterization of a chimeric rabbit/human Fab for co-crystallization of HIV-1 Rev. *J Mol Biol.* 2010; 397(3):697–708. doi: 10.1016/j.jmb.2010.01.061. [PubMed: 20138059]
15. Mattia MA, Watts NR, Stahl SJ, Rader C, Wingfield PT, Stuart DI, Steven AC, Grimes JM. Implications of the HIV-1 Rev dimer structure at 3.2Å resolution for multi-meric binding to the Rev response element. *Proc Natl Acad Sci U S A.* 2010; 107(13):5810–5814. doi:0914946107 [pii] 10.1073/pnas.0914946107. [PubMed: 20231488]
16. Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol.* 1986; 189(1):113–130. [PubMed: 3537305]
17. Andris-Widhopf, J.; Steinberger, P.; Fuller, R.; Rader, C.; Barbas, CF. Generation of antibody libraries: PCR amplification and assembly of light- and heavy-chain coding sequences.. In: Barbas, CF.; Burton, DR.; Scott, JK., editors. *Phage display: a laboratory manual.* Cold Spring Harbor; New York: 2001.
18. Rader C. Generation and selection of rabbit antibody libraries by phage display. *Methods in molecular biology.* 2009; 525:101–128. xiv. doi: 10.1007/978-1-59745-554-1_5. [PubMed: 19252834]
19. Beckett D, Kovaleva E, Schatz PJ. A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein Sci.* 1999; 8(4):921–929. doi: 10.1110/ps.8.4.921. [PubMed: 10211839]

Fig. 1.

HIV-1 Rev. (**a**) Functional domains of Rev include two oligomerization sequences, an arginine-rich motif (ARM) that partially coincides with a nuclear localization sequence (NLS), and a nuclear export sequence (NES). Residues 9 to ~65 are ordered, the C-terminal domain is predicted to be intrinsically disordered. (**b**) The helix-turn-helix structure of the N-terminal domain, shown as the dimer observed by crystallography. (**c**) Dimers associate via AA interactions and are proposed to oligomerize via BB interactions. (**d**) Fab-Rev complex

Fig. 2.

Assemblies of Rev. (**a**) In vivo, Rev monomers associate with the viral mRNA, first at the site of the Rev response element (RRE) and then at secondary sites, usually up to 10–12 monomers. In vitro, in the presence of heterologous RNA, Rev can form irregular filaments with a length proportional to the mass of the RNA. (**b**) In vitro, in the absence of RNA, Rev forms dimers that polymerize into regular hollow filaments. Such filaments are of indeterminate length and tend to aggregate, preventing protein crystallization. (**c**) Rev monomers can associate in an antiparallel manner via the A-faces of their helix-turn-helix N-terminal domains and then further associate via their B-faces to form polymers. When Rev is mixed with a monoclonal Fab directed against the B-face of the N-terminal domain a 1:1 Rev–Fab complex forms and Rev can no longer polymerize. These Rev–Fab complexes can then associate via Fab:Fab interactions and thus be crystallized, as can the corresponding Rev–scFv complexes

Fig. 3.
Flow diagram for crystallizing Rev with a Fab chaperone

Fig. 4.

A single T7 promoter drives synthesis of both the Fab light-chain *V_L*-*C_L* (*white*) and heavy-chain fragment *V_H*-*C_H* (*grey*). Transport of both chains to the periplasm is mediated by the leader peptides *ompA* and *pelB*. A His-tag (H6) on C-terminus of *V_H*-*C_H* is used for affinity purification

Fig. 5. SDS-PAGE of affinity purification of Rev-Fab complex. (Lane 1) molecular weight standards, (Lane 2) Rev, (Lane 3) Rev-Fab mixture applied to Ni-affinity column, (Lane 4) low-imidazole wash, (Lane 5) Rev-Fab complex eluted with high-imidazole buffer

Table 1

Primer pairs for chimeric rabbit/human Fab libraries

	5' Sense primer	3' Sense primer	Product
1	RSCλ1	RHybL-B	V _λ
2	RSCVK1	RHybK1-B	V _K
3	RSCVK1	RHybK2-B	V _K
4	RSCVK1	RHybK3-B	V _K
5	RSCVK2	RHybK1-B	V _K
6	RSCVK2	RHybK2-B	V _K
7	RSCVK2	RHybK3-B	V _K
8	RHyVH1	RHyIgGCH-1B	C _H
9	RHyVH2	RHyIgGCH-1B	C _H
10	RHyVH3	RHyIgGCH-1B	C _H
11	RHyVH4	RHyIgGCH-1B	C _H

These are chimeric Fabs and have variable regions from immunized rabbits and light- and heavy-chain constant regions from cloned human Fab.
 See [17], Chapter 9, Protocol 9.6