Effects Of Rev Protein On Microtubule Arrays In Living Cells

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ABSTRACT

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The HIV protein Rev regulates the expression of essential viral proteins during the course of infection by a mechanism that is well understood. It promotes nuclear export of viral transcripts, normally retained in the nucleus owing to the presence of introns, by interacting with host cell transport factors. However, over-expression of Rev in cells leads to defects in cell cycle progression, specifically slowing growth and impairing progression through mitosis (43). While it is possible that Rev may be altering the proteins in transport pathways, cell cycle defects may be attributed to Rev’s interactions with other proteins.

In vitro experiments show that highly purified Rev has a high and specific affinity for α and β tubulin present either as free heterodimers or polymerized into microtubules (MTs) (65). Moreover, Rev rapidly depolymerizes MTs in vitro producing intermediates that closely resemble the products of depolymerization reactions triggered by a variety of experimental conditions. Owing to structural similarities, Rev hypothetically depolymerizes MTs by a mechanism used by Kin-13 proteins that are potent MT depolymerizing enzymes.

To determine whether Rev is interacting with MTs in a Kin-13-like manner, point mutations were previously introduced into Rev substituting alanine for
amino acids shared with Kin-13. Mutant proteins were tagged with YFP, over-expressed in HeLa cells and cell cycle progression was monitored by Chang and Miller (27). In contrast to expression of Rev, which lengthened doubling times and all stages of the cell cycle, each point mutant partially corrected the defect. These results are consistent with Rev acting in a manner similar to Kin-13. To determine whether Rev over-expression affects MT dynamics in cells, MT arrays were experimentally depolymerized and allowed to recover. If Rev inhibits MT nucleation or promotes depolymerization, then MT arrays in cells expressing Rev should require more time to recover.

Results show that MT arrays recover from depolymerization equally well in presence and absence of Rev. Because wild-type Rev accumulates largely in the nucleus and nucleoli, we used Rev mutants M4, M6, and Rev2.2 with mutations that impair Rev multimerization and nuclear import, and Rev attached to glucocorticoid hormone receptor respectively. These mutants typically maintain higher cytoplasmic expression levels than wild type Rev. However, exogenous expression of Rev mutants does not affect MT recovery after depolymerization. Furthermore, the ability of MTs to recovery after cold-treatment in Rev expressing metaphase cells was also studied when Rev localizes perichromosomally and is in a position to affect spindle behavior. However, similar defects were observed in control cells suggesting there was no consequence attributable to Rev. These results suggest that the cell cycle defects observed in Rev-expressing cells are not mediated by Rev’s ability to alter the polymerization state of MT. It therefore seems likely that cell cycle defects caused by Rev must be mediated by its interactions with other proteins, possibly B23 or Ran.
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**Introduction**

The human immunodeficiency virus (HIV) is a causative agent of Acquired Immune Deficiency Syndrome (AIDS) (66). It kills CD4+ T helper cells and weakens the immune responses permitting opportunistic infections. It also inhibits humoral immunity and B-cell functions (25). The HIV viral infection cycle is closely linked to the cytoskeleton of the host cell and infection can disrupt cytoskeletal arrays including both actin and microtubule arrays. Epithelial cells infected with HIV have altered cytoskeletal arrays, especially those associated with the plasma membrane. These alterations result in junctional leakage and cell injury (7, 14, 38, 70).

One or more viral proteins may cause cytoskeletal alterations. Nef (Negative Factor) is a regulatory protein that leads to a loss of actin stress fibers, increases lamellipodia and causes HIV-associated nephropathy (36). The regulatory protein Tat down-regulates the expression of several cytoskeletal proteins including tubulin (9). Tat causes a decrease in microtubule-associated protein 2 (MAP2) and the collapse of neuronal cytoskeletal filaments in biopsies of patients exhibiting HIV-induced encephalopathy (1). Tat expression in lymphoid cells shortens microtubules (MTs) and leads to apoptosis (26). In vitro experiments however show that Tat can stimulate MT polymerization (26). Watts et al. suggest that some cytoskeletal defects seen in HIV-infected cells might be caused by the regulatory protein Rev as Rev binds tubulin and depolymerizes MTs in vitro (65). Over-expression of Rev impairs cell cycle progression leading to defects in mitosis (43) suggesting that Rev-MT interactions may be important.
Microtubule Dynamics

MTs are an important component of the cytoskeleton and play crucial roles in the development and maintenance of cell shape, cellular transport and cell division. They are polymers of α- and β-tubulin heterodimers. Heterodimers assemble into linear protofilaments that laterally associate with each other to form hollow tubules. MTs have an intrinsic ability to spontaneously grow and shrink. One end, denoted as the (+) end and terminating with β-tubulin, is more dynamic than the (-) end. Plus end polymer growth and shrinkage rates are several-fold faster than at the opposite end. Whether a MT will grow or shrink depends on two factors: the guanine nucleotide bound state of the β subunit and the concentration of free heterodimers available for polymerization.

Both α- and β-tubulin are GTPases. However, α-tubulin is always bound to GTP (or GDP•P_i) owing to the structure of the heterodimer (33). The nucleotide-bound state of the β subunits is key. When heterodimers newly incorporate into a MT polymer, they activate GTP hydrolysis in underlying β subunits. Consequently, β-tubulin may be bound to either GTP or GDP. When β-tubulin is bound to GTP, tubulin heterodimers assume a ‘straight’ conformation allowing it to incorporate at MT ends and associate laterally with adjacent protofilaments. In the GDP-bound state β-tubulin assumes a more curved conformation that cannot be incorporated into MT ends. The majority of β-tubulin within the body of a MT is bound to GDP owing to its GTPase activity. However, it is constrained to lie ‘straight’ due to its interactions with adjacent protofilaments. Essentially, MTs are tensioned and primed to disassemble, held intact only by the β-tubulin•GTP cap that forms at the MT end. Loss of the ‘GTP cap’ exposes β-tubulin•GDP that leads to
depolymerization. The concentration of heterodimers available for polymerization also determines whether MTs will grow or shrink. The critical concentration (Cc) is that concentration of free heterodimer where rates of growth and shrinkage are equal at both ends of MT. When the concentration of α-/β-tubulin is greater than Cc, growth occurs.

Although purified MTs will exhibit spontaneous growth and shrinkage in vitro, anti-mitotic drugs like Taxol, maytansine and colchicine are effective because they affect the nucleotide-bound state of β-tubulin, the conformation of the heterodimer, or the concentration of soluble heterodimers. Taxol (paclitaxel) promotes polymer assembly (6). It has a high affinity for tubulin (K_d = 10^{-8}M) and binds two patches in a nucleotide-sensitive helix of β-tubulin (19, 29). Its binding enhances MT polymerization by binding β-tubulin on the inside of the MT where it stabilizes interactions between adjacent subunits (45).

On the other hand, maytansine, vinca alkaloids and colchicine depolymerize MTs. Vinca alkaloids and maytansine disassemble MTs by binding tubulin polymers (8) at or near the nucleotide-binding site of β-tubulin (53). MTs treated with vinca drugs depolymerize forming spiraling protofilaments and other curved MT structures. At higher concentrations, vinca alkaloids inhibit MT polymerization (28).

Colchicine depolymerizes MTs by a different mechanism. It binds at the interface of α and β subunits and is not inhibited by Taxol and only marginally inhibited by maytansine (8). It depolymerizes MT in two different mechanisms. First, it binds to soluble tubulin and reduces the concentration of tubulin heterodimers available for polymerization. This shifts the
equilibrium away from polymerization and towards depolymerization (4). Second, it binds to tubulin heterodimers and forms tubulin-colchicine complexes, which incorporate at the MT ends (28). When concentration of colchicine-tubulin dimers is more than free tubulin, it binds to MT end and prevents the further addition of other free tubulin subunits at the end of a MT. Thus it poisons MT ends.

Temperature also affects the polymerization state of MTs. At unphysiologically cold temperatures, MTs depolymerize releasing tubulin heterodimers and curved oligomers and rings (42). Warmer and physiological temperatures promote polymerization (in presence of GTP) (33). Obviously cells do not regulate MT dynamics by regulating cell temperature. They rely on cellular proteins called MT-associated proteins (MAPs). Some MAPs like Tau and Map2 are important for stabilization of MT. These MAPs causes cross-linking of protofilaments and formation of bundles of microtubules via their N-terminal projection domain results into stabilization of MT (13). Other proteins including some kinesins promote depolymerization. Kinesins are motor proteins involved in most MT activities and are essential for spindle function during cell division.

One well-studied kinesin is xMCAK, a member of the Kin13 family of motor proteins distinguished from motile kinesins by their ability to depolymerize MTs. Its depletion in the *Xenopus* egg extracts in presence of chromatin suppresses the bipolar spindle formation and results in abnormally large MTs (15, 59, 62, 65). Conversely, addition of xMCAK can completely suppress MT formation. xMCAK does not act as a conventional motor protein that translocates along microtubules. Instead it diffuses along the microtubule lattice targeting the ends of the microtubules and causes depolymerization
During depolymerization, it induces a conformational change in the microtubule resulting in protofilament peeling similar to the conformation change when GTP of β-tubulin undergoes hydrolysis. This leads to the release of many tubulin dimers and a small number of tubulin dimer/xMCAK complexes (63). xMCAK consist of three domains. An N-terminal domain targets xMCAK to kinetochores. A middle motor domain and neck region are essential for depolymerization. The C-terminal tail is essential for tight MT binding in the presence of excess tubulin heterodimer and regulates homodimerization and influences ATPase activity (40, 46, 21).

The mechanism by which xMCAK depolymerizes MTs is well understood. Only the motor domain and neck are required for depolymerization. The neck is positively charged and binds to the acidic C-terminus of tubulin. It makes MT depolymerization more efficient by disturbing lateral interactions between the MT protofilaments at MT ends (40, 46, 21). The α4 helix of the motor domain of the murine ortholog of xMCAK Kif-2C binds to the curved MT end and links the poly-glutamate tail of β-tubulin with α-tubulin (46). This crosslinking of the poly-glutamate tails stabilizes the curved structure of the intradimer interface and causes the initiation of ATP hydrolysis. Crosslinking also facilitates the insertion of a KVD finger (Lys293Val294Asp295-absent in motile kinesins) in the interdimer interface and stabilizes the curved conformation of the protofilament. Therefore the α4 helix is thought to stabilize the curved conformation of MT ends. Moreover, mutation of KVD and KEC (Lys268Glu269Cys270) residues of the α4 helix inhibits xMCAK’s ability to depolymerize MTs (46, 56). In this way, xMCAK specifically binds to the ends of MTs and stabilizes an already bent conformation or induces a
curvature at the MT end (15, 46, 21, 56). This curved protofilament within the MT is unstable and is thought to cause the MT polymer to depolymerize (62).

**Rev**

*Rev function*

Rev is 13 kDa basic protein with high affinity for RNA (11, 20, 67, 70). It is produced early in infection and is essential for late stages of viral infection. During the early phase, the provirus integrates into the host genome where it transcribes a 9 kb primary transcript. This 9 kb mRNA can be spliced into the various classes of transcripts: fully spliced 2kb transcripts, 4kb transcripts containing one intron, and 9kb unspliced transcripts containing two introns. Of these three transcripts, only the 2kb mRNAs are exported into the cytoplasm where they translated into one of three proteins, one of which is Rev.

Rev enters into the nucleus in a NLS-dependent fashion (70). The NLS (nuclear localization signal) contains an arginine-rich motif with sequence $^{30}\text{TRQARRNR}^{50}$. Mutating this sequence (M6: $^{41}\text{RRRR}→\text{DL}$ and M5: $^{38}\text{RR}→\text{DL}$), results into a significant level of Rev accumulation in the cytoplasm (65). Cytoplasmic accumulation is not absolute as Rev is small enough to diffuse into the nucleus. The NLS binds importin β, a cytoplasmic importing receptor and Ran•GDP docks Rev to nuclear pore complexes (57). The exchange of GDP for GTP releases Rev from the importin β-pore complex allowing import into the nucleus. Nuclear exchange occurs because RCC1, Ran’s nucleotide exchange factor, is bound to the chromatin (44, 47). Conversely, the Ran•GAP (RanGTPase activating protein) is localized in the cytoplasm ensuring hydrolysis of GTP so that Ran is bound to GDP in...
cytoplasm (51). This gradient of Ran•nucleotide, Ran•GTP in the nucleus and Ran•GDP in the cytoplasm, ensures the directionality of Rev transport.

As Rev expression level rises during the early phase of infection, it promotes the gene expression of late phase proteins like gag, pol, env, vif and vpr without activating transcription (65). In the nucleus, Rev binds to the Rev Response Element (RRE), ≈351 nucleotide sequence present in the 3ʼ intron of 4kb and 9kb transcripts. The RRE forms the intramolecular double stranded stems and single stranded loops. Rev multimerizes on the RRE by the arginine-rich motif (ARM, amino acids 37-50) that overlaps with the NLS. Rev-RRE binding triggers RNA export although binding of a single-Rev monomer is insufficient to export of transcripts-multimerization at least four Rev monomers is essential (11, 70).

The ability to multimerize on the RRE is mediated by amino acid sequences 9-26 and 51-65 that straddle the arginine rich motif. Rev-RRE multimers are then exported into the cytoplasm owing to the nuclear export sequence (NES) (51). Mutation of 9-26 and 61-65 residues impairs multimerization and reduces Rev affinity for the RRE, resulting in inhibition of formation of high molecular weight complexes on the RRE (16).

The NES is present near the C-terminus, amino acids 75-83. It is rich in leucine residues. The export factor Crm1 and Ran-GTP bind to the NES and the newly formed complex consisting of Rev-RRE, Crm1, Ran-GTP is targeted to NPCs (60). The complex is disrupted by the GTP hydrolysis stimulated by cytoplasmic RanGAP. The released RRE-containing transcript is then engaged by ribosomes and late viral proteins are expressed. Mutation in its NES inhibits Rev’s ability to bind export factor Crm1 (51). The M10
(78LE→DL) mutant Rev localizes almost exclusively in nucleoli due to defective nuclear export.

Unliganded Rev re-enters the nucleus by using NLS (37). Once Rev is back inside the nucleus, it is again ready for export of other intron containing transcripts. Therefore Rev Protein is important for the nuclear export of intron-containing transcripts.

*Rev interactions with MTs*

While attempting to determine the three-dimensional structure of Rev, Watts et al. found that Rev interacts with tubulin (65). When highly purified Rev is mixed with MTs, MTs rapidly depolymerize forming rings they called Rev-tubulin toroidal complexes (RTTs). RTTs are 3-4 MDa, double-ringed structures with 28, 30 or 32 Rev-tubulin dimers (65). RTTs are similar to rings when MTs depolymerized by cold (39, 42, 65) or exposed to certain antineoplastic drugs belonging to the maytansine family of anti-mitotics, Dolstatin-10, cryptophycin, hemiasterlin (2, 3, 65). These drugs with great anti-cancer potentials have ability to produce curved tubulin structures e.g., rings, spirals, and bracelets (2, 15, 22, 29, 65). Curved tubulin structures are thought to be important events that initiate depolymerization (12, 15, 22). Therefore formation of rings of tubulin by Rev *in vitro* suggests that Rev may affect the polymerization state of MT in cells during HIV infection.

Watts et al. (65) predicted that Rev and tubulin interacts via simple electrostatic interactions: Rev is a basic protein (pI = 9.2) and possesses an arginine-rich region whereas tubulin is acidic (pI = 4.8-5.2) and both α- and β-tubulin have glutamate-rich C-terminal tails. RTTs and/or Rev-tubulin
complexes, however, still form under experimental conditions that abrogate simple acid:base interactions e.g., presence of salt, changes in pH, and removal of tubulin tails by subtilisin. This shows that Rev-tubulin interactions are more complex than simple charge interactions. Rev-tubulin interactions require Mg\(^{2+}\). RTTs formed when Rev is mixed with either taxol-stabilized MTs and colchicine-depolymerized tubulin. Maytansine however inhibits RTT formation suggesting that Rev binds at or near the vinca site of \(\beta\)-tubulin. Collectively these data suggests the interaction between Rev and tubulin or MTs is specific.

Watts et al. looked for sequence similarities between Rev and other MT depolymerizing proteins. A statistically significant similarity exists between Rev’s ARM (amino acids 34-70) and the motor domain of xMCAK (amino acids 506-543). Rev’s ARM amino acids E57, R42, and R50 are similar to the amino acids in \(\alpha_4\) helix of the motor domain of xMCAK. \textit{In vivo}, mutants of these amino acids appeared to have the corrected mitotic defects which was not seen in wild type Rev expressing cells. Therefore by considering the similarities between Rev’s ARM and xMCAK motor region, the ARM may act like the xMCAK \(\alpha_4\) helix that binds the tubulin intradimer interface. There are additional similarities between Rev- and xMCAK-mediated MT depolymerization. Both Rev and xMCAK cause depolymerization from both ends of MTs (15, 65). Neither xMCAK nor Rev require ATP hydrolysis for depolymerization, although xMCAK relies on ATP hydrolysis to release tubulin heterodimer thus allowing the enzyme to recycle for another round of depolymerization. xMCAK-mediated depolymerization is more efficient than that mediated by Rev as Rev lacks a recycling mechanism (15, 65). Depolymerization of stabilized MTs by both proteins results into tubulin rings
(15, 65). It is possible that multimerization of Rev occurs at the tubulin intradimer interface as ATP state does for xMCAK. Taken together, these data suggest that Rev might multimerizes and induces the curved conformation of MT ends similar to xMCAK. Also both proteins affect the cell cycle progression. Kin13 alters spindle assembly and chromosomal movement (62), whereas overexpression of Rev accumulates the cells in prophase and metaphase (27, 43). Therefore Rev may be a useful model for understanding how MCAK depolymerizes MTs.

**Rev-tubulin interactions in vivo:**

Previous data show that Rev has the potential to affect the cell cycle progression by affecting spindle function through its ability to bind tubulin and depolymerize MTs. Rev inhibits aster formation in frog egg extracts that recapitulate the multiple cell cycles *in vitro* showing that Rev and tubulin can interact in the presence of cellular constituents (14, 65). Transient over-expression of Rev expressing COS and Hela cells slows cell growth (43, 27). Rev expression leads to chromosomal abnormalities and accumulation of cells in G2/M specifically before the spindle checkpoint (27, 43). These defects may result from Rev’s ability to alter spindle dynamics by depolymerizing MTs, interfering with MT polymerization by sequestering tubulin heterodimers, or interfering with centrosome duplication (43). Its over-expression can produce changes in ploidy. Cells divided into more than the normal number of daughter cells where MTs are shared between the three forming daughter cells (27). The nuclei of these cells were abnormally larger and the MT cytoskeleton was highly perturbed (27).
To test the hypothesis that Rev depolymerizes microtubules in living cells leading to growth defects, the rates MT arrays recover after depolymerization in the presence and absence of Rev were measured. If Rev expression promotes MT depolymerization, either by active depolymerization or by tubulin sequestration, then recovery should be slower in Rev cell lines than control lines.
**Materials and Methods**

*Cell culture:*

HeLa cells were maintained at 37°C in Dulbecco’s Modified Eagle medium with high glucose supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/L streptomycin, and in the humidified incubator with 5% CO₂. Cells were grown in 100 mm x 20 mm polystyrene cell culture dishes. When growth was confluent, cells were passaged into new cultures by treatment with 0.25% of trypsin with 0.5 mM EDTA. Briefly, media was removed and cells were washed with PBS (Phosphate Buffered Saline-137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄). 1 mL of 0.25% of trypsin with 0.5 mM EDTA was added for 2 minutes at 37°C. Trypsin activity was quenched with the addition of 9 mL of media. New 10 mL cultures were seeded with 1 ml of cells liberated by trypsin-EDTA treatment. HeLa cells stably expressing YFP, Rev-YFP, M6-YFP, M4-YFP and Rev-GR-GFP (2.2) were also maintained for further study.

*Plasmid Isolation:*

YFP-C and YFP-Rev plasmid containing E. coli (DH5α) were grown in LB/Amp broth overnight. Bacteria were then plated on LB/Amp plates and individual colonies were used to inoculate LB/Amp broth cultures. After 18-24 hour of growth, bacteria were collected by centrifugation at 5000 x G for 15 min at 4°C. Plasmids were isolated by using QIAfilter Mini Kit (Qiagen) as per manufacture’s instruction.

*Transfection:*

Prior to transfection HeLa cells were grown in 6 well plates. After 50% growth, cells were transfected with 1.6µg of YFP-Rev and YFP-control, by
using Polyfect transfection reagent (Qiagen). Cells were incubated for 24 hours for transient expression of YFP and Rev-YFP.

*Cold depolymerization and recovery of MTs*

To compare the recovery of MTs in Rev and YFP expressing cells, ‘trypsinized cells’ were seeded onto poly-L-lysine coated coverslips in 1 mL media per well in a six well plate. When cells were 70-80% confluent, the media was removed and replaced with ice-cold L-15 media. Cells were maintained in an ice-cold water bath for three hours.

To induce MT recovery, cold media was removed and replaced with warm L-15 media and incubated at 37°C with 5% CO₂. At different times, cells were fixed with freshly made 4% paraformaldehyde dissolved in PBS for 90 m.

*Colchicine Depolymerization*

Cells were grown on coverslips placed in 6-well dishes. After cultures were 70-80% confluent, cells were used for colchicine depolymerization of MT. 100 mM Stock colchicine was prepared in ethanol and stored at 4°C. Colchicine was freshly diluted 1000-fold from 100 mM ethanol stock in warm DMEM. 2 ml of 100 nM Colchicine was added in each well and plate was incubated at 37°C for 15 m. Cells were rinsed twice with warm media to remove bound and excessive colchicine and then incubated in warm media for 3, 6, 12 and 24 hours. At these time points cells were fixed in paraformaldehyde.
Immunofluorescence Microscopy

Fixed cells were washed with PBS for four times and then permeabilized by 5 m treatment with 0.1% Triton X-100-PBS. Cells were washed four times with PBS and transferred to blocking buffer (2% BSA and 0.1% NaN₃ in 50ml of PBS) for 30 minutes. After additional washed with PBS, tubulin-specific antibody (DM1α) diluted (1:500) in blocking buffer was added for 1 hour at room temperature. Excess antibody was washed away by six washes with PBS. To detect MT-DMA1α complexes, goat anti-mouse IgG conjugated to Texas Red diluted (1:500) in blocking buffer was added to cells and incubated an additional hour at room temperature. Excess antibody was removed by dilution with six PBS washes. Coverslips were mounted on glass slide by using DAPI and PPD fluorescence mounting media. Images were then analyzed and processed using the Metamorph software program (Metamorph Meta Imaging Series 6.1).

Quantifying MT Recovery After Depolymerization

MT recovery was quantified by two methods: visual array index (VAI) and microtubules array assay (MAA). In the former method, two independent judges scored images of randomly selected cells in a blinded manner. The extent of recovery was scored using the rubric outlined in Figure 1. At least twenty random cells were scored and scores were averaged for each time point. To determine the statistical difference if one exists, a Ranked order analysis, Rfit, was performed comparing time, sample, and trial. Statistically significant results were subjected to paired t-test with Bonferroni corrections. The MAA method measured the area within a cell covered by MTs. Twenty cells from randomly chosen fields of viewed were quantified using ImageJ software. To locate the boundaries of MTs easily, images were converted into
gray scale and converted to a binary image using the staining intensity of MTs to set the threshold gray value. ImageJ was used to measure the area of MT fluorescence. The MT area of 20 cells was averaged per time point. There were three independent trials. To determine the statistical difference if one exists, a multiple ANOVA was performed comparing time, sample, and trial. Statistically significant results were subjected to paired t-test with Bonferroni correction. The triplicate samples of each time point was averaged and separate graphs were plotted for Rev-YFP, M6, and M4 with their control.
Figure 1: Estimating MT recovery by determining a Visual Array Index. Cells are scored from 0 to 4 depending on phenotype of MT arrays. A. Cytoplasmic microtubules are depolymerized with no intact MTs. Most tubulin is present as dots of fluorescence. B. Cells have small asters with few, short MTs. C. Asters are small with MTs roughly spanning the radius of a cell. It is impossible to manually count MTs. D. MTs are shorter than untreated cells but cover roughly 50% of normal arrays. E. MTs cover most of the cytoplasm but arrays are not as extensive as untreated cells. F. Cells possess large population of long MTs extending to the periphery. Bar = 5µm.
Visual Array Index

Score 0

Score 0.5

Score 1

Score 2

Score 3

Score 4
Results

To test the hypothesis that Rev depolymerizes microtubules in living cells, one would ideally measure MT dynamics in living cells expressing Rev or a control protein. This is difficult due to the overwhelmingly large number of MTs present. However measuring the dynamics of individual MTs at the cell periphery using time-lapse video microscopy is a common solution. Before committing to such laborious methodology, bulk MT dynamics can be estimated by monitoring the ability of MT arrays to recover from cold-induced depolymerization. Consequently HeLa cells transiently expressing YFP and Rev were placed on ice to depolymerize MTs. Unfortunately, after cold treatment cells were not viable. Therefore, Hela cells stably expressing YFP and Rev were used. Since HeLa cells do possess cold-stable MTs (33), all MTs are depolymerized by this treatment (Figure. 1A). Cells were then allowed to recover different times at 37°C before fixed and the extent of recovery was quantified using two assays as described in the “Materials and Methods.” If Rev depolymerizes MTs, then MT recovery should be slower in cells expressing Rev.

Microtubule recovery in Rev expressing cells

Two assays were used to estimate recovery. The first calculates a Visual Array Index (VAI) score, a qualitative assessment of recovery. Cells are given a score of 0-4 depending on extent of MT polymerization and cytoplasmic coverage using a rubric listed in figure 1.
Figure 2: Cellular localization of Rev. Photomicrographs of cells expressing YFP (A), Rev-YFP (B), RevM6-YFP (C), RevM4-YFP (D), and Rev2.2 (E). Bar = 5μm.
Rev localization
Figure 3 shows the representative images of fluorescence in YFP and Rev-YFP expressing cells at different times of recovery. MT arrays in both control and experimental cells recover as function of time. After three hours of cold treatment, MT depolymerization was essentially complete in Rev and YFP controls (Fig.3 A, B). This is consistent with published research (42). After 5 m of recovery, YFP cells show average VAI$_{t5}$ of 0.8, the VAI$_{t5}$ of Rev cells is 0.9 (Fig. 3 panel C, D). After 15 m recovery, MTs in both YFP and Rev expressing cells are short segments covering rough half of the cytoplasmic area (VAI=2.4) (Fig. 3 E, F). At later time points, there was extensive MT recovery in both cell types (YFP VAI$_{t30}$=2.9, Rev VAI$_{t30}$= 2.8, and YFP VAI$_{t60}$=2.9 and Rev VAI$_{t60}$=2.6). MT recovery was time-dependent (p<0.001). There is no statistically significant difference between the recovery rates in YFP and Rev expressing cells (p>0.05). To avoid biased scoring, the same photomicrographs were re-scored by a second, blinded analyst. The results obtained by the second analyst confirmed the original data set (data not shown). Analysis of two scoring by Rfit model showed that Rev expressing cells does not inhibit MT recovery.

Given the intrinsic qualitative nature of VAI scoring, MT recovery was also measured using an assay less prone to subjective error. The MT Area assay attempts to measure the surface area covered by fluorescently labeled MTs. The areas of MT at different stages of recovery are shown in Appendix 1. MT recovery was time-dependent (p<0.001). However analysis of data by multiple ANOVA showed that there was no statistical significant difference between experimental and control cells. Together with VAI data, these observations are collectively inconsistent with hypothesis that Rev expression inhibits the recovery of MT arrays following depolymerization.
Figure 3: YFP and Rev expressing cells have similar MT recovery rates.

The average VAI for cells immunolabeled with anti-α tubulin antibody are listed at right bottom of each panel. A and B: cells show complete depolymerization of MTs after three hours of cold treatment (0’). MTs start to polymerize at 5’ time point (C and D). In subsequent time points such as 15’ (E and F), 30’ (G and H), and 60’ (I and J), both Rev expressing and control cells show same increase in MTs. Bar = 5μm.
Time

0'

YFP

Rev-YFP

A

B

C

D

E

F

G

H

I

J

23
Figure 4: MT Area Assay. MT surface areas in Rev and YFP expressing cells recovering from cold treatment are similar. Cells are immunolabeled as above. Twenty cells were threshold and MT area was measured by using ImageJ computation method. Graph demonstrates the average of MT area (Appendix 1) of three trials on Y-axis and time of recovery on x-axis. The error bars represent standard deviation. Rev and YFP expressing cells show similar recovery after cold depolymerization at each time of recovery (P>0.05).
YFP and Rev-YFP

MT Area (Pixels)

Recovery Time (m)

Rev

YFP-C
**MT recovery in Rev mutants:**

One possible reason why Rev does not demonstrably affect rates of MT recovery may be due to an insufficient concentration of Rev in the cytosol. In Rev expressing cells, Rev primarily localizes to the nucleus with substantial localization in interphase nucleoli. Since tubulin exclusively localizes to the cytoplasm, there is limited opportunity for Rev and tubulin to interact during interphase. To increase the cytoplasmic concentration of Rev, Rev mutants M4, M6 and 2.2 were also studied.

M6 is a mutation in the NLS that inhibits Ran-dependent nuclear import of Rev in the nucleus (58). In contrast to wild-type Rev, M6 localizes equally in the cytoplasm and nucleus since Rev has an ability to diffuse into nucleus (Figure 2). M4, possessing a mutation that blocks homo-multimerization, predominately localizes to cytoplasm with reduced amounts in nucleolus (Figure 2). Both M6 and M4 have the ability to bind tubulin heterodimers *in vitro* (54). Moreover, transient and stable over-expression of M6 and M4 in HeLa cells leads to defects in cell cycle progression (55). Neither mutant are able to depolymerize MTs *in vitro* so if they are to have an effect in recovery assays, they are predicted to slow recovery by decreasing the concentration of tubulin available for polymerization.

The Rev2.2 HeLa cell line stably over-expresses Rev fused to the hormone-responsive element of the glucocorticoid receptor and GFP (34). In the absence of hormone, Rev2.2 protein localizes exclusively in the cytoplasm. Previous results show that Rev2.2 cells spend more time in mitosis similar to cells expressing Rev (41). Whether Rev2.2 retains the ability to depolymerize MTs *in vitro* is not known. Depending on these observations, it was
hypothesized that RevM6, RevM4 and Rev2.2 may show slower polymerization compared to control cells.

**RevM6 and control cells show similar rates of recovery after microtubule depolymerization by cold**

Figure 5 shows representative results comparing the recovery of MT arrays in RevM6 and YFP expressing cells. After depolymerization, control and M6 VAI values were 0.2 and 0.1, respectively. Later time points both RevM6 and control cells showed similar score. Rank order analysis of this scoring suggested that the rates of recovery in RevM6 and control cells were statistically similar to controls (p>0.05). Whereas blinded scoring demonstrated that RevM6 cells has faster recovery than control cells. Similar conflicting results were obtained during statistical analysis of MAA data, where point to point t-test showed that there was no difference in recovery whereas multiple ANOVA suggested that RevM6 cells has faster recovery than control cells (Figure 6). Comparable results of both VAA and MAA suggest that RevM6 does not inhibit the MT recovery.
Figure 5: RevM6 and control cells show similar rates of recovery after microtubule depolymerization by cold. Cells are immunolabeled with anti-DMA-1α antibody to visualize microtubules. Right bottom shows the average score of 20 cells of three trials. Similar to Rev expressing cells, RevM6 and YFP show no remarkable difference in recovery of MTs after depolymerization. Both RevM6 and YFP also show similar time dependent increase in polymerization of MTs during recovery periods (p<0.001). Bar = 5µm.
Figure 6: MT Area Assay. RevM6 and control cells show similar growth of microtubule. Twenty cells were threshold and MT was measured by using ImageJ. Graph demonstrates the average of MT area of three trials (Appendix 2) with the error bars of standard deviations. RevM6 and YFP expressing cells show similar MT area in successive time of recovery.
YFP and RevM6

MT Area (Pixels) vs. Recovery Time (m)

- M6
- YFP-C
**RevM4 and control cells show similar recovery after microtubule depolymerization by cold**

The recovery of MTs arrays after depolymerization in RevM4 and YFP expressing cells at different times is shown in Figure 7 and 8. MT arrays recover at similar rates in RevM4 and YFP expressing cells. Depolymerization was not as effective in these experiments as small aster-like structures were visible (YFP $VAI_{t0}=0.0$, RevM4 $VAI_{t0}=0.2$) (Fig. 7A, B). After 5 m of polymerization, YFP cells had growth of MTs segments originating at the centrosome ($VAI_{t5}=0.8$). M4 expressing cells often had short MT arrays not connected to the centrosome ($VAI_{t5}=1.4$). At later time points, most of YFP and RevM4 cells’ MTs appear like score “3” where most of microtubules segments are scattered in the cytoplasm (YFP $VAI_{t15}=2.4$, RevM4 $VAI_{t15}=2.6$, YFP and RevM4 $VAI_{t30/60}=2.8$) (Figure. 7). Measurement of MT areas showed that recovery was similar in M4 and control cells ($p>0.05$) by point-to-point t-test (Figure 8). Further analysis of this data showed similar results with Rev-M6.
Figure 7: RevM4 and control cells show similar recovery after microtubule depolymerization by cold. Cells are immunolabeled and visualized for microtubule presence. Right bottom shows the average score of 20 cells of three trials. After cold depolymerization RevM4 and control cells show the absence of MT arrays (0’). With successive increase in recovery time both cells show same rate of MT polymerization at each time points (p>0.05). Bar = 5\text{\mu}m
Figure 8: MT Area Assay. RevM4 mutant show polymerization of MTs similar to YFP. Graph demonstrates MT area average of YFP and RevM4 expressing cells of three trials at different recovery time (Appendix 3). Standard deviation (Appendix 3) is plotted as error bars at different time points. RevM4 and YFP expressing cells show similar increase in MT presence at consecutive time (p<0.05). Both RevM4 and control cells show similar increase in MT area.
YFP and RevM4

MT Area (Pixels) vs. Recovery time (m)

- M4
- YFP-C
Rev2.2 expressing cells show insignificant difference in recovery of microtubules compared to control cells

The recovery of MT arrays in Rev2.2 and control cells is shown in Figure 9. The extent of MT depolymerization is similar in both cells, VAI=0.0. Nucleation of MTs was evident after 5 m in both cells (VAI$_{t=5}$= 0.7). Recovery continued over the course of the next hour. During the 60 m of recovery, MTs become long and covered 50% of area of cell (YFP VAI$_{t=15}$= 2.3, Re2.2= 2.2, YFP VAI$_{t=30}$=2.7 and Rev2.2 VAI$_{t=30}$= 2.3). At 60 m, YFP expressing cells show MT presence similar to that of 30 m recovered YFP cells (YFP VAI$_{t=60}$=2.7). On other hand, at 60 m Rev2.2 showed average score “3.4” where most of the MTs are formed and present in bundles. VAI show that there is no effect of Rev polymerization of MTs. Microtubule area measurement showed that Rev2.2 expressing cells do not recover slowly compared control cells (Fig. 10). These data are consistent similar to those obtained when cells expressing RevM6 and RevM4 were used.
Figure 9: Rev2.2 and control cells show similar recovery after microtubule depolymerization by cold despite high levels of Rev in the cytoplasm. Rev2.2 cells express Rev, fused to the glucocorticoid receptor in the absence of hormone. Visual Array Assay. At different time points both cells show very similar recovery of MTs (A-J). Bar = 5µm.
Figure 10: MT Area Assay. Rev2.2 mutant show polymerization of MTs similar to YFP. MT Array Area Assay. Graph shows the average MT area versus time of recovery of single trial when Rev2.2 and YFP cell’s MTs were depolymerized and recovered. Rev2.2 and YFP expressing cells show similar increase in MT presence at respective time points.
YFP and Rev 2.2

MT Area (Pixels)

Recovery time (m)

- YFP-C
- Rev 2.2
**MT recovery in Rev expressing metaphase cells**

During metaphase, Rev localizes around the periphery of chromosomes where MTs that comprise the spindle apparatus are known to nucleate owing to the Ran-GTP gradient (27) (Figure 11). This suggests that Rev is temporally and spatially positioned to perturb the mitotic spindle. Since Rev expressing cells show slow cell cycle progression spending more time in metaphase (27, 43), it seems possible Rev-MT interactions are important during division. To test this, I measured MT arrays in mitotic cells after cold treatment. Only cells in metaphase were included in this study. MTs of Rev and YFP expressing metaphase cells were depolymerized by cold. At t=0 m, cells had chromosomes aligned at the metaphase plate but no MTs were present (Figure 12). After 60 m of recovery, defects were evident in both cells. Unaligned chromosomes (Figure 13, 14) and tripolar spindles (Figure 13, 14C) were common. However, the number of abnormalities seen in both cells was similar suggesting that Rev was not the cause of these abnormalities.
Figure 11: YFP and Rev expressing metaphase cells. YFP (A) and Rev (B) expressing metaphase cells were immunolabeled with tubulin specific antibody and DNA was stained with DAPI. Each of the panels (Left to right) displays DAPI, tubulin, YFP and merge channel that shows the spindle formation around the chromosome. Bar = 5µm.
A. YFP (no treatment)

- DAPI
- Tubulin

B. Rev-YFP (no treatment)

- DAPI
- Tubulin

- YFP
- Merge
Figure 12: YFP (A) and Rev (B) expressing Metaphase cell after cold depolymerization of spindle. YFP expressing Hela mitotic cells were depolymerized on ice cold water for three hours, immunolabeled with tubulin specific antibody, and DNA was stained with DAPI. Each of the panels (Left to right) displays DAPI, tubulin, YFP and merge channel. Merge panel shows the depolymerized spindle around the chromosome. Bar = 5µm.
Figure 13: YFP expressing Metaphase cells show chromosomal and spindle formation abnormalities after recovery. YFP expressing mitotic cells were depolymerized on ice cold water for three hours followed by recovery of spindle for 60 minutes at 37°C. Cells were immunolabeled with tubulin specific antibody and DNA was stained with DAPI. All panels display depicts the merge channel of the depolymerized spindle around the chromosome. Metaphase cells show lost chromosomes (A-F) with normal spindle formation after recovery of 60 minutes. Bar = 5µm.
YFP (60m recovery)
Figure 14: Rev expressing Metaphase cells show chromosomal and spindle formation abnormalities similar to YFP expressing cells after recovery. Similar to YFP expressing metaphase cells, Rev expressing mitotic cells were depolymerized on ice cold water and spindles were recovered. Cells were immunolabeled with tubulin specific antibody and DNA was stained with DAPI. Rev expressing cells showed chromosomal and spindle abnormalities similar to YFP expressing cells (A-D). Bar = 5µm.
Rev-YFP (60m recovery)
Discussion

Previous data clearly shows that Rev binds tubulin and depolymerizes MTs in vitro (65). That Rev inhibits aster formation in Xenopus egg extracts shows it has the potential to perturb MT dynamics under cell-like conditions. Such dramatic inhibition occurs only when Rev and tubulin concentrations are equal, a condition unlikely to exist in living or transformed cells. However, low levels of Rev may be sufficient to poison MT dynamics at the plus-ends to generate more subtle and potentially lethal effects (65). Certainly, many anti-mitotic drugs that obliterate MT arrays at high concentrations are lethal at concentrations 1000-fold less because they subtly alter MT and or spindle dynamics. The cell cycle defects seen in Rev-expressing cells are consistent with this hypothesis (27, 43).

This study aims to determine whether Rev has the ability to depolymerize MTs or otherwise affect their activity in living cells. Data presented here shows that over-expressing Rev does not inhibit recovery of MT arrays following depolymerization in interphase cells. These data are consistent with experiments using egg extracts—the cellular concentration of Rev is insufficient to elicit a detectable effect. It is important to recognize that the assays used in this study monitored bulk MT dynamics and may not be sufficiently sensitive to detect subtle effects. Future experiments should use time-lapse video microscopy to track the dynamics of individual MT dynamics measuring rates of catastrophe and rescue.

On the other hand, Rev might have subtle affects on MT that are visible only when following the complex movements driven the mitotic spindles. This is reminiscent of the action of anti-mitotic anti-cancer drugs. Moreover, Rev
localizes perichromosomally in metaphase cells so it is well positioned to affect MT behavior at the kinetochore. To this end, the ability of MTs to recovery after cold-treatment in Rev expressing cells was studied. Indeed, there are obvious defects in Rev expressing cells (Fig.13 & 14). Fewer but similar defects were observed in control cells suggesting there was no consequence attributable to Rev. Future work should repeat these experiments increasing sample sizes. Moreover, imaging of living mitotic cells would be useful to accurately track chromosomal movements and spindle activity.

With the exception of possible effects that occur during cell division, the results of these experiments suggest that the cell cycle defects seen in Rev expressing cells is not directly due to Rev:tubulin/MT interactions. How then is Rev affecting cell growth? Rev may affect the cell cycle progression through its interactions with B23 or Ran. Normally, B23 is present in nucleoli; however, in Rev expressing cells, B23 colocalizes with Rev in the cytoplasm as well as nucleoli (27). Moreover, nucleolar morphology is abnormal when levels of Rev expression are high. As B23 is important in assembly and maturation of ribosomes, it is possible that Rev is inhibiting B23 function reducing the ribosome synthesis and reducing levels of protein synthesis. This is consistent with the observation that Rev expression slows all stages of the cell division cycle (27). To test this hypothesis, future experiments should attempt to measure the rates of ribosome synthesis and protein synthesis in Rev expressing and control cells. In addition, mutations in Rev and/or B23 that block Rev-B23 interactions should restored cellular growth rates.

Given that Rev interactions with Ran in nuclear export and import, Rev may alternatively be altering cell growth through its interactions with Ran. Ran is a small GTPase, important in nucleocytoplasmic transport of many proteins...
and RNAs (18, 57). It is also important for microtubule nucleation (18, 57). Consequently Ran affects transport of proteins and RNAs important for cell proliferation and differentiation and regulates the structure and function of the mitotic spindle (52). It is significant that Rev concentrates around the periphery of metaphase chromosomes where Ran•GTP accumulates (57). Thus, Rev is positioned to both destabilize kinetochore MTs and affect the function of Ran•GTP. Future experiments should therefore attempt to follow Rev:Ran interactions, particularly during mitosis. Live cell microscopy in Rev expressing mitotic cells should be instructive.
References


# Appendices

## Table 1: YFP-C and Rev-YFP

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Table 2: YFP-C and RevM6-YFP

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