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Abstract

The HIV-1 Rev (Regulator of Expression of Virion) protein, an RNA-binding protein essential for viral replication, is imported into the host cell nucleus by human Importin β (Imp β). Rev is phosphorylated *in vivo* on serine residues by a nuclear kinase. In this study, we introduced glutamate substitution mutations that mimic phosphorylation at serine positions previously identified as potential phosphorylation sites and assessed their impact on the ability of Rev to bind Imp β in thermal shift, gel shift, and fluorescence polarization assays. Phosphomimetic mutations introduced in either the N-terminal tail, helical hairpin domain or C-terminal domain of Rev had a small but reproducible stabilizing effect on the Imp β /Rev complex. Moreover, the mutation of Rev residue Ser56, which localizes to one face of the helical hairpin domain, had a greater stabilizing effect than that of Ser54 located on the opposite face, suggesting that the helical hairpin orients its Ser56-containing face towards Imp β . Taken together, our results suggest that phosphorylation can significantly modulate the ability of Rev to associate with Imp β .

Introduction

Although current anti-retroviral therapy is effective at preventing HIV-1 infected patients from developing AIDS, there remains considerable interest in exploring new therapeutic strategies that target steps of the viral replication cycle not inhibited by currently available drugs [1]. One such step is the nuclear export of intron-containing viral RNA mediated by HIV-1 Rev (Regulator of Expression of Virion), an RNA-binding protein essential for viral replication. Rev is one of three viral proteins translated from the fully spliced HIV-1 RNA transcript that is exported to the cytoplasm by the host cellular mRNA export machinery [2]. Rev mediates the nuclear export of unspliced and incompletely spliced viral transcripts by specifically binding to a highly structured 350-nucleotide RNA motif, the Rev Response Element (RRE), located within incompletely processed transcripts. Newly translated Rev is recognized in the cytosol by the host cell transport factor Importin β (Imp β), which forms a heterodimeric complex with Rev and imports it into the nucleus [3] [4]. Rev then binds specifically to RRE-containing RNAs by a cooperative process that involves the association of several Rev monomers with the RRE, mediated by Rev-Rev and Rev-RNA interactions. In the nucleus, Rev is recognized by the host cell transport factor CRM1, which mediates the nuclear export of the Rev/RRE complex. In the cytosol, the viral RNA is released from Rev and translated into viral structural proteins or packaged into new viral particles. The reassociation of cytosolic Rev with Imp β allows Rev to shuttle back to the nucleus for another cycle of viral RNA export. *In vitro*, Rev binds various import factors including Imp β , Transportin-1, Transportin-5 and Transportin-7 [5]. Evidence suggests that the import pathway is cell-specific, with Rev preferring Imp β in T-cell lines [6].

Rev is a highly basic 13 kDa protein, which contains a short N-terminal tail followed by a helical hairpin domain and an intrinsically disordered C-terminal domain [7] [8] (Fig. 1A). The C-terminal domain contains a leucine-rich nuclear export signal (NES) responsible for recruiting CRM1 [9] [10]. The helical hairpin domain contains an arginine-rich motif that mediates RNA binding and acts as a nuclear localization signal (NLS) recognized by Imp β . Hydrophobic residues located on either side of the NLS, referred to as an oligomerization domain (OD), define hydrophobic surfaces on both faces of the helical hairpin, allowing Rev to multimerize by forming tail-to-tail (T/T) and head-to-head

(H/H) interactions [7] [8] [11]. Imp β is a 97 kDa acidic protein with a solenoid structure comprising 19 tandem HEAT repeats [12]. Whereas most macromolecular cargos bearing a basic NLS are recognized by Importin α , which in turn associates with Imp β to mediate nuclear import, the arginine-rich NLS of Rev is directly recognized by Imp β [3] [4]. Impairing Imp β /Rev complex formation compromises Rev nuclear import and blocks viral replication [4], raising the prospect of exploiting the Imp β /Rev interaction as a possible chemotherapeutic target for antiretroviral therapy.

While atomic structures are known for Imp β bound to various partners, the structure of the Imp β /Rev complex is unknown. Technical difficulties hampering structural analysis of this complex have led us to search for Rev variants with enhanced affinity for Imp β . In particular, we considered phosphorylated versions of Rev, since phosphorylation can modulate the nature and strength of protein-protein interactions, and frequently results in complex stabilization [13]. Indeed Rev exists as a phosphoprotein *in vivo* [14] [15]. Although the kinase responsible has not yet been identified, phosphorylation is known to take place in the nucleus and occurs exclusively on serine residues [14] [15] [16]. Of the 11 serine residues in Rev, 6 have been identified as potential phosphorylation sites [16] [17] [18] [19] [20]: 2 in the N-terminal tail (Ser5 and Ser8), 2 in the helical hairpin domain (Ser54 and Ser56), and 2 in the C-terminal domain (Ser92 and Ser99) (Fig. 1B). Interestingly, Rev sequence alignments from HIV-1 and HIV-2 strains show that 4 of these serine residues are highly conserved in the HIV-1 group M, which is responsible for the majority of the global epidemic, whereas none are conserved in the less-virulent HIV-2 (Fig. 1A). *In vitro* experiments indicate that Rev can be phosphorylated by casein kinase-2 (CK2), mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and cyclin-dependant kinase 1 (CDK1/cdc2) but not by protein kinase A (PKA) and casein kinase 1 (CK1) [14] [19] [21] [20]. However, the relevance of Rev phosphorylation *in vivo* is unclear, since phosphorylation is not required for Rev-mediated export of intron-containing viral RNA [16] [17].

The present study is motivated by the question of whether Rev phosphorylation can enhance the ability of Rev to interact with Imp β and form a stable heterodimeric complex. To address this question, we introduced phosphomimetic mutations at the serine positions previously identified as potential phosphorylation sites and assessed their impact on the ability of Rev to bind Imp β in thermal shift, gel shift, and fluorescence polarization assays.

Objective

Our aim was to determine whether mutations that mimic the phosphorylation of Rev can enhance the ability of Rev to interact with human Imp β .

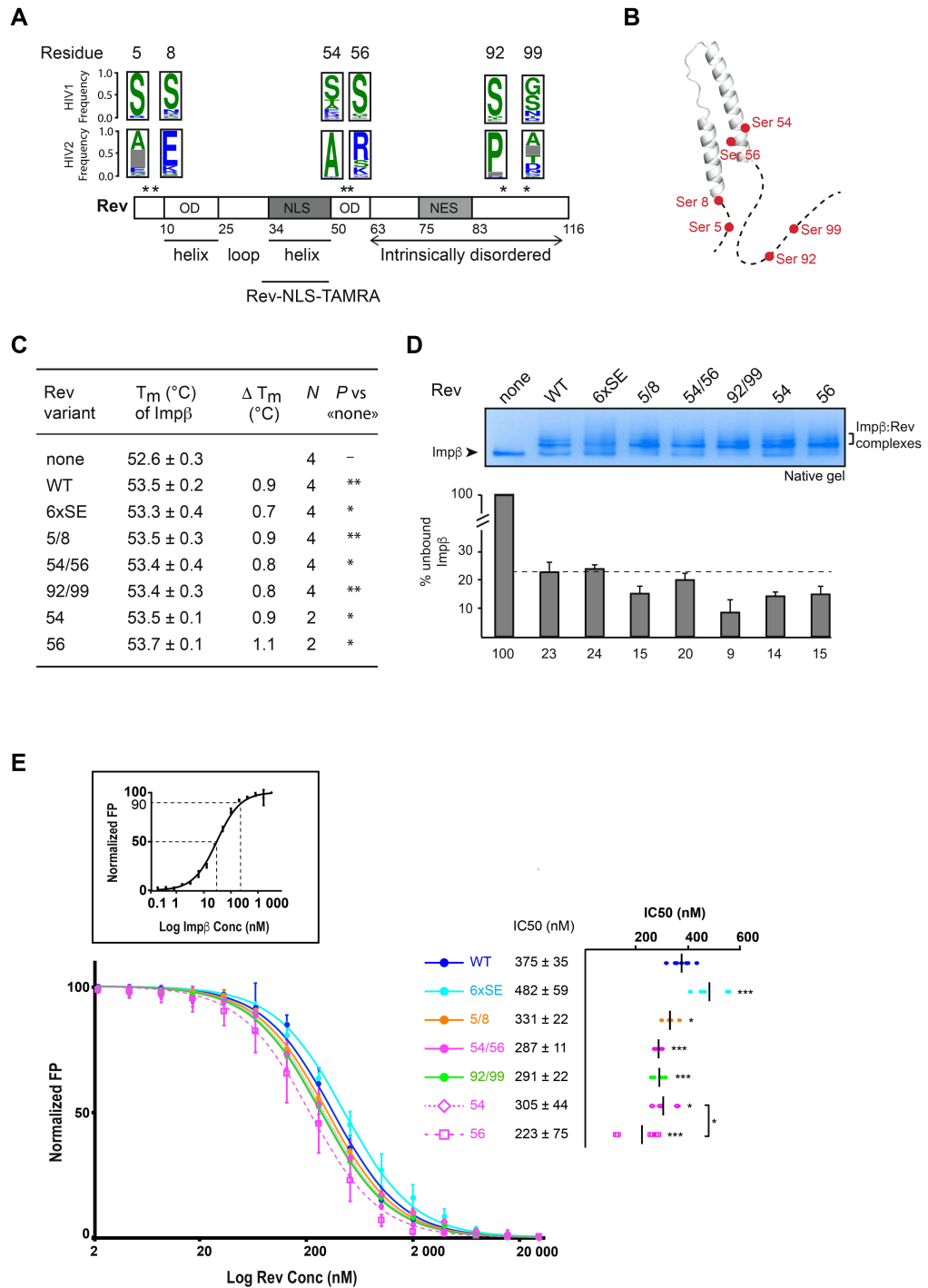


Figure Legend

Figure 1.**(A) Domain organization of Rev and location of phosphorylated serine residues.**

The oligomerization domain (OD), nuclear localization signal (NLS), nuclear export signal (NES) and the corresponding Rev-NLS-TARMA peptide used in this study are indicated. The serine residues mutated to glutamate are indicated by an asterisk. For each of these, the corresponding frequency histogram of residues in a Rev sequence alignment is shown for HIV-1 and HIV-2. Frequency histograms were generated with the AnalyzeAlign tool of the web server www.hiv.lanl.gov, using 2269 sequences from HIV-1 group M subtypes A-K and all 73 HIV-2 sequences available in the LANL HIV Sequence Database.

(B) 3D structure of Rev.

Crystal structure of the helix-loop-helix domain (residues 9–65) (PDB entry 3LPH). The disordered N- and C-terminal domains are indicated by dashed lines. The serine residues mutated to glutamate are shown as red circles.

(C) Effect of wildtype and mutant Rev proteins on the thermal stability of Imp β determined by NanoDSF.

Mean T_m and S.D. values were obtained from 2–4 independent experiments (N). P values for T_m values obtained in the presence of Rev proteins compared to those for Imp β alone (“none” Rev variant) were determined using a two-tailed Mann-Whitney test. * P < 0.05, ** P < 0.01. Rev mutants did not yield T_m values significantly different from the WT (P > 0.05). 6xSE stands for the Rev mutant in which all 6 serine residues were simultaneously mutated to glutamate.

(D) Gel shift analysis of the Imp β /Rev interaction.

Upper panel: Representative native gel showing the formation of Imp β /Rev complexes when Imp β and Rev proteins were mixed in a 1:1 molar ratio. Bands were visualized by Coomassie staining. The intensity of bands was quantified and the indicated fraction of unbound Imp β was estimated by comparing the intensity of the unbound Imp β species to the total intensity of all bands. *Lower panel:* Averaged fraction of unbound Imp β and S.D. obtained from 3 independent native gel shift assays. The averaged value for each Rev variant is reported below the histogram.

(E) The Relative binding affinity of Rev proteins for Imp β as assessed in a competitive fluorescence polarization assay.

Inset: Binding of a fluorescent Rev peptide by Imp β . The fluorescence polarization (FP) of Rev-NLS-TAMRA was measured in the presence of increasing concentrations of Imp β . EC_{50} and EC_{90} values are indicated by dashed lines. *Main panel:* Competitive binding assay. The FP signal of the Rev-NLS-TAMRA peptide was measured in the presence of 195 nM Imp β and an increasing concentration of WT or mutant Rev. The plotted FP data represent the mean and S.D. values obtained in 6 independent experiments. The corresponding IC_{50} values are shown in the scatter plot at the right. Results were highly reproducible, although mutants 6xSE and S56E showed higher variability. P values were determined using a two-tailed Mann-Whitney test between RevWT and phosphomimetic mutants. * P < 0.05, ** P < 0.01, *** P < 0.005.

Results & Discussion**Phosphomimetic mutants of Rev retain the ability to bind Imp β**

To study the impact of HIV-1 Rev phosphorylation on its interaction with Imp β , we replaced each of the 6 phosphorylatable serine residues of Rev by a glutamate residue, whose negatively charged side chain mimics that of phosphoserine. We constructed a mutant (6xSE) in which all 6 serine residues were simultaneously mutated, as well as 3 double point mutants (S5E/S8E, S54E/S56E, and S92E/S99E) that address the effect of phosphorylation within the N-terminal tail, helical hairpin or C-terminal domain,

respectively. To pinpoint observations concerning the helical hairpin domain, we also constructed single point mutants (S54E and S56E) involving residues Ser54 and Ser56, which localize to opposite faces of the hairpin (Fig. 1B). Human Imp β , wildtype (WT) Rev and the 6 phosphomimetic mutants were then recombinantly expressed in bacteria and purified to homogeneity, and the purity and expected mass of each protein were confirmed by mass spectrometry, as detailed in the Methods.

As an initial test to verify whether our purified Rev proteins could associate with Imp β , we determined the thermal stability of Imp β in the presence and absence of Rev by nano-differential scanning fluorimetry (NanoDSF), which exploits the change in intrinsic fluorescence that occurs as a protein unfolds over an increasing temperature gradient [22]. Whereas the isolated Rev proteins gave very little fluorescence signal, isolated Imp β gave a clear thermal transition characterized by melting temperature (T_m) of 52.6°C (Fig. 1C). For all Rev proteins tested, the addition of WT or mutant Rev yielded a modest but statistically significant (0.7–1.1°C) increase in T_m , which we attribute to the stabilization of Imp β because of the lack of observable signal for Rev. This finding confirms that all Rev variants retain the ability to associate with Imp β and suggests that none of the phosphomimetic mutations grossly perturb the overall structure of Rev. Because the small shifts in T_m and comparatively large measurement errors did not allow us to reliably determine whether the Rev mutants bind Imp β better or worse than WT Rev, we turned next to a more sensitive binding assay.

Phosphomimetic mutations enhance the ability of Rev to bind Imp β in a gel shift assay

To visualize how Rev mutations affect Imp β /Rev complex formation we compared the ability of the different Rev proteins to bind Imp β in a gel shift assay. In the absence of Imp β , the highly basic Rev proteins do not enter the gel, whereas, in the absence of Rev, Imp β migrates as a single discrete band (Fig. 1D, upper panel, lane 1). The addition of WT Rev results in the appearance of two distinct, more slowly migrating bands as well as faint, more poorly defined bands of even lower mobility (lane 2). These species presumably correspond, in order of decreasing intensity and mobility, to Imp β /Rev complexes containing 1, 2 or more Rev monomers, respectively, consistent with the known ability of Rev to multimerize. A similar band pattern was observed upon addition of the different phosphomimetic mutants of Rev (lanes 3–8). However, notable differences in the relative intensities of unbound and bound Imp β species indicate that the various Imp β /Rev complexes differ in their degree of stability. In particular, compared to WT Rev, the 6xSE mutant binds more weakly to Imp β (as indicated by a more smeary appearance of the bound species and the slightly increased intensity of the unbound Imp β species), suggesting that the simultaneous mutation of all 6 serine residues destabilizes the Imp β /Rev complex. By contrast, the stability of the Imp β /Rev complex appears increased for all the single and double point mutants (as shown by the enhanced disappearance of the unbound Imp β species), with the highest affinity observed for the double mutant involving the C-terminal domain (S92E/S99E). Thus, the results suggest that the addition of one or two phosphomimetic mutations enhances the ability of Rev to associate with Imp β irrespective of the Rev domain harboring the mutation.

Rev mutants displace a Rev-derived peptide from Imp β more efficiently than WT Rev

To more quantitatively assess the impact of phosphomimetic mutations on the Imp β -Rev interaction, we measured the ability of the different Rev mutants to displace a fluorescently labeled peptide from Imp β in a fluorescence polarization (FP) assay. In this assay, the observed FP signal reflects the tumbling rate of the fluorophore in solution: the FP signal recorded from the unbound (rapidly tumbling) fluorescent peptide increases dramatically upon Imp β binding, which causes the peptide to tumble more slowly. We used a fluorescently labeled peptide spanning the arginine-rich NLS of Rev (Rev-NLS-TAMRA). Incubating the peptide with an increasing amount of Imp β revealed EC₅₀ and EC₉₀ values (concentrations that yield 50% and 90% maximal binding) of 29 and 195 nM, respectively (Fig. 1E, insert). We then performed a competition assay by fixing Imp β at the EC₉₀ value and adding an increasing amount of WT or mutant Rev, which dis-

places the peptide from Imp β and causes a reduction in FP signal. The Rev concentration yielding a 50% reduction (half-maximal inhibitory concentration, IC₅₀) provides a useful estimate of Imp β binding affinity, with lower IC₅₀ values corresponding to tighter binding. WT Rev efficiently displaced the peptide from Imp β , yielding an IC₅₀ of 375 nM (Fig. 1E). By comparison, peptide displacement was less efficient with the 6xSE mutant (IC₅₀ \approx 480 nM) and more efficient with the single and double point mutants of Rev (IC₅₀ values between \sim 220 and 330 nM), in general agreement with the results of the gel shift assay. Also in agreement, the single point mutant S56E was significantly more efficient than S54E at displacing the Rev peptide from Imp β (IC₅₀ values of \sim 220 versus 305 nM, respectively).

Conclusions

This work presents the first preliminary results concerning the impact of Rev phosphorylation on the interaction with Imp β . Our results indicate that the introduction of one or two phosphomimetic mutations within either the N-terminal tail, helical hairpin or C-terminal domain of Rev detectably enhances affinity, whereas a combination of all 6 mutations has the opposite effect. Moreover, within the helical hairpin, a mutation at Ser56 stabilizes the Imp β /Rev complex to a greater extent than that at Ser54. Thus, it seems highly plausible that phosphorylation could enhance the binding affinity of Rev for Imp β .

Limitations

A limitation of this work is that the effect of phosphorylation was investigated using serine-to-glutamate mutations that mimic phosphorylation. Performing binding studies with *in vitro* phosphorylated Rev would verify whether this approach is valid. Also, there is a lack of evidence concerning which of the 6 serine residues investigated in this study are phosphorylated *in vivo*. Assessing the effect of phosphomimetic mutations on the intracellular localization of Rev in cell-based assays would help verify the conclusions and biological relevance of this work.

Alternative Explanations

Conjectures

The enhanced Imp β -binding affinity observed for Rev double point mutants regardless of whether the mutations localize to the N-terminal tail, helical hairpin or C-terminal domain suggests that the enhanced affinity may reflect an overall change in the electrostatic character or solution behavior of Rev, rather than the gain of a specific intermolecular contact within the Imp β /Rev interface. Similarly, the fact that the 6xSE mutation destabilized the complex leads us to speculate that the simultaneous introduction of 6 additional negative charges might perturb the conformational landscape of Rev in such a way as to hamper Imp β binding. Interestingly, of the two point mutations located on opposite faces of the structured helical hairpin domain, S56E enhanced Imp β -binding affinity to a significantly greater extent than S54E, suggesting that the Ser56-containing face of the hairpin may be oriented towards Imp β . Indeed, whereas Ser56 is buried in the homodimer interface of the H/H-type Rev dimer, it localizes to the outer face of the T/T Rev dimer, which is the dimeric form preferentially adopted by Rev in solution [2]. Furthermore, Ser56 localizes to the same side of the hairpin as residues Arg38 and Arg39, residues previously identified as critical for Imp β binding and Rev nuclear localization [3] [16]. Taken together these observations suggest that Ser56 localizes within or close to the Imp β /Rev interface. Additional structural data on the Imp β /Rev complex are required to confirm this hypothesis.

Additional Information

Methods

Mutagenesis

Rev serine-to-glutamate point mutations were introduced using a PCR-based protocol adapted from the QuikChange site-directed mutagenesis method (Stratagene). PCR amplification was performed with Phusion High-Fidelity PCR Master Mix (NEB) using the expression plasmid pET28a-His-GB1-TEV-Rev (see below) as a template and specific mutagenic primers. PCR products were digested by DpnI. Final mutations were selected by transformation and verified by DNA sequencing.

Protein expression and purification

Imp β

N-terminally His-tagged *Imp* β was expressed from a pETM-11 plasmid in *E. coli* strain BL21 (DE3). Cells were grown in LB medium containing kanamycin (50 μ g/ml) at 37°C until reaching an optical density at 600 nm of 0.5–0.6, induced with 0.5 mM IPTG and further incubated for 5 h at 30°C. Bacteria were collected by centrifugation (6,200 g, 20 min, 4°C) and resuspended in lysis buffer [50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM imidazole, 10 mM MgCl₂, 2 mM β -mercaptoethanol (BME)] containing nuclease and protease inhibitors [10 μ g/ml DNase I, 10 μ g/ml RNase A, 2 mM PMSF and 1 tablet/80 ml of complete EDTA-free protease inhibitor cocktail (Roche)]. Cell lysis was carried out by sonication (15 s pulses separated by 30 s, 5 min total sonication time) and the soluble fraction was recovered by centrifugation (50,000 g, 20 min, 4°C). The soluble fraction was then loaded onto a HisTrap FF NiNTA column (GE Healthcare) pre-equilibrated with Buffer A (50 mM HEPES pH 7.5, 200 mM NaCl, 2 mM BME, 10 mM imidazole). After extensive washing with buffer A, the bound protein was eluted in 2 steps, first with 40 mM and then with 300 mM imidazole. Fractions containing *Imp* β were further purified using a Superdex 200 16/60 gel filtration column (GE Healthcare) pre-equilibrated with 50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP). Fractions containing pure *Imp* β were pooled, concentrated to >70 mg/ml on an Amicon centrifugal filter (30 kDa cutoff, Millipore) and stored as aliquots at -80°C. The *Imp* β protein concentration was determined using a molar extinction coefficient of 79,051 M⁻¹cm⁻¹, which was experimentally determined by quantitative amino acid analysis.

HIV-1 Rev

Rev proteins were expressed from a pET-28a plasmid as fusion constructs bearing an N-terminal His tag followed by the acidic B1 domain of Streptococcal protein G (GB1 domain) and a tobacco etch virus (TEV) protease site. The use of the GB1 tag has been shown to improve Rev solubility and facilitate purification [23]. Transformed *E. coli* BL21 (DE3) cells were grown in Auto-Induction Medium [24] containing kanamycin (50 μ g/ml) for 12 h at 27°C. Bacteria were collected by centrifugation (6,200 g, 20 min, 4°C) and resuspended in lysis buffer (25 mM HEPES pH 7.5, 200 mM NaCl, 100 mM Na₂SO₄, 10 mM MgCl₂, 10 mM imidazole, 0.1% Tween 20) containing nuclease and protease inhibitors (as described above for *Imp* β). Cells were lysed and the lysate was centrifuged as described above for *Imp* β . The clarified lysate was incubated with RNase T1 (20 U/ml, Roche), RNase A (20 μ g/ml, Euromedex) in the presence of 2 M NaCl for 1.5 h at room temperature before performing a new centrifugation step (50,000 g, 10 min, 4°C). The soluble fraction was loaded onto a HisTrap FF NiNTA column (GE Healthcare) pre-equilibrated with high-salt buffer B (2 M NaCl, 50 mM Tris pH 8, 0.1% Tween 20, 2 mM BME and 10 mM imidazole). After extensive washing with high-salt buffer B followed by a second wash step with low-salt buffer B (containing 250 mM NaCl and no Tween 20), the bound protein was eluted in 2 steps with low-salt buffer B containing first 40 mM and then 300 mM imidazole. Fractions containing His-GB1-Rev protein were diluted with an equal volume of 50 mM Tris pH 8, 125 mM NaCl before loading onto HiTrap Heparin HP columns (GE Healthcare) pre-equilibrated in 50 mM Tris pH 8, 125 mM NaCl. After extensive washing with 50 mM Tris pH 8, 125 mM NaCl, elution was performed using 50% High Salt Buffer (HSB) (50 mM Tris pH 8, 200 mM NaCl, 400 mM (NH₄)₂SO₄, 100 mM Na₂SO₄, 2 mM BME) and 50% of 2 M NaCl. The His-GB1 tag was then removed from the Rev protein by overnight incubation with His-tagged

TEV protease (1:20 w/w TEV/Rev protein ratio) supplemented with 2 mM BME and 2 mM PMSF. The untagged Rev protein was recovered in the flow-through after passing the sample through a new NiNTA column pre-equilibrated as described above. The untagged Rev protein was then diluted with an equal volume of 50 mM Tris pH 8 and then concentrated by repeating the heparin chromatography step described above. Heparin elution fractions containing Rev were applied onto a Superdex 75 16/60 gel filtration column (GE Healthcare) pre-equilibrated with HSB containing 1 mM TCEP. Fractions containing pure Rev were pooled, concentrated on an Amicon centrifugal filter (10 kDa cutoff, Millipore) and stored at -80°C . Rev protein concentration was determined using a molar extinction coefficient of $9476\text{ M}^{-1}\text{cm}^{-1}$, which was experimentally determined by quantitative amino acid analysis.

TEV protease

N-terminally His-tagged TEV protease bearing the point mutation S219V was expressed from a pRK793 plasmid [25] in *E. coli* strain BL21 (DE3). Cells were grown in LB medium containing ampicillin (100 $\mu\text{g}/\text{ml}$) at 37°C until reaching an optical density at 600 nm of 0.8, induced with 1 mM IPTG and further incubated for 4 h at 30°C . Bacteria were collected by centrifugation (6,200 g, 20 min, 4°C) and resuspended in lysis buffer [phosphate buffered saline (PBS), 10 % glycerol, 25 mM imidazole]. Cell lysis was carried out by sonication (5 s pulses separated by 15 s, 5 min total sonication time). DNA and RNA were precipitated by the addition of 0.1% (v/v) polyethyleneimine (PEI) and the resulting precipitate removed by centrifugation (50,000 g, 20 min, 4°C). The soluble fraction was then loaded onto a HisTrap FF NiNTA column (GE Healthcare) pre-equilibrated with lysis buffer. After extensive washing with lysis buffer containing first 25, then 50 mM imidazole, the bound protein was eluted with PBS, 10% glycerol, 500 mM imidazole. Fractions containing TEV were further purified using a Superdex 200 16/60 gel filtration column (GE Healthcare) pre-equilibrated with PBS, 10% glycerol, 2 mM EDTA, 10 mM DTT. Fractions containing pure TEV were pooled and the concentration was adjusted to 1 mg/ml as determined in a Biorad Bradford Assay. Aliquots were stored at -80°C .

Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC/ESI-MS)

LC/ESI-MS was performed on a 6210 LC-TOF spectrometer coupled to an HPLC system (Agilent Technologies). All solvents used were HPLC grade (Chromasolv, Sigma-Aldrich). Trifluoroacetic acid (TFA) was from Acros Organics (puriss., p.a.). Solvent A was 0.03% TFA in water; solvent B was 95% acetonitrile–5% water–0.03% TFA. Immediately before analysis, protein samples were diluted to a final concentration of 5 μM with solvent A. Protein samples were then desalted on a reverse phase-C8 cartridge (Zorbax 300SB-C8, 5 μm , 300 μm ID \times 5 mm, Agilent Technologies) at a flow rate of 50 $\mu\text{l}/\text{min}$ for 3 min with 100% solvent A and subsequently eluted with 70% solvent B for MS detection. MS acquisition was carried out in the positive ion mode in the 300–3200 m/z range. MS spectra were acquired and the data processed with MassHunter workstation software (v. B.02.00, Agilent Technologies) and with GPMW software (v. 7.00b2, Lighthouse Data, Denmark).

The masses of the full-length proteins determined by LC/ESI-MS were in excellent agreement with the expected masses (shown in parentheses) and were as follows : 100,298.91 \pm 2 Da for Imp β (100,298.91 Da); 13,174.51 \pm 0.67 Da for WT Rev (13,175.85 Da); 13,427.53 \pm 0.55 Da for 6xSE (13,428.08 Da); 13,259.81 \pm 0.12 Da for S5E/S8E (13,259.93 Da); 13,258.78 \pm 0.87 Da for S54E/S56E (13,259.93 Da); 13,259.61 \pm 0.32 Da for S92E/S99E (13,259.93 Da); 13,216.74 \pm 0.86 Da for S54E (13,217.89 Da) and 13,216.74 \pm 0.86 Da for S56E (13,217.89 Da). The spectra showed an absence of any contaminating proteins or degradation products.

Differential scanning fluorimetry (NanoDSF)

Imp β and Rev proteins were first independently diluted to 50 μM in 50 mM HEPES pH 7.5, 100 mM NaCl, and then incubated in a 1:1 Imp β :Rev ratio (5 μM each, 15 μl final volume) for 5 min at 25°C . Tryptophan fluorescence at 330 nm and 350 nm were monitored between 20°C and 90°C with a $1^{\circ}\text{C}/\text{min}$ increasing temperature ramp on

a Prometheus NT.48 instrument (Nanotemper) using standard grade capillaries (Nanotemper ref. PR-Coo2). T_m values were determined at the inflection points of the first derivative of the tryptophan fluorescence ratio (F_{350 nm}/F_{330 nm}) curves.

Native gel shift assay

Imp β and Rev proteins were initially diluted in phosphate buffered saline (PBS) to 25 μ M, and incubated in a 1:1 Imp β :Rev ratio (3.7 μ M each, 10 μ l final volume) for 5 min. After adding 1 μ l of native loading buffer (62.8 mM Tris HCl pH 6.8, 40% glycerol, 0.01% bromophenol blue), 2.5 μ l of samples were analysed on a 10% TGX (Biorad) gel, run under native conditions (0.5x TAE buffer, 4°C, 110 V, 50 min). Gels were stained with Coomassie blue and scanned on a ChemiDoc MP gel imaging system (Biorad). Protein band intensities were quantified using Adobe Photoshop Version CS5 by integrating pixel intensities in a rectangle encompassing the band and subtracting the background signal determined from an adjacent region of the gel.

Fluorescence polarization binding and inhibition assays

A Rev-NLS-TAMRA peptide, corresponding to the Rev NLS (32-EGTRQARRNRRRRWRERQR-50) N-terminally fused to tetramethylrhodamine (TAMRA) was commercially synthesized (Peptide Specialty Laboratories, Heidelberg). Fluorescence polarization (FP) was measured in black 384-well plates (Greiner ref. 781076, Greiner Bio-One) on a Clariostar plate reader (BMG Labtech) using a 540 \pm 20 nm excitation filter, a 590 \pm 20 nm emission filter and an LP565 dichroic mirror. The FP values were determined using the equation $F = (F_{//} - F_{\perp}) / (F_{//} + F_{\perp})$, where $F_{//}$ and F_{\perp} are the fluorescence intensities parallel and perpendicular to the excitation plane, respectively. The concentration of the Rev-NLS-TAMRA peptide was determined using its specific molar extinction coefficient at 556 nm of 89 000 M⁻¹cm⁻¹.

Binding assay

30 μ l of Imp β (6.25 μ M) were prepared in Fluorescence Polarization (FP) buffer (20 mM Tris pH 8, 100 mM NaCl, 0.05% Tween 20, 1 mM DTT) and used to perform 14 two-fold serial dilution of Imp β (3.1 μ M to 0.2 nM) by mixing 15 μ l of sample with 15 μ l of FP buffer. A sample containing only FP buffer was used as a reference. 2x Rev-NLS-TAMRA dilution buffer was prepared containing 200 nM of Rev-NLS-TAMRA diluted in FP buffer and dispensed into 16 wells of a black 384-well plate (10 μ l/well). To each well 10 μ l of Imp β dilutions or reference buffer were added and mixed by pipetting. The plate was incubated for 20 min at room temperature before reading.

Inhibition assays

The concentration of Rev variants were adjusted to 100 μ M in HSB, before preparing a 30 μ l aliquot at 70 μ M using 2x Rev-NLS-TAMRA dilution buffer. Then 14 2-fold serial dilutions of each Rev variant (35 μ M to 4.3 nM) were performed by mixing 15 μ l of sample with 15 μ l of Rev dilution buffer. A sample containing only Rev dilution buffer was used as a reference. A 2x Imp β solution containing 390 nM Imp β and FP buffer was prepared and dispensed into 16 wells of a black 384-well plate (10 μ l/well). In each well, 10 μ l of Rev dilutions or reference buffer were added and mixed by pipetting. The plate was incubated for 20 min at room temperature before reading.

Curves obtained with each phosphomimetic mutant were independently fitted and the IC₅₀ values were determined with GraphPad Prism 6 software. The option “Log [Agonist] vs. response (3 parameters)” was used to fit binding data to the equation $F = F_{min} + (F_{max} - F_{min}) / (1 + 10^{\log EC_{50} - X})$, where F_{min} and F_{max} are the minimal and maximal values of fluorescence polarization and X is the common logarithm of Imp β concentration. The option “Log [Inhibitor] vs. response (3 parameters)” was used to fit inhibition data to the equation $F = F_{min} + (F_{max} - F_{min}) / (1 + 10^{X - \log IC_{50}})$, where X is the common logarithm of Rev concentration.

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Ethics Statement

Not applicable.

Citations

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