Protein structure and oligomerization are important for the formation of export-competent HIV-1 Rev–RRE complexes

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Abstract

The translation of the unspliced and partially spliced viral mRNAs that encode the late, structural proteins of HIV-1 depends on the viral-protein Rev. Oligomeric binding of Rev to the Rev response element (RRE) in these mRNAs promotes their export from the nucleus and thus controls their expression. Here, we compared the effects of hydrophobic to hydrophilic mutations within the oligomerization domain of Rev using assays for oligomeric RNA binding, protein structure, and export from the nucleus. Oligomeric RNA binding alone does not correlate well with RNA transport activity in the subset of mutants. However, protein structure as judged by CD spectroscopy does correlate well with Rev function. The oligomeric assembly of Rev-L18T is impaired but exhibits minor defects in structure and retains a basal level of activity *in vivo*. The prevalence of L18T in infected individuals suggests a positive selection mechanism for L18T modulation of Rev activity that may delay the onset of AIDS.

Keywords: HIV-1; Rev; RRE; nuclear export; viral latency

The Rev protein of HIV-1 controls gene expression by promoting the nuclear export of unspliced and partially spliced viral mRNA (Malim et al. 1989b; Pollard and Malim 1998; Hope 1999). Rev recognizes these mRNAs through both the binding of an arginine-rich RNA binding domain (RBD) to a specific binding site within the Rev

response element (RRE) (Malim et al. 1989a; Bogerd and Greene 1993) and the self-association of Rev monomers into higher-order oligomeric complexes on the RRE (Malim et al. 1989a; Bogerd and Greene 1993). It is this strong tendency to self-associate that has thwarted attempts to obtain structural information of full-length Rev at the atomic level. Understanding the essential contribution made by Rev oligomerization to influence the course of the viral infection (Malim and Cullen 1991; Madore et al. 1994; Mann et al. 1994) has been hampered by the resulting lack of detailed structural information on the oligomeric complexes. Many questions concerning the connection between Rev structure and oligomeric Rev–RRE assembly (Blanco et al. 2001; Havlin et al. 2007), the self-association tendency of Rev in the absence of its cognate RRE (Wingfield et al. 1991; Cole et al. 1993; Havlin et al. 2007), and the observation of Rev variants with

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Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immune deficiency syndrome; Rev, regulator of expression of virion proteins; RBD, RNA binding domain; RRE, Rev response element; CRM1, chromosome region maintenance 1; CD, circular dichroism; NES, nuclear export signal.

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similar assembly properties on the RNA but profoundly different levels of *trans*-activation activity (Thomas et al. 1997; Brice et al. 1999; Trikha and Brighty 2005; Churchill et al. 2007) remain to be answered.

Extensive mutagenesis studies (Malim and Cullen 1991; Madore et al. 1994; Thomas et al. 1998; Jain and Belasco 2001) have been used to map the oligomerization domain of Rev to sequences that flank the RBD, and both circular dichroism (CD) (Auer et al. 1994) and solid-state nuclear magnetic resonance (NMR) (Blanco et al. 2001) studies support a structural model that the combined oligomerization domain and RBD form an N-terminal amphipathic helix-turn-helix motif. Jain and Belasco (2001) used a combination of mutagenesis data, *in vitro* Rev–RRE assembly assays, and the putative N-terminal helix-turn-helix structural motif to propose a detailed model for the intermolecular interactions between Rev monomers bound to the RRE. In this model, multiple Rev proteins interact symmetrically through two distinct hydrophobic interfaces (i.e., “head” and “tail”) within the oligomerization domain (Fig. 1). Based on this framework, we analyzed the effects of a set of mutations on the protein structure using CD-spectroscopy and the functional consequences on the *trans*-activation activity of Rev using a Rev-dependent *in vivo* gene expression assay. The mutations V16D, L18Q, I55N, and L60R represent replacements of hydrophobic by hydrophilic residues. All four Rev variants show a gradient of oligomerization deficiencies.

Interestingly, the mutation-dependent changes in the CD spectra can be used to group the Rev variants into distinct classes that exhibit distinct *trans*-activation activities. In contrast, mutations that are nearly equally efficient at *in*

vitro assembly on the RRE have markedly different levels of *trans*-activation activity. The combined observations support the hypothesis that Rev function is dependent on both the ability of Rev oligomers to adopt a defined structure and the ability of Rev to oligomerize on the RRE.

Our search of the available HIV-1 Rev sequences isolated from patients revealed the frequent occurrence of the hydrophobic-to-hydrophilic L18T mutation ([Novitsky et al. 2002] www.hiv.lanl.gov). A threonine at the originally proposed tail position eighteen (Jain and Belasco 2001) reduces, but does not eliminate, *trans*-activation in our reporter construct assay. Previous studies have linked reduced *trans*-activation by Rev to both the viral latency period and escape from the immune system (Pomerantz et al. 1990; Iversen et al. 1995; Hua et al. 1996; Bobbitt et al. 2003). We suggest that modulation of Rev activity through changes in the oligomerization domain confers a positive selection advantage that might contribute to the slow progression of AIDS.

Results

Mutations of the Rev oligomerization domain result in a variety of Rev–RRE assembly defects

The formation of specific complexes between radiolabeled RRE RNA and Rev variants was monitored by an electrophoretic gel mobility shift assay over a range of protein concentrations. Head (V16D and L60R) and tail (L18Q and I55N) mutants previously described by Jain and Belasco (2001) were chosen for study and the related mutant, L18T, was selected because of its prevalence in

Figure 1. Model for oligomeric assembly of Rev on the RRE. (A) A model of stepwise binding was proposed based on the observation of oligomerization deficient mutants (Jain and Belasco 2001). These states of the model include free Rev, Rev monomer bound, Rev dimer bound, and Rev oligomer bound RRE. Tail mutants arrested at the monomer bound state and were proposed to interrupt the tail oligomerization interface (dark gray). Head mutants arrested at the dimer bound state and were proposed to interrupt the head oligomerization interface (white). (B) Tail and head mutations were mapped onto a helical wheel model of the N-terminal helix-turn-helix motif of the oligomerization domain.

clinical isolates (*vide infra*). Representative gels and corresponding nonlinear least square curve fits using a three-step sequential Rev–RRE assembly model are presented in Figure 2A, with the three observed K_d values summarized in Table 1. These gels exhibit discrete bands

at lower protein concentrations associated with monomeric and dimeric complex formation and poorly resolved bands at higher protein concentration indicative of higher-order oligomers.

Binding of the L60R Rev variant resembles wild-type Rev binding very closely with no observable difference in K_{D1} values, and K_{D2} and K_{D3} values increasing less than threefold. The first Rev molecule of all remaining variants bound with a lower affinity than wild type (i.e., K_{D1} is larger), with I55N and V16D being more affected than variants bearing mutations at position eighteen (i.e., L18T and L18Q). The binding of L18T and L18Q Rev variants was qualitatively similar, with differences from the determined wild-type K_{D1} values of less than threefold and fivefold, respectively. Interestingly, there is no distinguishable difference in K_{D2} between L60R and L18T, and only a fivefold difference in K_{D2} between L60R and L18Q. Based on the model in Figure 1A, head mutants V16D and L60R should exhibit specific increases in K_{D3} , while tail mutants L18Q and I55N should exhibit specific increases in K_{D2} . The I55N and V16D mutations sharply increased all three stepwise binding constants. For these variants, the K_{D1} values increase by over 7- and 12-fold, respectively.

In the data fitting analysis (Fig. 2B), systematic deviations in the residuals of the fits are observed. This is related to the oversimplification of treating the third binding step (K_{D3}) as a single binding step. Including additional binding steps and K_D values to fit the poorly resolved species in the higher-order band does improve the fits to the data. However, because of the poor resolution of these species on the gels, the estimates of the first three binding constants are not improved. The imperfection of the model does not mask the clear differences in binding that distinguish wild type from the mutated variants or the differences that distinguish I55N and V16D from the other variants.

Defects in Rev–RRE assembly and reporter gene expression are not directly correlated

The effects of the head and tail mutants on Rev function in human cells were not previously determined (Jain and Belasco 2001). To determine how the assembly defects affect Rev function, a reporter construct was prepared in which Gag expression in transiently transfected HeLa cells is dependent on functionally active Rev protein (Fig. 3A). The reporter plasmid carries the HIV-1 Gag/protease coding region under the control of a CMV promoter fused to a segment from the 3'-end of the HIV-1 genome that includes the RRE embedded within a portion of the *Env* gene (Wodrich et al. 2000). The transcript contains a 5' splice donor site but not a splice acceptor site, so it accumulates in the nucleus in the absence of Rev. When cells are cotransfected with plasmids encoding wild-type or mutant

Figure 2. In vitro assembly of HIV-1 Rev variants on the RRE. (A) Radiolabeled RRE RNA was titrated with purified variants of HIV-1 Rev at six different concentrations (left to right 6, 18, 60, 166, 500, and 1500 nM). Free RNA (green), monomeric Rev (black), dimeric Rev (red), and oligomeric Rev (purple) bound RRE radioactive bands are labeled. (B) Nonlinear least squares fits for the binding data to a model of stepwise binding are also shown. Fraction of RRE RNA bound by Rev at each binding step is plotted as a function of the log Rev concentration. Data points represent the measured percent of the total RRE RNA in each state. Error bars on the data points represent the standard deviation from at least three experiments. Solid curves represent the fits to the data. Each state is color coded to match the corresponding band on the gels. Systematic errors in the residuals result from the error introduced by correcting for background over gel areas of multiple sizes.

Table 1. Quantitative analysis of binding of HIV-1 Rev oligomerization domain variants to the RRE

	Value $\times 10^{-9}$	Error $\times 10^{-9}$
Wild type		
K _{D1}	40	20
K _{D2}	70	50
K _{D3}	80	60
L60R		
K _{D1}	40	10
K _{D2}	170	80
K _{D3}	200	200
L18T		
K _{D1}	100	20
K _{D2}	170	40
K _{D3}	400	100
L18Q		
K _{D1}	200	50
K _{D2}	300	100
K _{D3}	600	200
I55N		
K _{D1}	290	60
K _{D2}	900	300
K _{D3}	600	300
V16D		
K _{D1}	490	60
K _{D2}	2300	600
K _{D3}	1600	900

Rev constructs, the level of Rev-dependent nuclear export can be monitored by Gag immunostaining, which reflects translation of exported mRNA. In addition, HeLa cells were also transfected with a control plasmid expressing the *trans*-dominant repressor RevM10 mutant (Malim and Cullen 1991) with an impaired nuclear export signal (NES).

The results of immunofluorescence staining of Gag in cells cotransfected with the reporter construct and with plasmids encoding either wild-type or mutant Rev are shown in Figure 3B. Anti-Rev Western blotting of the transfected cell lysates (Fig. 3C) indicates that the Rev mutants are expressed at the same or higher levels than wild-type Rev in all cases. Gag expression is highly responsive to cotransfection with a plasmid that encodes wild-type Rev, as evidenced by intense Gag immunostaining (Fig. 3B). The oligomerization mutants, L60R, I55N, and V16D, produced little to no detectable stimulation of Gag expression in the export assay. The L60R and I55N Rev variants are inactive showing less than 2% of wild-type Rev activity while V16D is severely impaired (4% of wild-type Rev activity). In contrast, cells expressing both L18T and L18Q variants displayed measurable but reduced Gag expression (Fig. 3D). Both variants carrying mutations at position 18 are moderately impaired, exhibiting 24% (L18T) and 12% (L18Q) of wild-type Rev activity. Given the high degree of oligomeric binding of L60R, L18T, and L18Q on the RRE (Fig. 2), this result

clearly demonstrates the lack of a simple correlation between oligomeric assembly on the RRE and export function.

Point mutations within the oligomerization domain change the conformation of Rev oligomers

The CD spectra of Rev and Rev variants (Fig. 4) show characteristic double minima at 208 nm and 222 nm consistent with α -helical protein conformations (Greenfield 1996). The 222 nm/208 nm ratio relates to both the arrangement between helices in proteins containing helical bundles and to unfolded residues (Andersen et al. 1996; Greenfield 1996; Zhai et al. 2002), and the 222 nm/208 nm ratio for the wild type and Rev variants are reported in Table 2. In terms of 222 nm/208 nm ratio, L18T adopts a wild-type-like conformation. All other Rev variants analyzed exhibit altered secondary structure relative to the wild-type protein. Amino acid specific effects became apparent when we compared the L18Q and L18T mutations. A glutamine at position 18 alters the CD spectrum more than a threonine, but L18Q is not among the most disruptive of the mutations tested. In terms of their 222 nm/208 nm ratio, the L60R, V16D, and I55N variants are less structured than either the wild-type or position 18 variants of Rev.

Variation within Rev sequences indicates widespread diversity in Rev activity

The natural sequence diversity of the Rev oligomerization-mutant positions studied in this work has not been previously analyzed. The consensus sequence for the Rev protein was calculated using the alignment of sequences isolated from infected patients available from the Los Alamos HIV sequence database (Fig. 5). In addition to the RBD and oligomerization domain, Rev contains a nuclear localization signal (NLS) located within the RBD (Hope et al. 1990; Bohnlein et al. 1991; Hammerschmid et al. 1994) and a C-terminal NES spanning residues 75–83 (Hope et al. 1991; Hammerschmid et al. 1994). The longest stretches of highly conserved residues are the first five N-terminal residues, Helix 1 of the oligomerization domain, the arginine-rich RBD/NLS, and the leucine-rich NES. These stretches are connected by regions exhibiting more variability including the turn connecting Helix 1 of the oligomerization domain to the RBD/NLS, the polar residues of Helix 2 within the oligomerization domain, and a stretch of residues alternating from unconserved to conserved near the NES. Finally, the least-conserved region consists of the long C-terminal tail, which is dispensable for specific RRE recognition (Auer et al. 1994). Position 18 contains the highest percentage of naturally occurring hydrophilic side chains considering all hydrophobic-to-hydrophilic mutations originally modeled into head and tail positions. Approximately 6% of the sequences in this analysis contain threonine at

Figure 3. In vivo activity of putative tail and head mutations of HIV-1 Rev. (A) Diagrammatic representation of the 3-RRE reporter system: The reporter contains subgenomic fragments of HIV-1 and a CMV promoter, as indicated. Translation of Gag in transiently transfected HeLa cells from the reporter is dependent on interactions between Rev and the included RRE sequence. Splice donor site is indicated by the upward pointing arrow. (B) HeLa cells were cotransfected with wild-type or mutant pRev, p3-RRE, and pEGFPN1. Immunostaining for Gag expression (red) allowed for estimation of Rev export activity, and GFP fluorescence (green) provided a measure of transfection efficiency. (C) Rev expression in the transfected cells was detected by Western blot. (D) Nuclear export activity of wild-type and mutant Rev proteins in HeLa cells transfected with p3-RRE plasmid. Rev mutant activities are expressed as a percentage of wild-type Rev activity (set to 100%). Error bars represent the standard deviation of three independent experiments.

this position. Interestingly, in an independent comparison of sequences from the HIV-1C subtype, threonine was found in 10% of the sequences (Novitsky et al. 2002).

Discussion

Mutations to the oligomerization domain of Rev disrupt its structure and modulate trans-activation activity

Rev is essential for the viral life cycle, but many of the key details associated with Rev function remain to be

elucidated. In the previous study by Jain and Belasco (2001), the behavior of Rev mutations that disrupt oligomeric binding on the RRE was used to support a model of oligomerization wherein a helix-turn-helix motif undergoes oligomerization into groups of four-helix bundles. Their work was based on studies of Rev mutants that have established Rev oligomerization on the RRE as a prerequisite for export activity (Malim and Cullen 1991) and that have been used to map the Rev oligomerization interfaces (Malim and Cullen 1991; Thomas et al. 1997, 1998; Trikha and Brighty 2005). However, because the

Figure 4. CD of HIV-1 Rev variants. All CD spectra of 30 μ M wild-type and mutant Rev proteins were collected at 5°C. The double minima at 208 nm and 222 nm, characteristic of a helical protein, is apparent in all of the spectra. Visual inspection of the spectra demonstrates that the minimum at 208 is shallower for the L60R, I55N, and V16D mutations relative to the other Rev variants. The 222 nm/208 nm ratio for each variant are summarized in Table 1.

relationship between oligomeric binding and RNA-free Rev was untested and because equal changes in oligomerization have been related to different effects on *trans*-activation (Thomas et al. 1997; Brice et al. 1999; Trikha and Brighty 2005; Churchill et al. 2007), further investigation of the Jain and Belasco (2001) model was warranted. We have expanded the previous studies by probing the effects of mutation on Rev through a broad range of methodologies including cell biology and biophysical techniques. This provides an unusually comprehensive view of the relationship between Rev structure and function. As summarized in Figure 6, protein structure as assayed by CD correlates more closely with export activity than does measurement of oligomeric assembly of Rev on the RRE. We conclude that the mutations identified by Jain and Belasco (2001) do contribute to Rev self-association and activity, but the effects cannot be neatly grouped to support their detailed structural model. Instead, we propose a new classification scheme for Rev mutations that is based most importantly on changes in Rev export activity as assayed through the expression of a Rev-dependent reporter construct. Additional information can then be obtained through biophysical methods to

make predictions about the underlying causes for changes in activity resulting from mutation.

Mutating Rev results in changes to oligomeric binding on the RRE that cannot result solely from the disruption of oligomeric interfaces. Using the head (V16D and L60R) and tail (L18Q and I55N) mutants previously described by Jain and Belasco (2001) as well as the prevalent L18T, we investigated the structural and functional importance of these amino acid positions. Our gel shift assay utilized the full-length, 351-nt RRE, and a very low concentration for the radiolabeled RRE (0.05 nM) was deliberately chosen to satisfy equilibrium conditions. In the previous work, the V16D and L60R variants were both reported to have similar defects in trimer assembly and assigned to head positions, although measurements of K_D values were not provided (Jain and Belasco 2001). We observe that these two Rev variants are distinguishable. Furthermore, the observed 12-fold increase for K_{D1} of the V16D variant relative to wild-type Rev suggests that the mutation disrupts Rev structure in regions required directly for RNA binding that are distinct from the oligomerization interface. Equally surprising was a comparison between K_{D2} values of the head variant L60R with the tail variants L18Q and L18T. In the previous work of Jain and Belasco (2001), the effects of the L60R and L18Q mutations were used to define distinct head and tail positions, respectively. We observe that L60R and L18Q have distinctive values for K_{D2} , but, in contrast, the values of K_{D2} for L60R and L18T are the same. Apparently, the varied effects of mutation of L18 are related to subtle differences in the chemical nature of the substituted residue and do not arise from the simple disruption of a hydrophobic interface with a polar substitution.

The correlation between Rev secondary structure as assayed by CD and expression of the Rev-dependent reporter construct provide a reasonable means to group the effects of mutations on Rev structure and function. It should be noted that both solid-state NMR and CD data indicate that protein conformations of Rev and Rev–RRE complexes are indistinguishable (Daly et al. 1990; Havlin et al. 2007). The CD spectrum of wild-type Rev is indicative of a helical structure and is at least qualitatively

Table 2. Secondary structure data from circular dichroism of HIV-1 Rev variants

	222 nm/208 nm $\pm$ 0.01
Wild type	0.87
L18T	0.89
L18Q	0.84
V16D	0.78
L60R	0.76
I55N	0.73

Figure 5. Comparison of Rev sequences. Rev sequences isolated from two long-term nonprogressors (01zatm45 and 99zatm10) were aligned with wild-type Rev, the consensus sequence for HIV-1C (Novitsky et al. 2002), and the HIV-1 Rev consensus sequence calculated from the multiple sequence alignment from the Los Alamos Sequence database. The putative helix-turn-helix motif of the oligomerization/RBD is indicated graphically. An arrow marks the oligomerization domain mutations considered in this study, and their positions in the wild-type sequence are highlighted by a black oval. The consensus sequence represents the most probable character at each portion of the alignment. Sequence numbering does not include spaces where a gap is the most common character. A color-coded conservation score is depicted as a box under each position. Point mutations within nonprogressor sequences that are known to reduce Rev activity, and the additional inserts within the nonprogressor sequences are highlighted in gray.

similar to CD spectra of the four-helical bundle proteins interleukin-4 (Vaz et al. 2006), Annexin A2 (Aukrust et al. 2006), and Tropomyosin (Paulucci et al. 2002), consistent with the existing structural models (Thomas et al. 1997; Jain and Belasco 2001; Novitsky et al. 2002). However, CD spectra from V16D, I55N, and L60R reveal substantial alterations of protein secondary structure in addition to any local changes that might affect interfaces between self-associating monomers. Importantly, existing models for describing oligomeric Rev–RRE assemblies were largely based on the assumption that mutations in the oligomerization domain selectively interfere with specific interfaces that mediate oligomerization without alteration of global structure. At best, this assumption appears to only hold true for the position 18 variants.

The changes caused by mutations to position 18 are distinct from the other mutations and are not sufficient to completely abolish oligomeric binding and *trans*-activation activity. There are many proposed interactions between Rev and cellular cofactors that could require proper protein structure beyond oligomeric assembly on the RRE. Shutling Rev between the cytoplasm and the nucleus requires an interaction between unbound Rev and importins such as importin β , as well as an interaction between Rev–RNA complexes and the export receptor Crm-1 (Pollard and Malim 1998). Both L60R and M4-Rev with mutations to positions 23, 25, and 26 (Malim et al. 1989b) show an increased presence in the cytoplasm relative to wild type (Szilvay et al. 1997; Trikha and Brighty 2005). This pattern

of cytoplasmic localization has been cited as evidence for an oligomerization requirement for nuclear import (Trikha and Brighty 2005). Cytoplasmic accumulation of Rev also results from increasing the rate of Rev export (Soros and Cochrane 2001). Additional cellular factors affecting the *trans*-activation activity and cellular localization of Rev include DDX1, which affects cytoplasmic localization (Fang et al. 2005), B23, which promotes import (Szebeni et al. 1997), and the export-promoting proteins DDX3 (Yedavalli et al. 2004), eIF 5A (Ruhl et al. 1993; Bevec et al. 1996), hRIP (Sanchez-Velar et al. 2004), and Sam68 (Reddy et al. 1999). Altered interactions between Rev and these cellular cofactors might explain variable effects of Rev mutations.

Figure 6. Summary of effects of mutations to the oligomerization domain of Rev. For each type of measurement, a qualitative grayscale scheme is used to depict the change for each mutation relative to wild type. In general, the mutations cause a range of structural and functional changes with mutation to position 18 being the least disruptive.

Genetic modulation of HIV-1 Rev function provides opportunities for the virus to adapt to its environment

Genetic variation of position 18 of Rev, as in the L18T variant, may provide a selective advantage for the virus by reducing *trans*-activation activity without eliminating it. A recent model has been proposed wherein subtle modulations of Rev activity can provide protection from anti-Gag cytotoxic T lymphocyte (CTL) killing by reducing Gag expression (Bobbitt et al. 2003). The central idea of this model is that cells carrying attenuated Rev have increased chances of survival because they do not display large amounts of Gag epitopes to the CTLs. The continued survival and spread of HIV-1 with attenuated Rev activity is proposed to ensure the collapse of the immune system and promote the escape from latency. Consistent with this model, position 18 of Rev was recently identified as a positively selected position and a likely site of escape mutations through the use of codon-based substitution models and maximum-likelihood computation methods on a group of HIV-1 B and C sequences (de Oliveira et al. 2004). Alanine, tyrosine, phenylalanine, serine, and cysteine are all found with ~1%–2% frequency at position 18 in the Rev alignments. This chemical diversity is unique among the positions that are known to alter the oligomeric Rev assembly on the RRE and provides another opportunity to subtly modulate Rev activity.

Combining the genetic information from Figure 5 with measured changes in Rev activity makes it possible to make predictions of biological outcomes resulting from changes in the Rev protein sequence. Table 3 summarizes naturally occurring Rev point mutations that have been observed to affect Rev function and their frequency of occurrence in the aligned sequences. Intriguingly, the L18T mutation was observed in Rev sequences isolated from two HIV-1C-infected siblings who are long-term nonprogressors (Papathanasopoulos et al. 2003). In Figure 5, the sequences from two slow-progressing siblings are compared with the wild-type Rev consensus sequence and the consensus sequence of HIV-1 C Rev (Novitsky et al. 2002). The sequences of both long-term nonprogressors 01zatm45 and 99zatm10 contain the L18T mutation and the L78I mutation in the NES that were previously reported to reduce export activity (Iversen et al. 1995). Additionally, 01zatm45 contains a R39Q mutation in the RBD that would be expected to affect specific binding to the RRE. As reported previously (Papathanasopoulos et al. 2003), both of the Rev sequences isolated from the two asymptomatic siblings are lacking the C-terminal truncation found in the HIV-1C consensus sequence and contain the C-terminal EEC extension found in the Sydney Blood Bank Cohort of long-term nonprogressors (Oelrichs et al. 1998). Our results suggest that sequence variation at position 18 of Rev may contribute to the regulation of viral replication and con-

Table 3. Frequency of point mutations that are likely to affect Rev function

Mutation	% Occurrence	Result	Reference
R39K	42%	Reduction of specific RRE binding	Tan and Frankel 1994
L78I	34%	Reduction of export	Iversen et al. 1995
L18T	6%	Reduced oligomerization and reduced gene expression	This paper
T35A	1%	Reduction of specific RRE binding	Tan et al. 1993
R44K	1%	Reduction of specific RRE binding	Tan and Frankel 1994
L60F	1%	Reduced expression of Gag and Env	Bobbitt et al. 2003

tribute to the increased latency period of the HIV-1 infection in these siblings. This is consistent with the previous observation that attenuated alleles of Rev with reduced *trans*-activation activity and variable degrees of RRE binding persist in long-term nonprogressors of dominant (Churchill et al. 2007).

Conclusion

While the importance of Rev oligomerization has been previously established, there is no clear molecular basis for understanding the requirements for nuclear transport. A Rev–RRE complex that is competent for export from the nucleus consists of RNA, oligomerized Rev, and multiple host factors. Intramolecular folding of Rev, protein binding, and RNA binding all contribute to formation of this complex. Studies of Rev and Rev–RRE interactions alone cannot be used to define a biochemical system that reflects Rev function. However, by combining cell biology with multiple biophysical techniques, we have expanded the work of Jain and Belasco (2001) to describe subsets of mutations to the Rev oligomerization domain. The mutations within these subsets differ in the degree to which they affect Rev function and they have distinct biophysical properties. When combined with studies of Rev interactions with human factors, these mutations will provide an invaluable tool for dissecting the molecular basis for variability in Rev function. As the importance of variable Rev function to HIV-1 biology is increasingly becoming apparent, understanding the molecular basis for this phenomenon may be critical for understanding the course of AIDS infections.

Materials and Methods

Plasmid construction

Plasmid 3-RRE was constructed by Wodrich et al. (2000). Plasmids encoding wild-type Rev, pRev, and his-tagged Rev in

T7 expression vector, pSG003, have been previously described (Wodrich et al. 2000; Jain and Belasco 2001). Mutant plasmids of pHisRev/SG003 with amino acid changes were constructed by site-directed PCR mutagenesis. cDNAs encoding mutant proteins were amplified from mutant pHisRev/SG003 plasmids by PCR (*Pfu* polymerase, Invitrogen) with oligonucleotides 5'-GGGGGAATTCATGGCTGGTCGCTCTGGCGATTCTG-3' and 5'-GGGGGGGCCCTACTCTTTGGTTCCAGACTCCAGAAC-3', thereby introducing new *EcoRI* and *ApaI* restriction sites (underlined). PCR products were digested and ligated into pRev cleaved with the same enzymes to replace wild-type Rev cDNA. Constructs were verified by sequencing.

Protein characterization

Hexahistidine-tagged Rev variants were expressed in *Escherichia coli*, purified by affinity chromatography, and quantified as described previously (Jain and Belasco 1996). Protein samples of 1 mL were dialyzed against 4 L of buffer ~12 h and were centrifuged at 16,000g for 20 min prior to analysis.

CD spectroscopy

CD spectra were recorded on an Aviv 202SP spectrometer with 30 μ M Rev at 5°C in 50 mM K_2HPO_4 (pH 7.5), 500 mM KCl, and 500 μ M tris (2-carboxyl-ethyl) phosphine hydrochloride (CD buffer). Ellipticity was averaged for 3 s at 1-nm intervals. Percent helicity was estimated from the spectra using the program K2D (Andrade et al. 1993) at the Dicroweb homepage (Whitmore and Wallace 2004) (<http://public-1.crysl.bbk.ac.uk/cdweb/html/home.html>). Measurements of 222 nm/208 nm ratios for each variant were taken from three separate dilutions of protein, and the error represents one standard deviation of the measurements.

Electrophoretic gel mobility analysis

RRE transcripts were prepared by bacteriophage T7 RNA polymerase transcription from a linearized plasmid as previously described (Jain and Belasco 2001). The RNA transcript was dephosphorylated using Antarctic phosphatase (New England Biolabs), 5'-end labeled with [γ - ^{32}P]ATP and T4 polynucleotide kinase, and purified in a 6% polyacrylamide gel containing 7.5 M urea. RNA was the largest excised and extracted from the gel by crushing and soaking for 24 h in 20 mM Tris, pH 6.8, 1 M NaCl, 1 mM EDTA, then precipitated by adding 0.1 volumes of 3 M sodium acetate, pH 5.5, and 3 volumes of ethanol. The pellet was washed in 70% ethanol and suspended in water. The RNA was heated to 96°C for 1 min and then slow annealed in a heating block to room temperature. The radiolabeled RNA was diluted to 0.1 nM in QT buffer (10 mM HEPES, pH 7.5, 150 mM KCl, 5 mM DTT, 10% glycerol, 50 μ g/mL *E. coli* tRNA, 100 μ g/mL bovine serum albumin). Stocks of Rev variants (3 μ M) were stored in QT buffer and diluted to 12 nM through serial dilution into Falcon 96-well U-bottom plates containing QT buffer. Titrations were carried out by mixing equal volumes of RNA solution and protein solution at the various concentrations in the 96-well plates. This resulted in a final RRE–RNA concentration of 0.05 nM. These mixtures were incubated for 20 min at room temperature and then loaded into continuously running 7% polyacrylamide gels that were pre-run at 200 V for 1 h. The gels were run for 4 h and dried prior to exposure of a PhosphorImager screen.

Data were quantified as percentage of total radiolabeled RNA using ImageQuant software (Molecular Dynamics). The counts in the second band from the bottom were considered the singly Rev-bound species while the counts in the third band from the bottom were considered the doubly Rev-bound species (Fig. 2). All of the higher-order bands were grouped into the triply bound species. The data were fit using nonlinear least squares analysis using Igor software (Wavemetrics). Apparent dissociation constants K_{D1} , K_{D2} , and K_{D3} were determined by simultaneous solution of the following equations for a four-state model of stepwise binding:

$$\begin{aligned} R_f([Rev_f]) &= 1/P & R_f([Rev_f]) &= 1/P \\ R_1([Rev_f]) &= K_1[Rev_f]/P & R_1([Rev_f]) &= K_1[Rev_f]/P \\ R_2([Rev_f]) &= K_1K_2[Rev_f]^2/P & R_2([Rev_f]) &= K_1K_2[Rev_f]^2/P \\ R_3([Rev_f]) &= K_1K_2K_3[Rev_f]^3/P & R_3([Rev_f]) &= K_1K_2K_3[Rev_f]^3/P \end{aligned}$$

where $R_f([Rev_f])$ is the fraction of unbound RRE, and $R_1([Rev_f])$, $R_2([Rev_f])$, and $R_3([Rev_f])$ are the fractions of singly, doubly, and higher multimeric Rev-bound states, respectively. $[Rev_f]$ is the concentration of RNA-free Rev and is assumed to equal the total Rev concentration because radiolabeled RRE was added in trace. K_1 through K_3 are the binding constants:

$$\begin{aligned} K_1 &= [RRE \bullet Rev] / ([RRE_f][Rev_f]) \\ K_2 &= [RRE \bullet Rev_2] / ([RRE \bullet Rev][Rev_f]) \\ K_3 &= [RRE \bullet Rev_3] / ([RRE \bullet Rev_2][Rev_f]) \end{aligned}$$

where $[RRE \bullet Rev_1]$ through $[RRE \bullet Rev_3]$ are the concentrations of singly, doubly, and higher-order Rev–RRE complexes, respectively. Finally, P is the binding polynomial $P = 1 + K_1[Rev_f] + K_1K_2[Rev_f]^2 + K_1K_2K_3[Rev_f]^3$.

The binding constants were converted to dissociation constants by taking their inverse (i.e., $K_{D1} = 1/K_1$) within the fitting algorithm.

Cell culture and transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and supplemented with L-glutamine and antibiotics (penicillin–streptomycin). For transient expression, cells were seeded into 24-well plates and cotransfected 16 h later with either wild-type or mutant pRev, 3-RRE, and pEGFPN1 (Clontech) in a molar ratio 0.45:0.45:0.1 with a total of 1 μ g of pDNA per well using Lipofectamin 2000 (Invitrogen).

Viral Gag and Rev protein analyses

Twenty-four hours after transfection, cells were washed in ice-cold PBS, lysed (PBS, 500 μ M PMSF, 500 μ M benzamidin, 2.5 μ M pepstatin A, 2 μ M leupeptin, 1 μ M aprotinin), and sonified. Equal protein amounts were separated by sodium dodecyl sulfate 4%–20% polyacrylamide gel electrophoresis and transferred by electroblotting to nitrocellulose membranes, which were blocked in PBS supplemented with 5% milk and 0.05% Tween-20. For detection of Gag, membranes were probed sequentially with a 1:10,000 dilution of monoclonal mouse anti-human p24 (Gag) antibody (Chemicon International) and a 1:7,500 dilution of polyclonal HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). To detect

Rev expression, membranes were sequentially probed with polyclonal sheep anti-Rev (1:5,000 dilution; Fitzgerald Industries International) and HRP-conjugated anti-sheep (1:10,000; Jackson ImmunoResearch Laboratories). Immunoblots were developed using an enhanced chemiluminescence detection system (SuperSignal, Pierce).

Immunofluorescence analysis

For microscopic studies of Gag expression, cells were washed with PBS and fixed for 10 min in 4% paraformaldehyde in PBS 24 h after transfection. Cells were washed twice with 10 mM glycine in PBS, blocked with 1% FCS in PBS for 5 min, and incubated with mouse anti-human p24 (Gag) monoclonal antibody (Chemicon International) for 2 h. After washing, cells were blocked in 0.2% gelatin in PBS, incubated for 1 h in goat anti-mouse-rhodamine (Jackson), and washed again. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Images were obtained using PCI software (Compix Inc., Imaging Systems) on a Leica DM/IRE2 microscope. Three independent experiments were collected, and the number of Gag- and EGFP-expressing cells was manually determined to quantify the Rev variant's nuclear export activity. For each Rev variant, the number of Gag-positive cells was normalized according to EGFP-positive cells.

Conservation score calculations

A conservation score, $c(i)$, for each position, i , in the sequence of HIV-1 Rev was determined using the multiple sequence alignment available from the Los Alamos HIV sequence database (www.hiv.lanl.gov). This score was calculated as a combination of the normalized sequence entropy $e(i)$ and a gap probability $g(i)$ using the following expression (Valdar 2002):

$$c(i) = (1 - e(i))(1 - g(i))$$

$e(i)$ is the normalized sequence entropy $s(i)$:

$$e(i) = (\sum p(k) \ln(p(k)) / -1.79) = s(i) / -1.79$$

where $p(k)$ is the probability that the position in the sequence is occupied by a residue of type k . To be consistent with other calculations of sequence entropy, the 20 amino acids were grouped by the method of Mirny and Shakhnovich (1999) into the following six groups: (1) Arg, Lys; (2) Asp, Glu; (3) His, Phe, Trp, Tyr; (4) Asn, Gln, Ser, Thr; (5) Ala, Cys, Ile, Leu, Met, Val; (6) Gly, Pro. The probabilities were calculated over these six groups with a minimum value of -1.79 . $g(i)$ is the probability of a gap at position i . The result of combining the $e(i)$ and $g(i)$ values is a $c(i)$ score from 0 to 1. The lowest values are produced by positions that are gaps in all sequences except one, and the highest scores are in positions represented by a single amino acid.

Alignments for comparing Rev sequences isolated from long-term non-progressors to consensus sequences were calculated using ClustalW (1.8) with the default parameters (European Bioinformatics Institute: <http://www.ebi.ac.uk/clustalw/>).

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