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Characterization of Microtubule Depolymerization by the HIV Protein Rev

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Characterization of Microtubule Depolymerization by the HIV Protein Rev

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Shimpi Bedi ENTITLED Characterization of Microtubule Depolymerization by the HIV Protein Rev BE ACCEPTED AS PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

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Characterization of Microtubule Depolymerization by the HIV Protein Rev.

The HIV-1 Rev protein enables the nucleocytoplasmic export of unspliced or partially spliced mRNAs that is required for the synthesis of structural proteins. By doing so, it regulates the switch to the late phase of the viral replication cycle (Cullen, 1992). This regulatory control over viral replication makes Rev an attractive target for anti-viral intervention. The development of anti-viral remedies is hindered because the three-dimensional structure of Rev has not yet been solved by X-ray crystallography and NMR. Rev, which polymerizes into regular hollow filaments at high concentrations, forms side-to-side and end-to-side interactions making it prone to aggregation and precipitation (Wingfield *et al*., 1991). Watts *et al*. (2000) in an attempt to solve the solubility of Rev discovered a novel interaction between Rev and tubulin. They observed that Rev filaments react with microtubules (MTs) to form Rev-tubulin toroidal (RTT) complexes showing that Rev is a microtubule depolymerizing agent that possibly mimics the mechanism used by Kinesin-13 proteins, themselves potent microtubule depolymerases.

The first goal of the experiments conducted here was to develop a sedimentation assay capable of measuring Rev stimulated microtubule depolymerization. Under the conditions employed here, Rev tubulin toroidal complexes (RTTs) were not formed due to limiting concentrations of magnesium ions so that the amount of

tubulin released from microtubule polymers would not reform high molecular weight complexes that would sediment in our assays.

Initial experiments determined that bacterial expressed Rev was capable of depolymerizing GMPCPP stabilized microtubules. Depolymerization was not affected by the oligomeric state of Rev. Rev polymerized into filaments or maintained as monomers by the addition of high salt concentrations were equally able to depolymerize microtubules. Microtubule depolymerization appears to be partially dose dependent and occurs at concentrations as low as 300 nM. At low concentrations of Rev, more tubulin is released from the microtubule polymer than there is Rev present in the reaction. This suggests that Rev either has higher affinity for microtubule ends in the lattice or that Rev multimerization is important for depolymerization activity. Depolymerization occurs quickly which is consistent with the findings of Watts *et al*. (2000). In contrast to the findings of Watts *et al.* (2000) who demonstrated a complete disappearance of Taxol stabilized microtubules when treated with Rev, Rev was unable to completely depolymerize microtubule polymers stabilized by GMPCPP.

Key words: HIV, Rev, Kinesin-13, microtubules, tubulin

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Introduction

Human immunodeficiency virus-1 (HIV-1) causes human acquired immune deficiency syndrome (AIDS) (Cullen, 1991). Productive infection requires the expression of proteins encoded by singly spliced or unspliced viral mRNAs. Expression of these proteins requires Rev, a 116 amino acid long, 13 KDa viral transactivator that interacts with one or more cellular factors to promote the export of these RNAs (Cullen and Malim, 1991). In the absence of Rev, these mRNAs are retained in the nucleus and are either spliced to completion or are degraded (Pollard and Malim, 1998). Mutational studies and proviral rescue assays demonstrate that Rev is essential for virion production and viral replication (Feinberg *et al.,* 1986). This ability of Rev to regulate the replication of virus makes it a viable target for anti-viral drugs. Presently, reagents that reduce Rev expression (e.g. RNAi, ribozymes, and antisense oligodeoxynucleotides) or interfere with Rev function (e.g. decoys, antibiotics, and transdominant mutations) illustrate the potential for effectively inhibiting viral infection by inhibiting Rev function (Dayton *et al.*, 2000).

Rev is a 13 KDa RNA binding phosphoprotein that shuttles between the nucleus and cytoplasm of expressing cells. It mediates the early to late shift in viral gene expression (Cullen, 1998). Rev is imported into the nucleus due to the presence of arginine-rich motif (ARM) that contains a nuclear localization signal (NLS) (Figure 1). The NLS interacts directly with importin-β that targets Rev to nuclear pore complex (Truant and Cullen, 1999). This interaction is disrupted by Ran-GTP (Pollard and Malim, 1998) releasing Rev into the nucleus where it is free to bind the

Rev response element (RRE). The RRE is an RNA stem-loop structure present within the 3' intron present in incompletely spliced or unspliced viral mRNAs (Pollard, Malim 1998). Nuclear export of Rev-RRE complexes is mediated by a nuclear export signal (NES), a stretch of leucine-rich residues (residues 73-83) present towards the *C*-terminus of the protein. The NES interacts with the export factor Crm1 (Chromosome Region Maintainenance gene 1)/exportin 1 and Ran-GTP that together target Rev-RRE complexes to the cytoplasm. After the dissociation of the Rev/RRE/CRM1 complex in the cytoplasm, Rev re-enters the nucleus for another round of transport (Hope, 1999).

Multimerization of Rev on the RRE ensures that cellular concentrations must be sufficiently high and there is not a premature shift to late gene expression. Multimerization provides an explanation for the high incidence of latent infection by HIV-1 (Malim and Cullen, 1991). Mutagenesis has identified (residues 18-26 and 54-56) that flank the ARM (residues 34-50) as being important for multimerization (Malim and Cullen, Brice et al., 1991). Rev cooperatively binds the RRE through protein-protein and protein-RNA interactions (Heaphy *et al*., 1991). *In vitro* assays show that eight or more Rev monomers may be bound to a single RRE (Malim and Cullen, 1991). Studies on Rev-RNA co-assemblies show that Rev monomers first bind the RRE (Malim *et al*., 1990) and then multimerizes rather than Rev multimers forming as a requirement to bind the RNA. Consequently, Rev-RNA complexes form at lower protein concentrations than are known to be required to form multimeric filaments (Malim and Cullen, 1991). NMR spectroscopy of Rev peptide bound to stem-loop IIB of the RRE showed that the amino acids that make

important base-specific contacts with the RRE are Arg^{35}, Arg^{39}, Asn^{40}, and Arg^{44} (Battiste *et al*., 1996). Thr^{34} forms both peptide-peptide and RNA-peptide interactions and makes contact with the phosphate backbone of the RRE. In addition to Thr, six arginines at amino acid positions 38, 41, 42, 43, 46, and 48 make either hydrogen-bonding or simple electrostatic interactions with the phosphate backbone on the RRE (Battiste *et al*., 1996).

Biochemical characteristics of Rev

Depending on its concentrations, Rev may persist as monomer, an oligomer, or long hollow filament. In low salt buffers (50 mM NaCl), filament formation occurs at Rev concentrations greater than 80 µg/ml by a process that is temperature dependent (Heaphy *et al*., 1991). Rev filaments consist of dimers arranged in a six-start helical pattern in which *N*-terminal helical segments are present in the inner walls of the tubes (Wingfield *et al*., 1991, Watts *et al.,* 1998). Filament formation is not dependent on the multimerization domains present on either side of the ARM since mutants deficient in multimerization (M4 and M7) are capable of forming filaments (Watts *et al*., 1998). Multimerization refers to the oligomerization of Rev monomers at the high affinity site on the RRE whereas filament formation refers to Rev polymerization into long, stable tubes (Watts *et al.,* 1998). Rev filaments form side-to-side and end-to-side interactions making them prone to aggregation and precipitation (Wingfield *et al*., 1991).

The three-dimensional structure of Rev has not been solved by X-ray crystallography and NMR because of its tendency to aggregate and precipitate.

Nonetheless, there is substantial low-resolution structural information obtained by biochemical techniques and mutagenesis. Circular dichroism measurements of Rev mutants in which residues 68-112 or 93-112 were deleted indicated that the *N*-terminus contains most of the helical properties (Auer *et al.*, 1994). This confirmed the computer predictions suggesting Rev possesses an *N*-terminal $helix_1$-loop-$helix_2$ motif (Auer *et al*., 1994). There is approximately 50% α-helix and 25% β-sheet. The *N*-terminal of $helix_1$ and the *C*-terminal of $helix_2$ (corresponding to residues 9-24 and residues 34-62 respectively) likely contact each other in a manner exposing a hydrophobic patch comprised of residues Leu18, Ile19, and Leu22 on $helix_1$ and I52, I55, and I59 on $helix_2$ that is involved in intermolecular binding (Thomas *et al*., 1998). Thus, the two multimerization domains form a single exposed hydrophobic surface patch that form a surface for interactions with other Rev molecules (Thomas *et al.*, 1997). This model is confirmed by solid-state NMR analysis on Rev fibrils that further shows that both monomeric and filamentous Rev have similar helix contents (Blanco *et al*., 2001). Not much is known about the *C*-terminus except that the circular dichroism indicate that this half is partly helical (Havlin *et al*., 2007).

Rev-MT Interactions

To facilitate drug design, Steven and collaborators (Wingfield *et al*., 1991, Watts *et al*., 1998, 2000) attempted to determine the solution conditions suitable to study the structure of Rev. Rev normally tends to aggregate in solution at concentrations required for crystal growth (Thomas *et al*., 1997, Wingfield *et al*., 1991, Heaphy *et al*., 1991, Karn *et al*., 1991). Since the presence of arginine-rich region makes Rev highly basic (pI= 9.2), they predicted Rev would behave better with an acidic

partner or acidic environment. Watts *et al*., (2000) found that Rev filaments could be depolymerized effectively by polyanions such as poly G, poly dG, and polyglutamate. As polyglutamate is found in the *C*-terminus of the tubulin monomers that are positioned on the surface of MTs, Watts *et al*. (2000) suggested that solubility of Rev might be improved with the addition of tubulin. Tubulin heterodimers, of course can be polymerized into microtubules (MTs) that play important roles in cell division, cytoplasmic organization, and maintenance of cell polarity (Desai *et al.*, 1999). They predicted that there would be an interaction between the basic ARM of Rev and the acidic polyglutamate tracts on the tubulin monomers.

Preliminary investigations were made by mixing Rev filaments with taxol stabilized MTs or colchicine-treated tubulin heterodimers. Watts *et al*. (2000) observed that purified Rev depolymerizes MTs and forms bilayered ring like structures, called Rev-tubulin toroidal complexes (RTTs), comprised of Rev and tubulin (Watts *et al*., 2000). Rev monomers line the inside and tubulin monomers line the outside of these rings. Rev-tubulin rings are similar to those formed when MTs are exposed to cold temperatures (Melki *et al*., 1989) or certain anti-mitotic drugs, and in the presence of Kinesin-13 proteins, a unique sub-family of non-motile MT depolymerizing proteins (Moores *et al*., 2003). Reciprocal titrations showed that the excess protein remained in the filamentous form, indicating that depolymerization and formation of RTTs were not a buffer effect (Watts *et al*., 2000). The RTTs form even in the absence of tubulin's polyglutamate tail suggesting that this interaction is more specific than a simple electrostatic

interaction between the ARM of Rev and the acidic polyglutamate residues of the tubulin heterodimer (Watts *et al*., 2000).

The formation of RTTs was surprising since it implies that Rev, a small nucleolar protein, has the ability to interact with and depolymerize MTs. Furthermore, ring formation is not inhibited by the MT stabilizing drug taxol, the MT destabilizing drug colchicine, or microtubule-associated (MAPS) that usually stabilize and promote the assembly of MTs (Watts *et al*.,2000).

Mechanism of Rev-tubulin interaction

A possible explanation for the binding site of Rev on the microtubules comes from the observation that formation of RTTs complexes can be blocked by maytansine (Watts *et al*., 2000). Maytansine, and other drugs like the vinca alkaloids and vinblastine, compete for a different site near the exchangeable nucleotide site on the β-tubulin (Rai, 1998). Therefore, Rev might bind β-tubulin at or near the vinca site forming the basis of Rev-MT interactions. Depolymerization activity is encoded within Rev's first 59 amino acids where there is a sequence similarity to the motor domain of non-motile, MT depolymerizing Kinesin-13 proteins (Figure 2).

The kinesin superfamily is a class of motor proteins that use ATP to translocate along microtubules or to alter MT structure (Vale *et al*., 1997). These functions are mediated by a highly conserved 350 amino-acids force generating element called motor domain. This domain contains both an ATP binding/hydrolysis site and microtubule binding sites (Woehlke *et al.,* 1997, Ogawa *et al*., 2004).

Mutagenesis indicates that these highly conserved, positively charged MT-interacting residues are located in three loops on the surface of the motor (Woehlke *et al.,* 1997).

MCAK, the best-studied member of the Kinesin-13 family, shares a sequence similarity within the motor domain with other members of kinesin superfamily (Wordeman and Mitchison, 1995). Kinesin-13 proteins are characterized by the unique position of the conserved kinesin motor domain in the middle of the polypeptide (Desai *et al*., 1999). Mutagenesis has shown that *N*-terminus of MCAK is responsible for kinetochore binding but is not required for MT depolymerization (Walczak, 2003). Multimerization facilitates efficient depolymerization of MTs even though the monomeric protein can depolymerize MTs (Wordeman *et al*., 1995, Ems-McClung *et al*., 2007). The catalytic motor domain is capable of causing depolymerization on its own but the presence of neck (residues present *N*-terminus to motor) enhances the efficiency of activity (Wordeman *et al*., 1995, Walczak, 2003). The *C*-terminus is responsible for dimerization of MCAK and prevents lattice-stimulated ATPase activity (Desai *et al*., 1999).

Most of the residues that are highly conserved between Rev as well as XMCAK and KIF2C (murine ortholog of MCAK) reside in helix 2 of Rev and helix 4 (α4) of XMCAK. Studies done with KIF2C (ADP bound form) by Ogawa *et al*. (2004) showed that the main microtubule binding helix (α4) is stabilized by Glu521, Ile523, and Arg524 of α4 and residues 580, 581, and 583 of α6 by electrostatic and hydrophobic interactions. As seen in the alignment of Rev with the motile KIF1A and the non-motile MT depolymerizing Kinesin-13 proteins, there are some residues common between Rev and KIF1A (Figure 2). However, there is more homology with

non-motile kinesins than with motile kinesin KIF1A. This indicates that Rev might also behave like non-motile MT depolymerizing kinesin MCAK. Also, residues similar with MCAK and Rev but not KIF1A might be important for depolymerization. It is noteworthy that many of the residues shared between Rev and MCAK are not present in motile kinesins such as KIF1A suggesting that the shared residues may be important for MT depolymerization.

Two MCAK homologs- KIF2C and pKinI (*Plasmodium falciparum*) (Ogawa *et al*., 2004, Shipley *et al*., 2004) (Figure 3) specifically bind MTs at the ends (Desai *et al*., 1999) or reach the ends by one-dimensional diffusion (Hunter *et al.*, 2003). Once these proteins reach the MT ends, they cause a conformational change within the microtubule lattice leading to depolymerization. ATPase activity is not required in the early stages of depolymerization and is stimulated after the induction of curvature in the MT. The curvature is seen only when bound to curved tubulin either in GDP bound form or at the ends where depolymerization has started (Shipley *et al*., 2004). This is supported by the observation that KIF2C can be readily docked onto a curved MT structure but not a straight MT (Ogawa *et al*., 2004). By comparison, Rev also inhibits the formation of microtubule asters formed *in vitro* from mitotic frog egg extracts suggesting that Rev has the ability to interact with MTs and affect their polymerization state in a cellular environment (Watts *et al*., 2000). Therefore, it is likely that Rev-MT interactions might be similar to Kinesin-13 proteins.

Mutagenesis has identified regions containing highly conserved residues that play an important role in binding and depolymerization of MTs in Kinesin-13s. The mutant constructs were prepared by removing the *N*-terminal domain, the neck and/or

the *C*-terminal domain or by removing amino acids in catalytic domain of the protein. Two of the important regions were- KVD (Lys293-Val294-Asp295) and KEC (Lys268-Glu269-Cys270) (Shipley *et al*., 2004). The KVD motif in loop L2 makes a rigid, finger-like structure extending out from the catalytic core. Each residue in KVD is critical for causing MT depolymerization since mutating even one of the residues results in reduction of activity by 30-50% (Ogawa *et al.*, 2004). Deletion of all three residues results in 11-fold reduction in activity (Ems McClung *et al*., 2007). However, these mutations have no effect on MT binding. Mutations in KEC, however, results in lower MT binding (Ogawa *et al*., 2004). Mutating one of the residues within KEC motif (E529A) causes 4.5-fold reduction in depolymerase activity of MCAK. Another mutation (R522A) within α4 causes 2-fold reduction in depolymerase activity of MCAK (Ems McClung *et al*., 2007).

Residues 34-70 in Rev correspond to residues 506-543 in XMCAK that reside in helix 4 and the region responsible for MT binding (Watts *et al*., 2000). The helix 2 in Rev contains most of the Rev-XMCAK shared residues. The corresponding region of Kinesin-13 that is similar to Rev is L11-α4-L12, a region that provides anchorage around the switch II cluster on the MT surface and plays an important role in interaction with MTs (Shipley *et al*., 2004) (Figure 3). Residues shared between Rev and XMCAK are exposed to the surface and therefore are free to interact with microtubules. RevE57A has a high probability of being surface exposed as crystallographic data suggests that the corresponding residue in XMCAK (E529) is surface exposed (Ogawa *et al*., 2004). This residue is conserved in Kinesin-13 proteins and upon mutation causes 4.5 fold reduction in depolymerization activity

(Ems-McClung *et al.*, 2007). RevE47 is common to most Kinesin-13 proteins and may play a role similar to RevE57 (Ogawa *et al.*, 2004). Rev R50 corresponding to XMCAK R522 might have a role in MT binding rather than depolymerization since mutation of this residue in XMCAK caused two-fold reduction in the activity (Ems-McClung *et al.*, 2007). Both E57 and E47 might be capable of destabilizing microtubules since the microtubules are destabilized by their own E-hooks (*C*-terminal polyglutamate tracts of tubulin monomers exposed on the surface).

If Rev binds and depolymerizes MTs in a mechanism comparable to that of Kinesin-13 proteins, shared residues should play similar role of either MT binding or depolymerization in both proteins. Both cause curved structures when mixed with stabilized MTs and depolymerization occurs from both ends of the MT. A limited region of Rev shows statistically significant 36 amino acid similarity with the catalytic domain of XMCAK and KIF2C. Some of these residues are not conserved in motile kinesin KIF1A indicating that these residues may play an important role in MT depolymerization since motile kinesins lack depolymerase activity.

Specific Aims

The long-term goal of this research is to identify residues of Rev that are responsible for binding and depolymerization of MTs. By comparing the depolymerization activity of wild-type Rev with Rev that has been mutated in the area of sequence similarity with Kinesin-13 proteins, it should be possible to identify residues that are directly involved in MT binding and depolymerization. To do this, the assays for measuring interactions with MTs need to be developed and optimized. I propose to optimize microtubule depolymerization assays using wild-type Rev that will allow us to test the hypothesis that Rev depolymerizes MTs by a mechanism comparable to Kinesin-13 proteins. Specifically, I propose to duplicate the experiment done by Watts *et al*., 2000 whose experiment demonstrated the formation of Rev-tubulin rings when taxol-stabilized GTP treated MTs were mixed with Rev. Dissection of the Rev-induced depolymerization of MTs required (a) purification of tubulin (b) purification of Rev (c) adapting sedimentation assay to measure the time-dependence and dose-dependence of Rev-MT interaction.

Materials and Methods

SDS-PAGE and immunoblotting

Protein samples were prepared by mixing 10 µl of protein with 5 µl of 2 X SDS-PAGE protein sample buffer, boiled for 3 min, and the entire volume was loaded and proteins separated by size on polyacrylamide gel (%T=15%) using SDS-PAGE at constant current of 30 mA/gel for 60 min. The gels were recovered and stained overnight and destained with gel destaining solution until the protein bands were visible without background. Images of the gels were captured using Adobe Photoshop and analyzed using NIH-image to determine protein concentration.

For immunoblotting, protein samples were boiled in SDS-PAGE 2 X protein sample buffer, resolved by SDS-PAGE and transferred to a nitrocellulose membrane equilibrated in 1X transfer buffer at 50W, 100 mA, and 50V for two hours. The membrane was stained using ponceau stain and destained using 5% acetic acid solution until the protein bands were seen on the membrane. The blots were washed with TBST until the pH was 7.4 and incubated in 5% milk TBST blocking solution overnight. The blocking solution was replaced with 1X TBST, washed three times every 10 minutes (22˚C) on a rocking table. The blot was allowed to incubate for 1 h in 10 ml of primary antibody [1^0 Ab, Tubulin-specific DM1α-1:5000 and Rev specific rabbit antiserum- 1:2000] solution prepared in 1X TBST. After 1 h, the primary antibody solution was recovered and replaced with 1X TBST and washed three times every 10 min. After the washes, the blot was allowed to incubate in 10 ml of secondary antibody solution [1:100,000 HRP-conjugated

goat antimouse, 1:100,000 goat antisheep] prepared in 1X TBST (pH 7.3) for 1 h. After the blot was washed three times using 1X TBST pH 7.3, it was removed to a dish containing 10 ml of Pierce SuperSignal West Pico Chemiluminescent substrate for detection of HRP for five minutes. Image of the blot was captured using chemiluminescence and protein bands quantified using Fuji Image Gauge software (V2.02).

Rev expression and purification

a. Plasmid isolation and test expression of Rev

The plasmid pET11d-Rev was kindly supplied by Francisco Blanco (Blanco *et al*., 2001). Plasmid DNA was transformed into chemically competent DH5α cells (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Cells were grown overnight at 37˚C with 225 rpm shaking in Luria-Bertani (LB) broth supplemented with 100 µg/ml ampicillin (LB/amp). Stocks of overnight cultures of transformed cells were stored in 15% glycerol at -80˚C. Plasmid DNA was purified from 10 ml LB/amp cultures using plasmid isolation kits Qiagen (Valencia, CA).

pET11d-Rev DNA was transformed into chemically competent BL-21(DE-3) cells (Invitrogen, Carlsbad, CA) as described above. 10 ml LB/amp cultures with single ampicillin-resistant colonies were grown at 37˚C with 225 rpm shaking. Expression was induced for two hours with the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. Cells were harvested by centrifugation at full speed in a benchtop clinical centrifuge at 4˚C. The recovered pellet was resuspended in 1 ml of Buffer A (400 mM NaCl, 50 mM Tris-HCl pH

8.0, 1mM EDTA, 1 mM DTT, 0.5 mM phenyl-methylsulfonylfluoride (PMSF), sonicated (3 x 30 seconds), subjected to centrifugation at 4˚C. Both soluble and insoluble fractions were diluted with an equal volume of 2X gel sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue), boiled for 3 min and resolved by 15% protein gels. Gels were stained with 0.1% coomassie brilliant blue (CBB) in buffer and destained with 35% methanol, 10% glacial acetic acid or immunoblotted. Protein fractions from induced and non-induced cultures for both DH5α and BL-21(DE-3) cells were seen on a coomasie stained gel and western blot to check the expression level (Figure 3). Once high level of expression of Rev was seen in the induced BL-21(DE-3) cells, a larger bacterial culture (1-3L) LB broth (100 µg/ml ampicillin) was set up at 37˚C.

b.Large scale expression of Rev

A large scale (1-3 L) LB broth-ampicillin bacterial culture was inoculated from a 50-150 ml overnight culture and grown with constant shaking (225 rpm) at 37˚C. The growth of the culture was checked every 30 min in the spectrophotometer by measuring absorbance at 600 nm length until it measured 0.6. Cells were then induced for two hours by the addition of 1 ml of 1M stock of IPTG (1 mM final concentration) and harvested by centrifugation by spinning at 4,225 x g (Sorvall rotor GS-3) at 4^{o}C. Recovered cell pellets were frozen in -20˚C until cell lysis.

c.Purification of Rev

Lysis: Cell pellets were weighed, thawed on ice, mixed with 11 ml of chilled buffer A (400 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF), mixed thoroughly by vortexing, and sonicated 3 x 30 sec. The mixture was frozen in dry ice, thawed, and sonicated as described above. Lysate was subjected to centrifugation at 11,952 x g in a Sorvall centrifuge at 4˚C and the supernatant was recovered that was either frozen at -80˚C or applied to a Q-sepharose column.

FPLC: Rev was purified using an AKTA FPLC with Frac-950 as fraction collector (Amersham Biosciences). Clarified cell extracts were loaded into a 50 ml superloop and applied to a HiPrep 16/10 Q FF column equilibrated in 100 ml of buffer A. After washing the column with five column volumes of buffer A (100ml) and when the $O.D_{280}$ of the eluant reached below 0.05, Rev was eluted with three column volumes of buffer B (800 mM NaCl, 50 mM Tris- HCl pH 8.0, 1 mM EDTA, 1 mM DTT). Column fractions were resolved by SDS-PAGE (%T=15%) to confirm the presence of Rev. Peak fractions containing Rev (25-30 ml total volume) were pooled together and applied to a HiPrep 16/10 Heparin FF column equilibrated in 80 ml of buffer B. The column was washed with five column volumes of buffer B or C (1 M NaCl, 50 mM Tris- HCl pH 8.0, 1 mM EDTA, 1 mM DTT) until the $O.D_{280}$ of the eluant reached below 0.05. A step gradient was applied and Rev was eluted with 3 column volumes of buffer D (2 M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT). Fractions containing Rev were

pooled. In some instances, Rev was concentrated by ultrafiltration using Centricon-30.

In case of mutants, Rev was subjected to ammonium sulfate precipitation by adding concentrated stock of the salt to Rev from the heparin column to concentrate the protein. After centrifugation at 4˚C at full speed in a microfuge and resuspending the pellets in Karn D buffer (100 µl), the concentration of Rev was determined spectrophotometrically by measuring the absorbance at 280 nm wavelength and also densitomterically using ImageJ by comparing the intensity of Rev bands to BSA standards.

Rev refolding: Fractions containing Rev from the heparin column were pooled, denatured in 6M urea, and subjected to dialysis against dialysis buffer 1 (50 mM sodium phosphate, 150 mM sodium chloride, 600 mM ammonium sulfate, 50 mM sodium citrate, 1mM EDTA pH 7.0), dialysis buffer 2 (50 mM sodium phosphate, 150 mM sodium chloride, 50 mM sodium citrate, 1mM EDTA pH 7.0), and dialysis buffer 3 (20 mM Hepes, 100 mM sodium chloride, 50 mM sodium citrate, 1 mM EDTA pH 7.0) by changing the buffer solutions twice a day at 4˚C as described by Watts et al., 1998, 2000. Dialysis buffer 3 served as the storage buffer. The Rev filaments were concentrated using either centricon-30 (1,500 x g for 10 min) or by pelleting filaments at 90,000 rpm for four hours in TLA100.3 rotor at 4^{o}C (Watts et al., 1998). Rev filaments were stored at 4˚C at all times.

Concentration determination: The concentration of Rev solutions was detected both spectrophotomterically by measuring absorbance at 280 nm (E_{280}=8.34 mM) and densitometrically as mentioned above.

Purification of tubulin

Tubulin was purified by repeated cycles of polymerization at 37˚C and depolymerization at 4˚C cycles in the presence of ATP, GTP, $MgCl_2$, and glycerol and subsequently chromatographed over the phosphocellulose column (Desai and Walczak, 2001). Four fresh bovine brains were transported to the lab in a large cooler containing 3 L precooled 1.5% NaCl. Meninges and clots were stripped from the brains at 4˚C and the brains were weighed and homogenized with 1-1.1 volumes of PB (Pipes buffer: 0.1 M Pipes, pH 6.8, 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM ATP) twice for 15 s in a blender until homogeneous. The suspension was poured into SS-34 centrifuge tubes and spun for 45 min, K factor 714 at 4˚C. The supernatant was recovered and supplemented with pre-warmed glycerol (50% volume), ATP (final concentration 1.5 mM), GTP (final concentration 0.1 mM), and $MgCl_2$ (final concentration 3.5 mM). This mixture was mixed vigorously and incubated at 37˚C water bath for 30 min. The mixture was spun in a centrifuge at 35˚C in a GSA rotor at 25,805 x g for 306 min, K factor 1057.9 and the gelatinous pellets were transferred to a beaker on ice and resuspended in 10 ml cold PB with ATP and BME. Pellets were solubilized by douncing until homogeneous and transferred to a second chiller beaker. Douncing was repeated a second time and after incubation on ice for 40 min, the mixture was

spun for thirty min at 4˚C in 50.2 Ti rotor at 40,000 rpm for 30 min . The supernatant was decanted into a graduated cylinder, supplemented with pre-warmed 50% glycerol, GTP (0.5 mM final concentration), and $MgCl_2$ (4 mM final concentration), and allowed to polymerize at 37˚C for 40 min. The mixture was spun in a 50.2 Ti rotor at 40,000 at 35˚C for 30 min and the recovered pellets were kept warm until the supernatant was decanted from them. The pellets were scooped in a beaker set on ice and resuspended in CB (50 mM Pipes, pH 6.8, 1 mM EGTA, 0.2 mM $MgCl_2$), dounced until smooth and the protein concentration checked by Bradford using BGG as standard. After incubation on ice for 40 min, tubulin was centrifuged at 4˚C in a 50.2 Ti rotor at 40,000 for 30 min. Supernatant containing the tubulin heterodimer was recovered and chromatographed on phosphocellulose column (approx. 30 cm high x 3.2 cm ID-volume 200 ml) at a flow rate of 2 ml/min. Tubulin (phosphocellulose column or PC) eluted after 100 mls had passed through the column and the concentration of the fractions was checked again. Fractions that had greater than 1mg/ml tubulin were combined. The column was washed with 5 volumes of 1M KCl in CB to elute MAPS. The tubulin and MAPS were aliquoted in smaller fractions, frozen in liquid nitrogen, and stored at -80˚C. The PC column was equilibrated with 10 volumes of CB (0.1% NaN_3) for storage.

Rev-Microtubule Sedimentation assay

Single aliquot (200 µl) of PC tubulin was quickly thawed, diluted with MEM buffer (100 mM MES, 1 mM $MgCl_2$, 1 mM EGTA pH 6.4), and incubated on ice for 10 min, and spun at 4˚C using an ultracentrifuge in a chilled TLA100.3 rotor (350,000 x g for five min). The supernatant containing tubulin heterodimer was

transferred to an eppendorf tube, mixed with GMPCPP, and placed in a 37˚C water bath for 30 minutes. The rotor (TLA100.3) was warmed to 37˚C for the next spin. After an incubation time of 30 min, tubulin was transferred to an ultracentrifuge tube, and microtubules (MTs) were pelleted at 35˚C (350,000 X g for five minutes). The pellet was dissolved and brought up in 30-40 µl of MEM buffer (22˚C) by pipetting up and down and stored at room temperature until further use. A small volume (3 µl) of MTs was removed and mixed with 57 µl of chilled MES/$CaCl_2$, incubated on ice for 15 min, and the concentration of tubulin was determined spectrophotometrically using the coefficient (ε_{280} = 115,000 $M^{-1}cm^{-1}$). The stock microtubules were diluted to a final concentration of 3 µM in MEM buffer to set up reaction mixes with either different concentrations of wild-type Rev or buffer alone.

Wild-type Rev protein solution (stock concentration 45 µM) or buffer alone (MEM buffer) were added to polymerized microtubules to the final concentrations as indicated in the figures (0.3 pM to 3 µM). Rev was diluted just before addition to the MTs. Reaction was started by the addition of Rev (molar ratio of 1:1) to the diluted MTs. A volume of 20 µl of the reaction mixes was aliquoted in centrifuge tubes and allowed to incubate at 22˚C until spun in the ultracentrifuge. After an incubation time of 10-60 minutes, the tubes were placed in TLA100 rotor warmed at 22˚C, and centrifuged at 350,000 x g for five minutes. The supernatant (20µl) was carefully recovered to labeled eppendorf tubes containing 20 µl of 2X sample buffer, boiled for 3 min, and stored at -20˚C. The pellets were mixed with 40 µl of 1X sample buffer by pipetting up and down several times, transferred to an eppendorf tube, boiled for 3 min, and also stored at -20˚C.

The proteins in the supernatant and pellet fractions were resolved by SDS-PAGE (%T=15%). Gels were either stained with CBB or transferred electrically to a nitrocellulose membrane equilibrated in 1X transfer buffer as described above. The amount of percentage of tubulin in the supernatant of no Rev control was subtracted from the supernatant of MT-Rev mixtures. The percentage of tubulin released in the supernatant was plotted as a function of time and against different concentrations of Rev. Finally, the number of moles of Rev added was plotted against the number of moles of tubulin released in the supernatant for the concentration dependent experiments.

Statistical Analysis

All analyses were conducted using SAS version 9.2 by Mrs. Beverly K. Grunden, Statistical Consultant, at Wright State University. For time dependence, it was determined if the regression line for the date is significantly different from zero. A linear regression was run with percentage of tubulin as the dependent variable and time as the independent variable. For the dilution and time dependence, model assumptions could not be met by the raw data or by transformation. This was due to having too few observations at the higher levels of dilution, yielding a variance of zero at two of these levels. Therefore, a one-way ANOVA was used that ignored time, effectively pooling all the data at a given dilution level into one group. For the centrifugal speed analysis, a two-way ANOVA with percent of tubulin as the dependent variable and factor speed (5 levels) and group (2 levels: control or Rev+MT) was used. The model was tested by removing the interaction term as it

was not significant. Posthoc tests were run using Tukey's HSD to test the overall level of significance.

Results

This study attempted to measure MT depolymerization stimulated by the addition of Rev using sedimentation assay commonly used to quantify the depolymerase activities of Kinesin-13 proteins (Desai *et al*., 1999). The buffers used in this study were derived from Watts *et al*. (2000) who first demonstrated the formation of Rev-tubulin rings in MES buffers (see Materials and Methods). Briefly, Rev was mixed with GMPCPP-stabilized MTs and, after a period of incubation (10-60 min), Rev-MT mixture was centrifuged to separate tubulin released from the MT polymer. Proteins present in the supernatants and pellets were then subjected to SDS-PAGE and the amount of tubulin was quantified by densitometry as described in the Materials and Methods. Because this assay required microgram amounts of highly purified Rev and tubulin, my first specific aim was to purify proteins required for these assays: wild-type Rev protein and tubulin heterodimers.

Purification of wild-type Rev

Previous studies have shown that biologically active protein can be expressed in *Escherichia coli* cells (Wingfield *et al*., 1991, Watts *et al*., 1998, 2000, Brice *et al*., 1999). The expression plasmid encoding wild-type Rev used in this study (pET11d-Rev), a generous gift from Blanco et al. (2001), was transformed into competent BL-21(DE-3) cells (Invitrogen, Carlsbad, CA) and expression of Rev protein was confirmed using SDS-PAGE (Figure 4). To optimize Rev expression to maximize yields of full-length Rev while minimizing the yield of truncated protein, test

experiments were conducted with 10 ml cultures that showed that incubation longer than 3 hours resulted in an accumulation of Rev that appears proteolytically clipped (data not shown). With the conditions optimized, Rev was purified from 1-2 L cultures by FPLC methods described by Karn *et al*. (1995). Representative results are shown in Figure 5. Cell lysates clarified by centrifugation were chromatographed using a Q-Sepharose column that isolated Rev-RNA complexes. Column fractions were resolved by SDS-PAGE and typical results are shown in Figure 5 C. The vast majority of whole cell extract (WCE), a highly concentrated mixture of proteins of different molecular weights, did not bind the Q-sepharose column as indicated by the amount of protein in the flow-through (FT) fractions and the substantial peak on the chromatogram. After washing the column with three column volumes of buffer containing 400 mM NaCl (CW), there is a elution of proteins with 800 mM NaCl (EL) (Figure 5A, C) that contained Rev along with relatively small amount of contaminating proteins. The presence of Rev was confirmed by western blotting (not shown).

Rev containing fractions were pooled and applied to a heparin-sepharose column. Representative results are shown in Figure 5 B. The flow-through fractions (FT) that did not bind the column do not contain Rev showing that most of the Rev bound the column. Rev eluted from the heparin column in high salt buffer (2M NaCl) corresponding to the single peak on chromatogram and is seen as a single purified band on a coomassie stained gel at 19 kilodaltons (KDa) (Figure 5D). The anomalous gel mobility is consistent with published results (Malim and Cullen, 1989). The eluted protein is antigenically identical to Rev and can be stained using

Rev-specific antibodies.

Purified Rev was denatured with 6M urea and refolded according to the methods described by Watts *et al.* (1998). Under these conditions, Rev assembles into filaments that do not have a propensity to aggregate. Purified Rev appears as a single band when resolved by SDS-PAGE as seen in Figure 5E. The concentration of purified Rev was determined spectrophotometrically as mentioned in Materials and Methods. A representative image of the PAGE-based quantitation is shown in Figure 5E and the graph generated to calculate the concentration of Rev is shown in Figure 5F.

As a rule, 3.4-4.0 g of *E. coli* cells were harvested by centrifugation per liter of culture (Table 1). An average of 6 ml of 0.6 mg/ml Rev was recovered from the heparin-sepharose column and subjected to refolding. The summary of purification attempts is summarized in table 1 below. By comparison, the protein yield has been reported to be about 3.5 mg per gram wet weight of *E. coli* cells (Wingfield *et al*., 1991) and 100 mg/ml (Watts *et al*., 2000).

Table: 1

Yields of purified wild-type Rev from BL-21(DE-3) *E.coli* cells

Attempt	Volume of cell culture	Yield (mg)
1.	1L	1.5
2.	1L	1.1
3.	1L	1.4
4.	2L	2.3
5.	1L	0.6
6.	1L	1.4
7.	1L	0.3
8.	1L	0.9
9.	2L	3.1

Rev purified this way has been analyzed using mass spectroscopy that revealed that the protein is pure indicated by a single peak corresponding to 13 kDa and is free of contaminants (A. Sharma, personal communication). The protein yield has been in the range of 1-1.2 mg/ml and these results were reproducible from purifications of different lots.

Purification of Rev mutants

Attempts to purify mutants of Rev protein defective in multimerization (M4 and M7) or RNA binding (M5 and M6) using similar methods are discussed in detail in Appendix (Figure A1, A2). Purification was hindered by the inability of Rev to bind

the heparin-sepharose column resulting in low concentration of Rev and was associated with contaminating proteins that made it unsuitable for use in biochemical assay. These mutants were excluded from use in sedimentation assay due to the presence of contaminants.

Purification of tubulin

Tubulin used in this study was purified from bovine brains as described in Materials and Methods. Result of the purification is shown in Figure 6. The chromatogram and SDS-PAGE of purified tubulin is shown in Figure 6A. Tubulin is present in the flow-through fractions and appears a single band on a coomassie stained polyacrylamide gel migrating at 50 kDa (Figure 6C). The final concentration of pure tubulin was 22 µM and was aliquoted into 50-200 µl fractions, frozen on liquid nitrogen, and stored at -80^{o}C. Microtubule associated proteins (MAPS) were eluted from the column in 1M KCl corresponding to the peak in the chromatogram (Figure 6B).

Rev storage and aggregation

Rev eluting from the heparin-sepharose column was dialyzed to form filaments as described by Watts *et al*. (1998, 2000). Under the conditions employed, Rev filaments were stable for 4-5 months. As seen in Figure 10, Rev does not show precipitation and falling out of solution when present in elution buffer (2M NaCl) or storage buffer (Citrate buffer). Rev retains activity for 4-5 months at 4^{o}C but there is a loss of depolymerization activity afterwards. Samples stored for a period longer than 5 months show small Rev derived peptides (Figure 11) that correlates with the decrease in activity.

RTTs and the role of magnesium

Watts *et al*. (2000) observed that citrate ions, required to prevent aggregation of Rev in the storage buffer, chelates magnesium ions essential for the formation of RTTs. However, RTT formation is rescued if magnesium is supplemented in excess to the concentration of citrate ions (1-2 mM). When Mg^{2+} is limiting, a soluble Rev-tubulin complex forms. In the presence of 1-2 mM Mg^{2+} excess, RTT complexes form (Watts *et al*., 2000). Under my experimental conditions, there was an excess of citrate relative to magnesium (3.0-12.5 mM and 0.7-0.9 mM respectively). Therefore, RTTs are not likely to be formed under our conditions and instead a soluble Rev-tubulin complex is predicted. The omission of magnesium and therefore the failure to form RTTs should simplify interpretation of results from our sedimentation assays. Since RTTs will not form, all tubulin released should not be pelleted by high speed centrifugation and will not be confused with pelletable MTs.

Measurement of Rev: Microtubule Interactions

With purified proteins in hand, the next aim was to adapt sedimentation assays used to measure MT depolymerase activity of MCAK in order to quantify the ability of Rev to depolymerize MTs. These assays involve mixing MTs with a depolymerizing agent and after a period of incubation, separate high molecular weight MTs from low molecular weight tubulin released from the polymer by centrifugation (Desai *et al.,* 1999). The ultimate goal of this assay is to be able to measure difference in the amount of depolymerization by Rev protein harboring

mutations.

The assays used must have the ideal solution conditions that allows Rev function and allows the measurement of the time and concentration dependent properties of Rev-MT interactions. To quantify Rev:MT interactions in anticipation that mutants will exhibit different activities, It was necessary to develop the buffer conditions for the sedimentation assay and then test the hypothesis that Rev depolymerizes MTs in a time-dependent and dose-dependent manner. Study of Rev induced MT depolymerization required the ability to form functional MTs from tubulin heterodimers that are stable for the time period used in this assay. Wild-type Rev and MTs had to be stable in control buffers to make statistically significant conclusions. Figure 7A shows that when Rev and MTs are separately added to reaction buffers, the percentage of pelletable proteins is constant for one hour. This indicates that Rev filaments dissociate into different sized complexes and does not aggregate during the reaction. Similarly, it shows that GMPCPP stabilized MTs do not depolymerize during the reaction unless a depolymerizing agent is added. Lastly, neither Rev nor tubulin is degraded during the reaction as the total amount of both proteins remains constant in each sample (supernatant and pellet). The mixing of Rev and MTs, however, results in the release of tubulin from the polymer as the fraction of Rev present in the supernatant increases with time (Figure 7C). This confirms the results of Watts *et al*. (2000) that Rev depolymerizes stabilized MTs.

Preliminary experiments were done using a microtubule compatible buffer BRB80/DTT. This buffer was used since sedimentation assays done to characterize

the depolymerase activity of Kinesin-13 used BRB80 as reaction buffer. However, there was poor polymerization of stable MTs in this buffer. This necessitated use of the buffers (MES and MEM) used by Watts *et al.* (2000) (see Materials and Methods). The percentage of tubulin present in supernatant when stabilized MTs were placed in MEM buffer alone was 12-15% indicating that MTs were not destabilized in this buffer. This buffer was subsequently used in the time-dependent and dose-dependent experiments.

Another consideration in the sedimentation assay was to study whether the rate of tubulin release varies with initial state of Rev when it is added to the MT. Rev exists in monomers in high salt buffer. The protein assumes filamentous structure when refolded using the methods described by Watts *et al*. (1998). Therefore, it was imperative to determine if the Rev storage buffer affected MT depolymerization. At low concentrations or in the presence of high salt (>750 mM NaCl), Rev exists predominantly as a monomer. At high protein concentrations or in low salt solutions, Rev forms long hollow filaments that tend to aggregate unless carefully refolded (Wingfield *et al*., 1991, Tycko *et al*.). After Rev is denatured and refolded following the methods of Watts *et al*. (1998), Rev assembles into filaments that do not aggregate. Results show that Rev depolymerizes microtubules regardless of the starting form or the storage buffer the protein is derived from (data not shown). These results also confirm that depolymerization is not due to buffer effect as the buffers that the two forms of Rev are stored in have different salt concentrations. Regardless of the form of Rev added to the reaction, Rev filaments do not completely pellet upon centrifugation and there is a high percentage (45%) of

soluble Rev that stays in the supernatant (Figure 7A). It is possible that there is equilibrium between Rev filaments and oligomers that causes only about half of the protein to pellet. Alternatively, there might be a formation of filaments of non-uniform lengths and centrifugation probably pellets only the larger ones. To further characterize Rev-MT interactions, attempts were made to see whether the products of Rev-MT interactions remain in the supernatant or co-sediment with MT polymers.

To determine whether Rev-MT interactions were time dependent, GMPCPP stabilized MTs were mixed with equimolar wild-type Rev, allowed to incubate for 10-60 min, and Rev-MT mixture was centrifuged to separate the supernatant from pellet. In these experiments, the concentration of MTs was kept the same (3 μM) while titrating against different concentrations of Rev. At this concentration, tubulin shows as a prominent band on a coomassie brilliant blue stained gel. Rev is barely detected with coomassie brilliant blue stained gel but is readily seen with immunoblotting (Figure 7C).

Investigation of time dependence of Rev-MT interaction will demonstrate if the reaction is more complex than the reactants simply interacting together to form products, e.g. rings, spirals etc. Analysis of the data from time dependent experiments (Figure 7A, B) indicates that there is a range of 12-18 % soluble tubulin that stays in the supernatant when MTs are placed in buffer alone. With the addition of equimolar Rev to microtubules, there is a net increase of soluble tubulin heterodimer in the supernatant over 60 min (30-35%) ($p<0.0001$), but the increase in soluble heterodimer is seen as early as 10 min after the addition of Rev (Figure

7C, D). This result is consistent with the observations of Watts *et al*. (2000) who observed transient turbidity immediately after mixing of taxol stabilized MTs with wild-type Rev. Depolymerization can be attributed to the activity of Rev alone since GMPCPP stabilized MTs are stable in buffer for the indicated time period (60 min) (Figure 7A).

To demonstrate that Rev-MT interaction is dose-dependent, increasing concentrations of wild-type Rev were mixed with GMPCPP stabilized MTs, incubated for 10-40 min, and soluble heterodimer and MT polymer were separated by centrifugation. Analysis of the stoichiometry required for Rev to depolymerize microtubules should indicate whether the interaction is enzymatic (like MCAK) or not and further help speculate on the mechanism of Rev induced depolymerization of MTs. Results indicate that this interaction is dose dependent. As seen in Figure 8A, addition of increasing concentrations of Rev the reaction mixture causes increase in the percentage of liberated tubulin in the supernatant. The lowest concentration of Rev that results in a statistically significant amount of tubulin released is 300 nM when mixed with 3 μM MTs (molar ratio 1:10) ($p<0.05$) (see Materials and Methods for statistical test used). The reason for the 30 nM concentration of Rev not causing a stastically significant depolymerization of MTs compared to 0 nM Rev is that the sample size had an N of 1. This suggests that Rev can depolymerize MTs at concentrations as low as 30 nM. Higher concentrations of Rev result in saturation seen at a tubulin: Rev molar ratio of 1:4 (Figure 8). Depolymerization saturates at 37% net tubulin released and the rate of tubulin release slows down gradually at all concentrations of Rev after 10 min. More

tubulin is released than the number of moles of Rev added to the reaction at lower concentrations of Rev (Figure 8A). The average rate of tubulin release is 0.67 pmoles/minute.

It is formally possible that the depolymerization described above might be dependent upon the starting oligomerization state of Rev and this may explain the failure of Rev to depolymerize 100% of the MT polymer. It is also possible that Rev never appears to completely depolymerize the MT polymer because the RTTs are formed and pelleted. Attempts were therefore made to keep the products of depolymerization e.g. rings in the supernatant by reducing the centrifugal force at which the mixture was spun. If rings were formed under our experimental conditions, then at a reduced centrifugal force, the MT polymer is expected to pellet due to higher mass but rings, 3.4-4.2 MDa in mass, are expected to stay in supernatant. To test this, Rev was added to MTs, incubated at room temperature for 20 minutes, and the reaction mixture was subjected to centrifugal forces ranging from 20,000 x g to 350,000 x g (Figure 9). The percentage of tubulin in the supernatant at each centrifugal force was calculated and plotted against the centrifugal forces. Figure 9 indicates that both the controls and the Rev-MT mixture show increase in the pelletable proteins as centrifugal forces are increased. For the analysis of the data, a two-way ANOVA with percent of tubulin as the dependent variable and factor speeds (5 levels) and group (2 levels: control or Rev+MT) was used. The model was strongly significant, but the interaction term was not significant. The final model with main effects only was strongly significant ($p=0.0001$) (See Methods and Materials for the test used). None of the centrifugal

forces showed a sharp drop in the percentage of tubulin that would have indicated the pelleting of Rev-tubulin rings. This is consistent with our data that indicates that RTTs are not formed.

Discussion

The long term goal of these experiments was to quantify the results of Watts *et al*. (2000) and to test the hypothesis that Rev depolymerizes MTs by a mechanism similar to Kinesin-13s postulated by Watts *et al.* (2000). This necessitated the purification of large amounts of wild-type Rev and comparison of depolymerization ability of wild-type Rev with mutant forms of Rev. Specifically, by comparing the activity of wild-type Rev with the mutant form that has point mutations in regions important for either multimerization or RNA binding, it will be possible to identify residues important for its function.

The substrate used in the sedimentation assays was GMPCPP-stabilized MTs unlike the GTP and taxol treated MTs used in the experiments by Watts *et al*. (2000). Because the hydrolysis is negligible over the course of polymerization, any depolymerization of MTs observed can be attributed to the presence of Rev. The buffer used in the sedimentation assays was a MT compatible buffer MEM (100 mM MES, 1 mM $MgCl_2$, and 1 mM EGTA). When GMPCPP stabilized MTs were placed in buffer alone, there was a range of 12-18% soluble tubulin heterodimer present in the supernatant that stayed within this range over 60 min (Figure 7D). Upon addition of purified Rev to stabilized MTs, the net increase of soluble tubulin in the supernatant was about 30% over the same period of time indicating that the assay can measure Rev-induced depolymerization of MTs. This was clearly not a buffer effect since MTs were stable in buffer alone and showed increase in soluble tubulin in supernatant only after the addition of Rev (Figure 7).

There is evidence that there is statistically significant time dependence and some

dose-dependence in Rev induced MT depolymerization (Figure 8). Rev filaments retained activity for 4-5 months when stored in Rev storage buffer; followed by decrease in depolymerization activity that correlated with the appearance of smaller Rev derived peptides seen in western blotting and coomassie stained gel (Figure 11). This was further confirmed by mass spectrometry that showed the presence of smaller peptides in Rev samples stored for long periods of time (Deacon Sweeney, personal communication). These smaller peptides seemed to inhibit the activity of wild type Rev (data not shown).

The MT substrate and depolymerization products had different sizes based on the results of the sedimentation assay done with different centrifugal forces. In the event, the sizes were the same, the MT substrate and the products would not have exhibited differential pelleting. Attempts were made to keep the product of Rev-MT mixture e.g. rings in the supernatant by spinning Rev-MT mixture at a lower centrifugal force (20,000 x g to 350,000 x g) (Figure 9). The expectation was that reduced centrifugal force would separate RTTs from intact MT polymer. None of the centrifugal forces showed a sharp drop in the percentage of tubulin that would have indicated the pelleting of Rev-tubulin rings. The explanation is that the product of depolymerization under these buffer conditions are not ring intermediates. Also, the citrate concentrations are present in molar excess that chelates magnesium ions and prevents the formation of rings. It is likely that spirals are formed as reported by preliminary experiments done by mixing GMPCPP stabilized MTs with Rev (Watts et al.,2000) . It would be useful to test the effects of increasing concentrations of magnesium on the products of depolymerization and

correlate these results with experiments done with taxol stabilized GTP treated MTs.

There are several means by which Rev can bind MTs and then depolymerize them. In one set of models, Rev exhibits a preference for MT ends. Once bound, it depolymerizes them. Alternatively, Rev may bind the MT ends and lattice equally. That means that Rev must target MT ends before it depolymerizes them. If this is true, then one would expect either a very low concentration of Rev to target the ends or Rev has the ability to track to the ends. MCAK, for example, exhibits a higher affinity for MT ends and also binds the lattice. MCAK bound to the lattice is known to diffuse along the lattice. Whether Rev has the ability to diffuse along the lattice is unknown but seems less likely. In the case of MCAK, a relatively larger protein, there is present a neck region that sterically hinders tight binding to the MT lattice. This hindrance is removed once it reaches the MT ends. Alternatively, Rev has the ability to multimerize meaning that it might have the ability to reach the MT end by multimerizing along the surface of the MT.

If depolymerization depends on the ability of Rev to form oligomers, mutants of Rev that exhibit multimerization deficiency in the two regions flanking the RNA binding site (M4 and M7) will not have a depolymerizing effect on the microtubules. *In vitro* analysis of Rev multimerization by Brice *et al.*, 1999 indicates that Rev M4 and M7 do not bind the RRE with the same affinity as wild-type Rev and the binding of subsequent monomers is reduced. If the model proposed above is correct and Rev multimerizes along the microtubule lattice until it reaches the high affinity ends and causes peeling of the protofilaments, then the

mutants of Rev (M4 and M7) should show lower depolymerization activity because of reduced affinity for the microtubule lattice. Preliminary data indicates that multimerization may be important for Rev activity, but is not required for depolymerization (A. Sharma, personal communication). Experiments done by mixing GMPCPP stabilized MTs with mutants of Rev deficient in multimerization have the ability to depolymerize microtubules, but not much is known about the ability of mutants relative to wild-type Rev.

Attempts to purify mutants of Rev protein defective in multimerization (M4 and M7) or RNA binding (M5 and M6) using similar methods are discussed in detail in appendix (Figure A1, A2). Purification was hindered by the inability of Rev to bind the heparin-sepharose column resulting in low concentration of Rev and was associated with contaminating proteins that made it unsuitable for use in sedimentation assay. These mutants were excluded from use in sedimentation assay due to the presence of contaminants.

One of the puzzling observations was that Rev induced depolymerization of MTs never reaches 100% (Figure 8) suggesting that Rev either is aggregating under the experimental conditions or is inhibited at the ends. The maximum percentage of released heterodimer in supernatant is about 45-50%. However, Watts *et al*. (2000) did not report seeing any aggregation of Rev under similar buffer conditions which is confirmed by our experiments (Figure 10). The experiments with Rev alone in storage buffer and in MT dilution buffer agree with no aggregation under our experimental conditions. Alternatively, it is possible that ring intermediates (3.4-4.2 MDa) that are intermediate products of depolymerization are pelleted upon high

speed centrifugation. This seems unlikely since the citrate ions in our buffer conditions are present in molar excess relative to magnesium meaning that rings are likely not formed (Watts *et al*., 2000). Furthermore, Watts *et al*. (2000) experiments show that GMPCPP stabilized MTs mixed with Rev results in the formation of spirals under similar buffer conditions. If Rev forms spirals with stabilized MTs, it is likely that only half of Rev forms these structures at the ends of MTs, and the rest forms collar like structure preventing further interaction with Rev. Preliminary data using transmission electron microscopy (TEM) to visualize the product of Rev-MT interaction does not show formation of ring-like structures that Watts *et al*. (2000) observed (Trombley, Hertzer, Miller, personal communication). Finally, it is possible that Rev is not as functional as it is thought to be. To test the functionality of Rev, EMSA can measure the ability of our Rev to bind RRE.

Proposed Model

In the event that Rev binds along the lattice with the same affinity as the ends, it likely binds the microtubule along the lattice as a monomer, multimerizes along the lattice due to Rev-Rev and Rev-tubulin interactions until it reaches the ends where it forms curved structures similar to the ones seen by Watts *et al*. (2000) (Figure 11). If this model is true, Rev will bind the MTs at lower concentrations without causing depolymerization and at medium or high concentrations of Rev, depolymerization activity should be seen. Whether Rev binds the middle of the MT lattice or a site closer to the ends will affect the critical concentration required to cause activity but in either case, multimerization likely plays an important role.

If, however, the binding affinity for the ends is higher than the lattice, the depolymerization activity should be observed at low concentrations of Rev. This is consistent with the results seen in our sedimentation assay in which maximal depolymerization activity was observed at a Rev:tubulin molar ratio of 4:1.

Rev likely binds the high affinity ends of the microtubule and stabilizes the curved protofilament leading to the depolymerization. This is the similar to that of Kinesin 13 induced depolymerization of microtubules. To determine what residues of Rev are responsible for binding and depolymerization, mutant forms of Rev that harbor mutations in regions responsible for Rev activity can be used in sedimentation assays to see their activity relative to wild type Rev.

To calculate the number of binding sites at the ends relative to those on the MT polymer, the length of MT polymer under our conditions had to be measured. The length of the MT is roughly 17.5 µm (Sharma, A. Personal Communication). Since the length of tubulin heterodimer is 8 nm, the concentration of tubulin at ends vs. the lattice is 35.6 nM and 2.9 µM respectively. In terms of ratio of the number of binding sites at the ends vs. along the lattice, it is 1:1092. Arguing that Rev binds the ends and lattice equally, and two Rev monomers bind one tubulin heterodimer, then the lattice could act as a sink. Therefore, low concentrations of Rev are not expected to cause depolymerization of MTs. However, data indicates that Rev is capable of depolymerizing at a molar ratio of 1:10. This raises the possibility that either two monomers of Rev release more than one heterodimer of tubulin or Rev has higher affinity for MT ends. Watts *et al*., 2000 have shown that RTTs contain equimolar amounts of Rev and tubulin monomers, therefore, it is unlikely that more

than one heterodimer is released. The preference for ends better explains the Rev-MT interactions under these conditions and is consistent with the data obtained from the sedimentation assay.

Future Experiments

Electron microscopy with the samples used in sedimentation assay can reveal curved structures to confirm depolymerization. Also, visual assay that relies on immunostaining using Rev and tubulin specific antibodies can show where Rev binds on the MT. Even if the MTs get shorter over time, it confirms the findings of sedimentation assays. Further characterization of Rev-MT binding could be seen by assessing the folded state of Rev using circular dichroism spectrum and RRE binding experiments that use electrophoretic mobility shift assays (EMSAs) to measure RNA affinities.

Figure. 1. Structure of HIV-1 Rev showing domains important during HIV function. The amino-terminal domain of the 116 amino acid protein consists of arginine-rich motif (ARM) that serves as the nuclear localization signal (NLS) and as the RRE-binding domain. The amino terminus is believed to assume a helix-loop-helix (Auer *et al*., 1994, Thomas *et al*., 1998) as is depicted in figure below. $Helix_1$ and $helix_2$ segments span residues 8-26 and residues 34-59 respectively. On either side of the ARM are regions important for multimerization of Rev monomers on the RRE. The *C*-terminal domain consists of leucine-rich region known as the activation domain containing the nuclear export signal (NES).

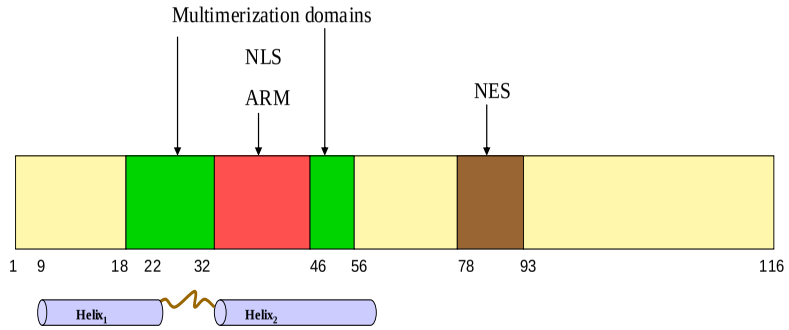
Multimerization domains
NLS
ARM
NES
1
9
18
22
32
46
56
78
93
116
Helix1
Helix2

Figure. 2. Alignment of Rev amino acid sequence with region of the catalytic domain of two Kinesin-13 proteins XMCAK and KIF2C and motile kinesin KIF1A to show sequence similarity between the proteins. Residues in **red** are identical, residues in **green** are conservative substitutions, and residues in **blue** are similar substitutions.

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Rev:      34 TRQARRNRRRRWRERQRQIHSISERILSTYLGRSAEP
XMCAK    506 TASADRITRMEGAEINRSLLALKECIRALGQNKSHTP
KIF2C    502 TSSADRQTRMEGAEINKSLLALKECIRALGQNKAHTP
KIF1A    260    AKGTRLKEGANINKSLTTLGKVISALAEMDSGPN
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Figure 3: Top: Catalytic domain of motile kinesin KIF1A showing MT-binding regions in green and region homologous to part of Rev in cyan (Niederstrasser *et al.*, 2002). Below: X-ray crystal structure (nucleotide not present) of MT depolymerizing Kinesin-13 protein pKinI showing important areas (adapted from K Shipley *et al*., 2004). Part of **MT-binding surface is** shown in **green (L12), loop L11 and helix4 which contacts both the MT and the nucleotide are shown in red**. Loop L11, helix α4, and loop L12 are the main MT-binding elements.

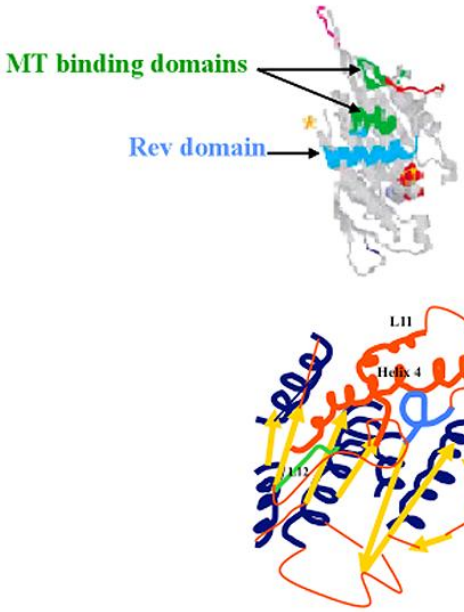

MT binding domains
Rev domain
L11
Helix 4
L12

Figure 4: SDS-PAGE showing expression of wild-type Rev in BL-21(DE-3) cells before and after induction with 1 mM IPTG during mid-log phase. Rev migrates to position that corresponds to 18 kDa on the gel.

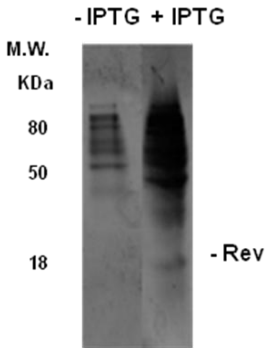
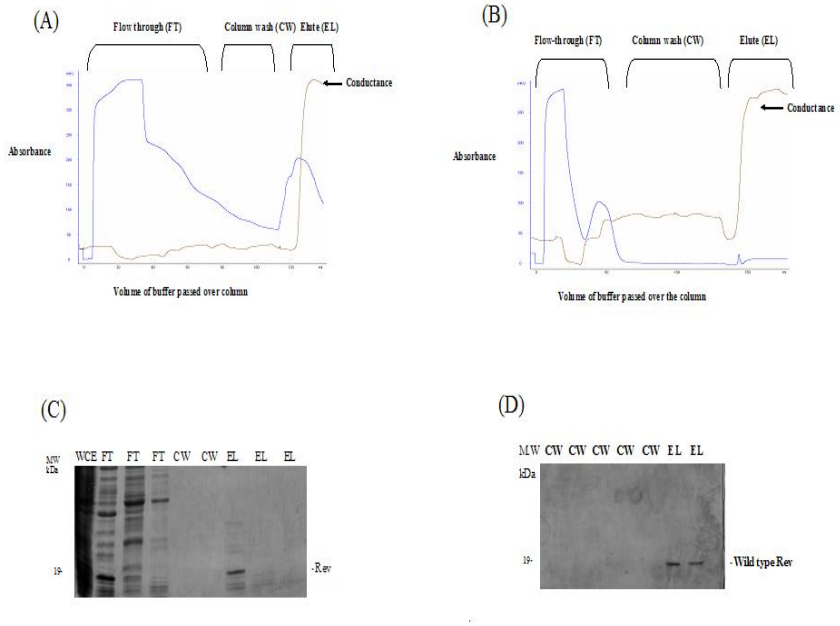
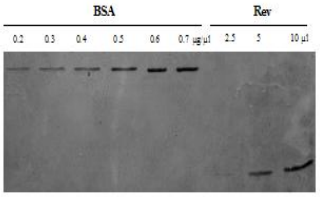

- IPTG
+ IPTG
M.W.
KDa
80
50
18
- Rev

Figure 5: Purification of wild-type Rev. Clarified cell extracts of Rev expressing BL21(DE3) cells were FPLC purified by a combination of Q-Sepharose and heparin-Sepharose columns. (A) Q-Sepharose column FPLC chromatogram. (B) Heparin-Sepharose column chromatogram. (C) Protein fractions from the Q-Sepharose column resolved by SDS-PAGE showing WCE (Whole Cell Extract) that has high concentration of proteins of different molecular weights, FT (Flow-through) fractions consist of proteins that did not bind the column. CW (Column wash) fractions and EL (Elute) fractions show Rev eluted with 800 mM NaCl. (D) Fractions from the Q-Sepharose column containing Rev were pooled and chromatographed over heparin-sepharose column. Rev elutes as a single band as a purified protein. (E) Purified protein was denatured with 6M urea and dialyzed against citrate buffer to form Rev filaments. The protein was concentrated using centricon-30. Quantitation of Rev using BSA concentrations as standards (NIH image). (F) Graphical representation of the quantitation of wild-type Rev (R^2=0.9).


(A)
Flow through (FT)
Column wash (CW)
Elute (EL)
Conductance
Absorbance
Volume of buffer passed over column
(B)
Flow-through (FT)
Column wash (CW)
Elute (EL)
Conductance
Absorbance
Volume of buffer passed over the column
(C)
MW kDa
WCE FT FT FT CW CW EL EL EL
19-
-Rev
(D)
MW CW CW CW CW CW EL EL
kDa
19-
-Wild type Rev

(E)


BSA
Rev
0.2
0.3
0.4
0.5
0.6
0.7 ug/µl
2.5
5
10 µl

(F)

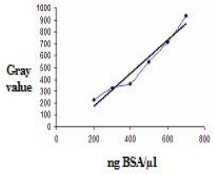
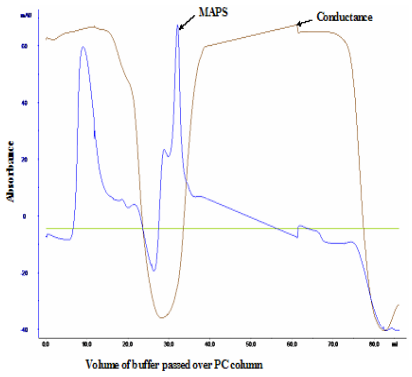

Gray value
1000
900
800
700
600
500
400
300
200
100
0
0
200
400
600
800
ng BSA/µl

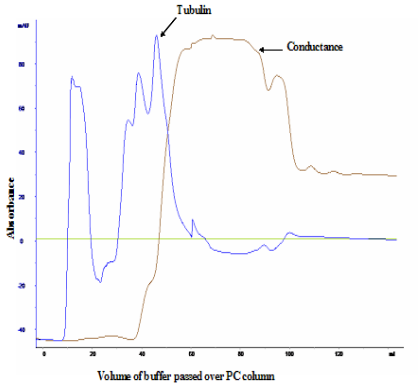
Fig 6: SDS-PAGE of purification of tubulin from bovine brains.

Chromatographic profile of purified tubulin (A) and microtubule associated proteins (MAPS) (B) as the proteins come off as flow-through and elute fractions respectively from the phosphocellulose (PC) column. The chromatographs are from the same purification separated by 24 hrs. Purified tubulin shows a single band that migrates to a position corresponding to 50 kDa on a gel (C).

(A)


MAPS
Conductance
Absorbance
Volume of buffer passed over PC column

(B)


Tubulin
Conductance
Absorbance
Volume of buffer passed over PC column

(C)

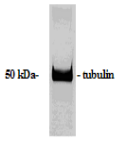
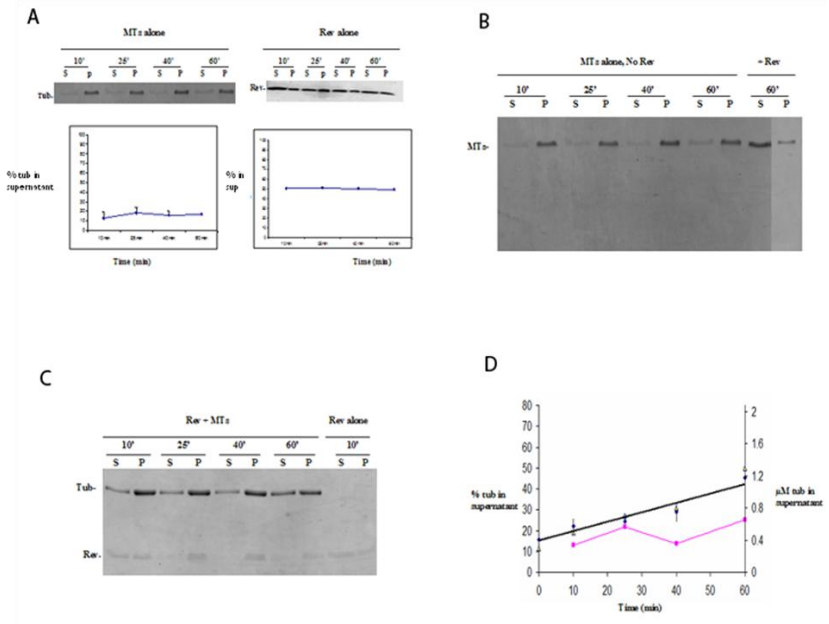

50 kDa-
- tubulin

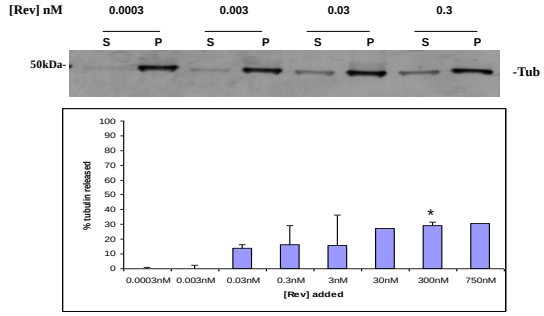
Figure 7: Rev induced time dependent depolymerization of microtubules. (A) Left: Western blot showing Rev alone placed in buffer alone (no MTs) and spun at the time (min) as indicated, and supernatant (S) and pellet (P) separated, and resolved by SDS-PAGE to calculate the percentage of pelletable protein during 60 min. Right: SDS-PAGE showing MT controls obtained by placing MTs in buffer alone (no Rev) at time points (min) as indicated. (B) SDS-PAGE showing no depolymerization of MTs in absence of Rev in lanes 1-8, indicating MTs are stable in MEM buffer. Lanes 9-10 show depolymerization by addition of Rev after 60 min in the same experiment. Last lane is from the same experiment but resolved on a different gel. (C) SDS-PAGE showing Rev induced depolymerization of MTs is time-dependent in lanes 1-8 at time points (min) indicated, lanes 9-10 are Rev controls in buffer alone, no MTs at 10 min. Also noticeable is the shift of Rev to the pellet when mixed with MTs in lanes 4, 6, 8. (D) Quantitation of depolymerization both in percentage of tubulin released and the number of micromoles of MT polymer left in the pellet (average of three independent experiments) with standard deviations. Bottom pink line indicates quantitation of MT controls for comparison. Analysis of data indicates that time is strongly significant ($p < 0.0001$), with parameter estimate 0.3942, indicating that the slope of this regression line is significantly different from zero.


A
MTs alone
Rev alone
10'
25'
40'
60'
S
P
Tub.
Rev.
% tub in supernatant
Time (min)
B
MTs alone, No Rev
+ Rev
MTs-

C
Rev + MTs
Rev alone
10'
25'
40'
60'
S
P
Tub-
Rev-
D
% tub in supernatant
µM tub in supernatant
Time (min)

Figure 8: Quantitation of tubulin released in supernatant at different Rev concentrations as indicated. (A) SDS-PAGE showing increased shift of tubulin to the supernatant with the addition of increasing concentrations of Rev after 10 min. (B) Depolymerization shows saturation at molar ratio of 4:1. Lines indicate time points at which depolymerization was measured (10 min-Blue, 25 min-Pink, and 40 min-Yellow). Statistical analysis was done using one-way ANOVA with factor dilution. This model ignored time, effectively pooling all the data at a given dilution level into one group. This was marginally significant ($p = 0.0648$). The data at concentration of 300 nM, indicated by asterisk, was found to be significantly different from control.

(A)



*** Indicates statistical significance when α = 0.08, p value is 0.064**

(B)

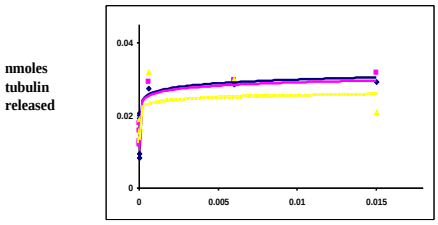
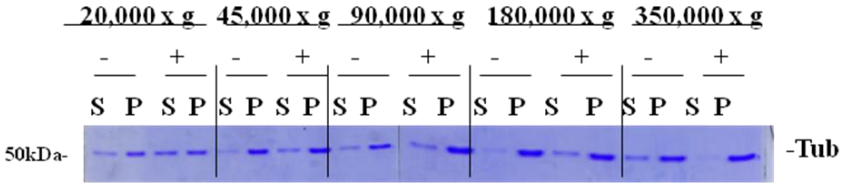


Figure 9: SDS-PAGE showing equimolar Rev and MTs (+) or MTs in buffer alone (-) incubated for 20 min and centrifuged at the g values as indicated.

Below: Quantitation of tubulin released in supernatant at the g values as indicated. Statistical analysis was done by a two-way ANOVA with percentage of tubulin as the dependent variable and factors speed (5 levels) and group (2 levels: control or Rev+MT). The model was strongly significant, but the interaction term was not significant. By removing the interaction term, the final model with main effects only was strongly significant ($p = 0.0001$) (See Materials and Methods).


20,000 x g
45,000 x g
90,000 x g
180,000 x g
350,000 x g
- + - + - + - + - +
S P S P S P S P S P S P S P S P S P S P
50kDa-
-Tub

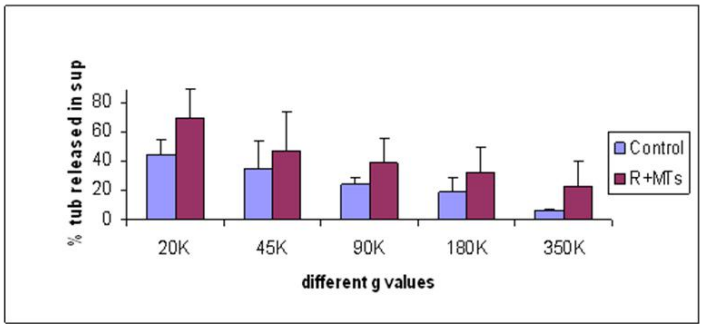

% tub released in sup
80
60
40
20
0
20K
45K
90K
180K
350K
different g values
Control
R+MTs

Figure 10: Western blot showing that Rev exists as a monomer in high salt elution buffer and show primarily in supernatant (S) upon centrifugation at 10 min. Rev filaments, when stored properly according to Watts *et al*., 2000, are present mainly in the pellet (P) fraction after centrifugation at time points indicated.

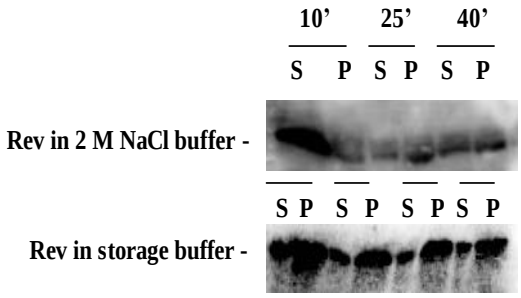

10’
25’
40’
S P S P S P
Rev in 2 M NaCl buffer -
S P S P S P S P
Rev in storage buffer -

Figure 11: Rev undergoes degradation upon storage for 4-5 months. SDS-PAGE (1) and western blot (2) showing purified Rev as a single band from the heparin-sepharose column. SDS-PAGE (3) showing the appearance of Rev derived smaller peptides (shown as small arrows) in addition to the full-length protein (larger arrow) after storage for 5 months. This degradation correlates to loss of depolymerization activity as seen in sedimentation assay.

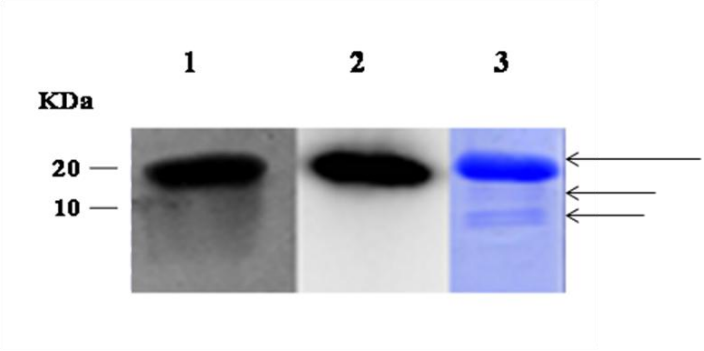
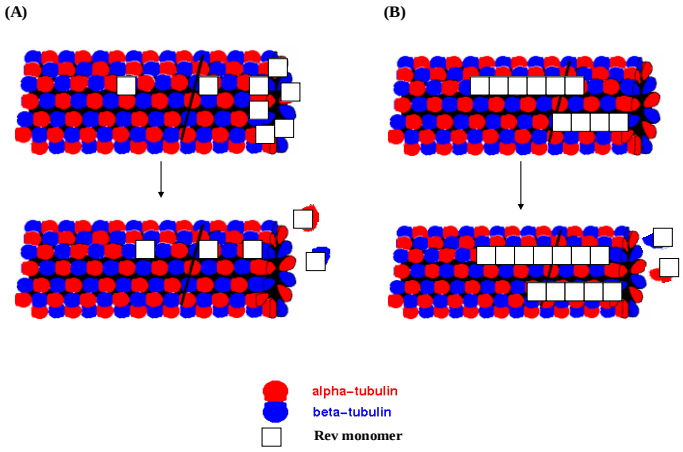

1
2
3
KDa
20
10

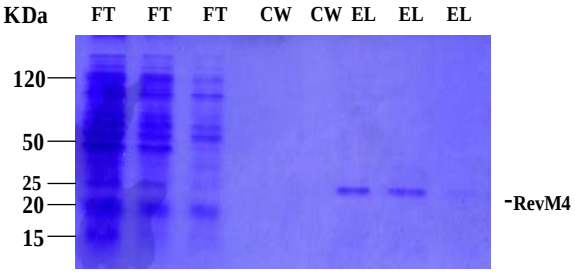
Figure 12: Proposed models to explain Rev induced depolymerization of MTs. Structure of a single microtubule, indicating slant in the diagram, tubulin heterodimer shown in red and blue. (Modified from microtubule structure at www.med.unibs.it/marchesi/pps97/sec11/assembli.html). Rev is shown as white squares. (A) Rev can either bind high affinity ends of MTs like Kinesin-13 to remove heterodimers from the ends. (B) Alternatively, Rev can bind along the MT lattice, multimerize to reach the ends, and cause release of heterodimers.


(A)
(B)
alpha–tubulin
beta–tubulin
Rev monomer

Appendix

Figure A1. Purification of Rev mutant M4: SDS-PAGE showing fractions from the Q-sepharose column (top) and heparin sepharose column (bottom). Contaminating proteins came off the Q-sepharose column as flow-through (FT) and Rev-RNA eluted in 800 mM NaCl (EL) that was chromatographed over the heparin column. Rev came off the column in 1M NaCl with the contaminants and did not show a significant band in elute (EL) fractions. Ammonium sulfate precipitation did not yield high concentration of purified Rev.

A



B

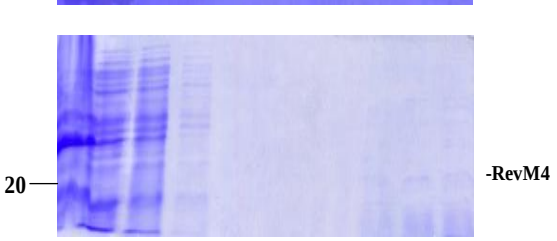
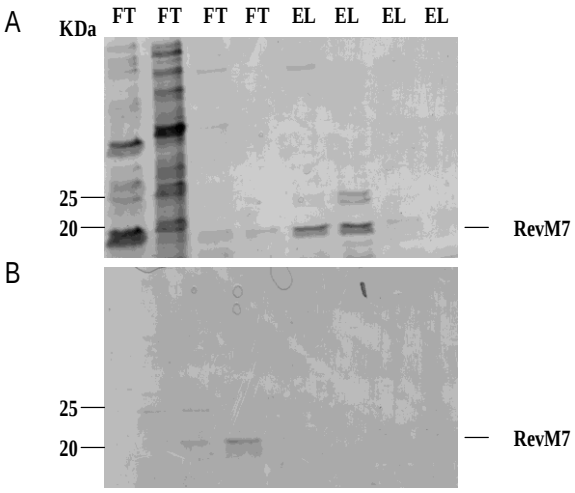


Figure A2. Purification of Rev mutant M7. SDS-PAGE showing fractions from the Q-sepharose column (top) and heparin-sepharose column (bottom). Clarified cell lysate was loaded on the Q-sepharose column (L) that resulted in the removal of most of the contaminating proteins (FT) and Rev-RNA complexes eluted in 800 mM NaCl (EL). Fractions containing Rev (S) were chromatographed over the heparin column that resulted in Rev not binding the column and coming off with the contaminants in 1M NaCl (FT) without any peak detected on the chromatograph or any protein band detectable on coomassie stained gel.


A
KDa
FT FT FT FT EL EL EL EL
25
20
RevM7
B
25
20
RevM7

Purification of mutant form of Rev that harbors point mutations in multimerization domain was attempted using the same method that was used to purify wild-type Rev. Multimerization is important for Rev function and mutant M4 and M7 are unable to multimerize on the mRNAs. Brice et al., 1999 have shown that these mutations are present in the exposed hydrophobic surface on Rev and likely disrupt the multimerization interfaces leading to defects in forming oligomers on the RRE,

Clarified cell lysate was prepared similar to that described for wild-type Rev purification (see Materials and methods) and chromatographed over the Q-sepharose column and heparin-sepharose column (Amersham Biosciences). The majority of the contaminant proteins did not bind the Q-sepharose column and came off in the flow-through (400 mM NaCl) as indicated in lanes 1-4 in the coomassie stained gel above. A step gradient was applied with 800 mM NaCl containing buffer B and Rev eluted as fairly purified proteins as seen by the presence of a single band in lanes marked EL on a gel. Fractions containing Rev were chromatographed over the heparin column equilibrated in buffer B (800 mM NaCl), washed with five column volumes of buffer C (1M NaCl) until the conductance leveled to the base line. A step gradient was applied in 2M NaCl containing buffer D for 4 column volumes. As seen in the gel, most of the contaminants and Rev came off in the flow-through (FT) and a faint band containing Rev is seen in the elute fractions. Attempts were made to collect the flow-through from the heparin column, and its salt concentration and pH adjusted to that of heparin column binding buffer (800 mM NaCl), and chromatographed again over the heparin

column. There was no peak detected on the chromatogram or protein band detected on a coomassie stained gel indicating that Rev M4 either did not bind the heparin column or the concentration was very low.

Solutions

~~1.~~1. Staining Solution- 0.1% Coomassie Brilliant Blue, 50% MeOH, 10% HoAc
45% methanol
9% acetic acid
0.25% (weight) Coomassie

Formatted: Bullets and Numbering

2. Destaining solution- 35% methanol
10% glacial acetic acid

3. 10 X Running Buffer- 30g Tris, 144g Glycine, 10g SDS. Add water to 1L

4. 10X Transfer Buffer- 15.1 g Tris, 72g Glycine, Add water to 1L

5. Blocking solution- 5% Non-fat dry milk in TBST
0.1% NaN_3
Stored at 4^0C

6. 20% ammonium persulfate (APS)- Dissolve 2.0 g ammonium persulfate in 10 ml water and aliquot into 1 ml tubes and freeze at -20^oC.

7. Antibodies used in sedimentation assay

Rev detection
Primary antibody- Rabbit antiserum, 2% BSA, 0.1% NaN_3 (1:1800) in 1X TBST
Secondary antibody- HRP Goat antirabbit in 5% BSA, 1X TBST

Rev and tubulin detection
Primary Ab- Rabbit antiserum (1:1800), DM1A (1:5000) in 1X TBST
Secondary Ab- HRP donkey antimouse (1:100,000), Goat antisheep (1:100,000) in 5% BSA, 1X TBST

8. Lower resolving Gel

ddH_2O	2.95 ml
4 x lower gel buffer	2.50 ml
Lower gel Acrylamide	4.45 ml
20% APS	41.25 µl
Temed	10 µl

9. Upper stacking Gel

ddH2O	3.1 ml
4 x Upper gel buffer	1.25 ml
Upper gel Acrylamide	0.65 ml
20 % APS	16.5 µl
Temed	5 µl

10. 10X TBST-1.5 M NaCl, 200 mM Tris, pH 7.2-7.5, 0.1% Tween-20

11. Blot stripping buffer- 25 mM Glycine, 1% SDS pH 2.0

12. 2X Protein sample buffer (1X=62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2 mercaptoethanol, 0.025 % BFB)

4 x Upper gel buffer	2.5 ml
Glycerol	2.0 ml
10% SDS	4.0 ml
Me	1.0 ml
1% BFB	0.5 ml
Total volume	10.0 ml

13. Rev purification Buffers (FPLC)

Karn A- 400 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 0.5 mM phenyl-methylsulfonyl fluoride (PMSF)

Karn B- 800 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT

Karn C- 1M NaCl, 50 mM Tris-HCl pH8.0, 1mM EDTA, 1mM DTT

Karn D- 2M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT

Dialysis buffer 1 (50 mM sodium phosphate, 150 mM sodium chloride, 600 mM ammonium sulfate, 50 mM sodium citrate, 1mM EDTA pH 7.0)

Dialysis buffer 2 (50 mM sodium phosphate, 150 mM sodium chloride, 50 mM sodium citrate, 1mM EDTA pH 7.0

Dialysis buffer 3 (20 mM Hepes, 100 mM sodium chloride, 50 mM sodium citrate, 1 mM EDTA pH 7.0), also acts as storage buffer

14. Pipes buffer- 0.1 M Pipes, pH 6.8, 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1% B-ME, 1 mM ATP

15. Column Buffer- 50 mM Pipes, pH 6.8, 1 mM EGTA, 0.2 mM $MgCl_2$

16. MEM buffer- 100 mM MES, 1 mM $MgCl_2$, 1 mM EGTA pH 6.9

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