A *Drosophila* model of HIV-Tat-related pathogenicity

Piero A. Battaglia[1], Santina Zito[2], Antonella Macchini[1] and Franca Gigliani[2,*]

[1]Laboratorio di Biologia Cellulare, Istituto Superiore di Sanità, Rome, Italy
[2]Dipartimento di Biotecnologie Cellulari ed Ematologia, Università La Sapienza, Rome, Italy
*Author for correspondence (e-mail: Gigliani@bce.med.uniroma1.it)

Accepted 1 May 2001
Journal of Cell Science 114, 2787-2794 (2001) © The Company of Biologists Ltd

SUMMARY

To analyze the mechanism of Tat-mediated HIV pathogenicity, we produced a *Drosophila melanogaster* strain transgenic for HIV-tat gene and induced the expression of the protein during *Drosophila* development. By in vitro and in vivo experiments, we demonstrated that Tat specifically binds to tubulin via the MAP-binding domain of tubulin, and that this interaction delays the polymerization of tubulin and induces a premature stop to microtubule-dependent cytoplasmic streaming. The delay in the polymerization of microtubules, the tracks for the transport of the axes determinants, alters the positioning of the dorso-ventral axis as shown by the mislocalization of Gurken and Kinesin in oocyte of *Drosophila* after Tat induction. These results validate the use of *Drosophila* as a tool to study the molecular mechanism of viral gene products and suggest that Tat-tubulin interaction is responsible for neurodegenerative diseases associated with AIDS.

Key words: Tat-tubulin interaction, Microtubule polymerization, Tat, Cell polarization

INTRODUCTION

Drosophila provides an outstanding opportunity to study the biological and genetic bases of several human pathologies, such as the molecular bases underlying ethanol-induced behaviors (Moore et al., 1998) and the genetics of human neurodegenerative diseases (Warrick et al., 1998; Feany and Bender, 2000; Fortini and Bonini, 2000), and allows for the identification and characterization of genes involved in tumor formation and development (Potter et al., 2000). In this work, we used *Drosophila melanogaster* as a model to investigate the mechanism underlying the pathological effects of the HIV-Tat protein (Karn, 1999).

A wealth of emerging evidence points to the involvement of host cell cytoskeleton in HIV infection (Cenacchi et al., 1996; Delezay et al., 1997; Malorni et al., 1997; Bukrinskaya et al., 1998). HIV-encoded proteins such as gp120 and Rev appear to affect cytoskeleton organization either by inducing cellular ultrastructural changes and massive distruption of microtubules (Cenacchi et al., 1996; Delezay et al., 1997; Malorni et al., 1997) or by depolymerizing microtubules via a specific Rev-tubulin interaction (Watts et al., 2000). Furthermore, it has been suggested that the degenerative neuronal changes described in HIV-infected people are caused by neuronal cytoskeletal changes (Jacobson et al., 1997). The HIV transactivator factor Tat, which can also be released by infected cells and which plays a number of extracellular roles (Rubartelli et al., 1998), affects several cellular functions by inducing angiogenesis (Mitola et al., 2000; Benelli et al., 2000), cell proliferation and apoptosis (Chang et al., 1995) and by affecting the immune response of the host (Goldstein, 1996). Also, Tat appears to be involved in AIDS-associated neurodegenerative diseases (Cupp et al., 1993; Conant et al., 1998) and oncogenesis (Delli Bovi et al., 1986; Ensoli et al., 1999).

The *Drosophila* model allows us to use a novel approach to study the action of viral gene products by analyzing their effects within a territory and not just in the single cell; this is similar to the study of gene expression restricted to a well defined territory during developmental process (Garcia-Bellido et al., 1973; Lawrence, 1973). Any expansion or restriction of the territory in which the gene is expressed results in mutated phenotypes. This novel concept offers unique advantages, allowing the analysis of the gene product interactions and the effects of the ectopic gene expression in the developmental context. Thus, to examine the effects of Tat, we considered this protein, expressed in *Drosophila*, as a gene expressed in a foreign territory (i.e. as a gene ectopically expressed).

To test whether Tat is involved in the cytoskeleton organization, we produced Tat transgenic fly lines and analyzed the effect of Tat (under the control of the hsp70 promoter) by expressing it during *Drosophila* oogenesis. The oocyte of *Drosophila* is, in fact, a highly polarized cell and genetic, molecular and cytological studies have shed light on the specific functions of the cytoskeleton during oogenesis.

In this paper we show that: (1) Tat expressed during *Drosophila* oogenesis results in embryos with only one dorsal appendage, indicating that Tat affects oocyte polarization; and (2) this oocyte depolarization appears to be a consequence of a delay in the microtubule polymerization process caused by the specific interaction of Tat with the MAP binding domain of tubulin. These results indicate that Tat can interact with tubulin to alter the MT polymerization rate in HIV-infected cells and further our understanding of the molecular mechanism underlying Tat-mediated pathogenesis.

MATERIALS AND METHODS

Drosophila stocks and transgenic line

Stocks were: $w^{67}c^{23}$, used to produce Tat-transgenic lines and control;

KZ503 yw; Pin/CyO; khc:lacZ (w+) (Clarck et al., 1994). Tat transgenic lines were generated by injection of purified DNA of pCasPer:Tat and pp25.7wc helper plasmids at a concentration of 400 μg/ml and 100 μg/ml, respectively, into embryos of strain $w^{67}c^{23}$ using standard procedures (Spradling, 1986). Several independent transformant lines were generated. The line used in this study contains one insertion on the second chromosome at position 21B1-2.

Flies homozygous for both hsp:tat and khc:lacZ constructs were obtained by crossing the WG hsp:Tat line (yw; tatw+; TM3/Gl), which carries the hsp:tat construct on the second chomosome, with the KZ503 strain (Clarck et al., 1994).

Recombinant plasmids

The pCasPeR:Tat plasmid was constructed as follows: full-length Tat cDNA (amplified by PCR) from pCMV-Tat plasmid, was cloned into the P-element vector pCaSpeR-hs *Eco*RI-*Xba*I sites. The pCI-Tat plasmid was constructed as described (Longo et al., 1995). The two pcI*-tub plasmids were constructed from pC169 plasmid (Longo et al., 1995) by replacing the *rop* gene (excised as a *Hin*dIII-*Bam*HI fragment) with the PCR products of the I and the II+III domains of α-tubulin, respectively.

Expression of HIV-Tat protein by heat shock treatment

In all experiments the expression of Tat (under the control of hsp70 promoter) was induced by subjecting adults or embryos to heat shock treatment for 1 hour at 37°C.

Immunoprecipitation and western blotting

To detect the expression of Tat in Tat-transgenic *Drosophila*, 18-hour-old embryos were subjected to heat shock, and protein extracts were fractionated by 15% SDS-polyacrylamide slab gel (PAGE) electrophoresis and transferred to nitrocellulose sheets. Membranes were incubated with 5% nonfat dry milk in NET buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.05% Triton X-100, pH 7.5) for 1 hour at room temperature. After incubation, the sheets were washed three times with NET and then incubated with anti-Tat anti-serum (kindly supplied by G. Imerio) at a dilution 1:500 for 2 hours at room temperature. After three washes, blots were incubated with a secondary antibody (goat anti-rabbit) conjugated to horseradish-peroxidase (Biorad, 1:15,000) and, after the final washing, the reaction was visualized by incubation with ECL chemiluminescence reagent (Amersham). For the immunoprecipitation experiments, protein extracts were immunoprecipitated with anti-α-tubulin antibody, using protein A/G plus-agarose (Santa Cruz) as recommended by the manufacturer. Precipitated proteins were resolved by 15% SDS-PAGE and immunoblotted with both anti-α-tubulin and anti-Tat antibodies. The secondary antibodies used to detected Tat and α-tubulin were goat anti-rabbit (Biorad, 1:15,000) and goat anti-mouse (Biorad, 1:5,000), respectively.

Immunofluorescence microscopy

Testes from adult males were dissected in PBS plus 5% glycerol. Spermatocytes were fixed in methanol for 10 minutes at –20°C and then in acetone for 5 minutes at –20°C, washed in PBS and observed under a phase-contrast microscope. Cytological preparations fixed on glass slides were extensively washed in PBS plus 3% BSA, incubated for 40 minutes at room temperature with anti-goat antibody, and then washed for 1 hour. PBS plus 3% BSA was also used both for the antibody incubations and for washing. The detection of Gurken in ovaries was as described (Neuman-Silberger and Schüpbach, 1996). All preparations were incubated overnight at 4°C in appropriate primary antibody dilutions. After washing in PBS plus 3% BSA, samples were incubated for 1 hour with secondary fluorescein- or rhodamine-conjugated antibodies and then extensively washed in PBS plus 3% BSA. Primary antibody dilutions were: monoclonal mouse anti-Tat, 1:200; monoclonal mouse anti-α-tubulin (Amersham), 1:150; anti-Gurken antibody, 1:3000. Secondary antibody dilutions were: fluorescein-conjugated goat $F(ab')_2$ fragment to mouse IGG (Cappel), 1:100; rhodamine-conjugated goat $F(ab')_2$ fragment to mouse IGG (Cappel), 1:300; fluorescein-conjugated goat $F(ab')_2$ fragment to rat IGG (Cappel) 1:100. All preparations were examined with a Nikon optiphot fluorescence microscope equipped with the Biorad MRC1024ES laser scanning confocal attachment.

In vitro tubulin polymerization assay

To polymerize microtubules, a solution containing 2 mg/ml of tubulin (Sigma), 10^{-4} M GTP, 10^{-2} M sodium phosphate, 10^{-3} M EGTA, 1.6×10^{-2} M $MgCl_2$ and 3.4 M glycerol at pH 7 was incubated at 37°C for 30 minutes. The absorbance was continuously monitored at 350 nm. Tat was added to a final concentration of 0.10 mM. Aprotinin (Sigma) control protein was added to a final concentration of 0.10 mM.

Phage immunity test

Bacterial cells transformed with plasmids expressing different λ repressor fusion proteins were tested for sensitivity to λ phages. Phages of different virulent phenotypes were assayed by spot tests, at concentrations varying from 10 to 10^6 phages per spot, on lawns of transformed bacteria. The λ phages used are as described (Longo et al., 1995).

Microtubule-dependent streaming

Bulk ooplasmic movements within living oocytes were assayed as follows: adult females were transferred to a cover glass and covered with halocarbon oil, and egg chambers were removed and dissected. The cover glass was then transferred to the confocal microscope, and autofluorescent yolk granules were directly imaged with a BHS filter set provided with the Bio-Rad MRC1024ES laser scanning confocal microscope. Single frame images were collected at 10 second intervals with the use of a confocal microscope with fluorescent filters. Temporal projections were created by summing 10 frames from a time-lapse sequence with the project (maximum) utility of the COSMOS software provided with the Bio-Rad confocal microscope. Each projection represents 100 seconds of total elapsed time.

Characterization of mutated phenotypes

To analyze chorion and embryonic cuticular phenotypes, embryos from both $w^{67}c^{23}$ and $w^{67}c^{23}$ strains that are transgenic for Tat were processed as described (Wieschaus and Nusslein-Volhard, 1986). *Drosophila* virgin females (5-6-days old) were subjected to heat shock for 1 hour at 37°C and then mated with males (5-6-days old) on apple juice-agar plates at 25°C for 39-40 hours (Wieschaus and Nusslein-Volhard, 1986). Embryos were then collected and observed under a microscope.

RESULTS

Detection of HIV-Tat protein by western-blotting

We performed the immunoblotting experiment to verify whether Tat is actually expressed in the pCaSperhs-Tat transgenic *D. melanogaster* lines. As Fig. 1 shows, anti-Tat immunoreactive band of ~15 kDa is observed only with protein extracts from Tat-transgenic embryos following heat shock treatment (lane 1). There is no immunoreactive band detectable in proteins separated from extracts of non-heat-shock treated and non-transgenic embryos (lanes 2 and 3, respectively).

Expression of Tat during *Drosophila* oogenesis causes abnormality in embryo dorsal appendage formation

Homozygous Tat transgenic lines of *D. melanogaster* were used to test whether HIV-Tat protein, expressed at different

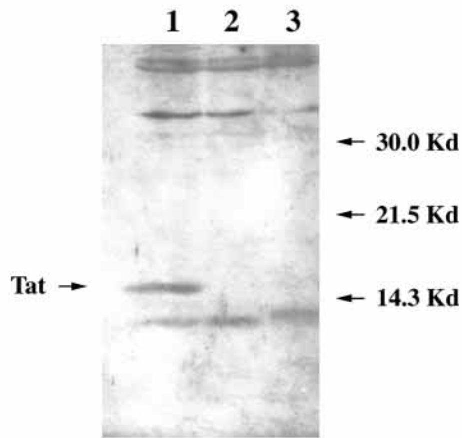

Fig. 1. Immunoblot of anti-Tat anti-serum with proteins separated by SDS-PAGE. Lane 1, protein extracts from Tat-transgenic embryos following heat-shock treatment. Lane 2, protein extracts from Tat-transgenic embryos without heat-shock treatment. Lane 3, protein extracts from non-transgenic embryos. Molecular weights of marker are indicated by arrows.

developmental stages by heat shock promoter, is capable of inducing particular phenotypes. To this end, Tat was expressed during *Drosophila* oogenesis (stages 8-10) by subjecting *Drosophila* females to heat shock treatment. 5000 fertilized eggs from different experiments were collected and 10% to 13% (depending on experiments) of eggs showed only one fused dorsal appendage (Fig. 2A) instead of the two normally present (Fig. 2B). In the control sample (non-transgenic line subjected to the same heat shock treatment) <1% of eggs exhibited this phenotype. This phenotype resembled that observed in mutations that alter the dorso-ventral patterning of the egg shell (Nilson and Schüpbach, 1999) and may be caused by either mislocalization of determinants for oocyte axis specification (Theurkauf et al., 1993) or inhibition of the microtubule polymerization process (Koch and Spitzer, 1983).

To shed light on the molecular mechanism by which Tat interferes with two apparently different mechanisms to produce this particular phenotype, we first tested whether Tat interacts with microtubules. We used *Drosophila* spermatocytes for these experiments since they are relatively large and the organization of the microtubules differs during the cell cycle stages.

Tat was induced by heat shock in adult males, testes were dissected and fixed, and spermatocytes at interphase and anaphase were immunostained with both anti-α-tubulin and anti-Tat monoclonal antibodies. Immunochemical fluorescent confocal microscopy showed that Tat colocalizes to microtubules both in interphase (Fig. 3A-C) and in division spermatocytes (Fig. 3D-F).

Delaying effect of Tat on tubulin polymerization process

Since Tat appears not to affect the microtubule structural organization of spermatocytes at interphase or anaphase (Fig. 3), we wished to determine the effect of the Tat-tubulin interaction by testing the reaction rate of tubulin polymerization in the presence or absence of Tat. Tubulin (the main component of microtubules) was polymerized in vitro in the presence of GTP (Mitchison and Kirschener, 1984) and Tat, and the polymerization reaction rate was monitored on the spectrophotometer. The results show that Tat causes a delay in the tubulin polymerization process by negatively affecting the cooperative effect of α and β tubulin monomers in the polymerization reaction (Fig. 4). Tat appears to affect the sigmoid trait of the curve, but not the lag phase or the final concentration of polymerized tubulin. The protein aprotinin (a protease inhibitor that, in common with Tat, has low molecular weight and cysteine residues) used as control does not affect the polymerization rate of tubulin (Fig. 4). This result demonstrates that Tat binds to tubulin and that this binding acts on microtubule assembly by delaying the tubulin polymerization process.

Tat and tubulin co-immunoprecipitation

To confirm that Tat associates with tubulin *in vivo*, we carried out a co-immunoprecipitation assay (Fig. 5). We incubated protein extracts from *Drosophila* embryos with anti-α-tubulin monoclonal antibody for immunoprecipitation, followed by immunoblotting with anti-Tat polyclonal antibody. As shown in Fig. 5 (lane 1), Tat is detected in immunoprecipitate obtained from Tat-induced transgenic embryos, whereas, both in non-Tat-induced embryos and in non-transgenic control embryos (lanes 2 and 3, respectively), no immunoreactive band is observed.

Tat and tubulin heterodimerization assay

To identify any domain(s) of tubulin involved in the linking between Tat and tubulin, we performed heterodimerization assays based on cI λ phage repressor properties (Longo et al., 1995). The cI repressor functions as a dimer; thus, the fusion of the cI DNA-binding domain to a heterologous protein region capable of forming dimers, is expected to produce a functional λ repressor, and render bacterial cells expressing it immune to λ phage infection. If no dimerization occurs the cells are phage sensitive. The α and β tubulin amino acid sequences are highly conserved among species (Theurkauf et al., 1986) and each monomer can be divided into three functional domains: the N-terminal domain I containing the nucleotide binding region, the intermediate domain II containing the taxol binding site and the C-terminal domain III, which appears to constitute the binding surface for motor proteins and for microtubule-

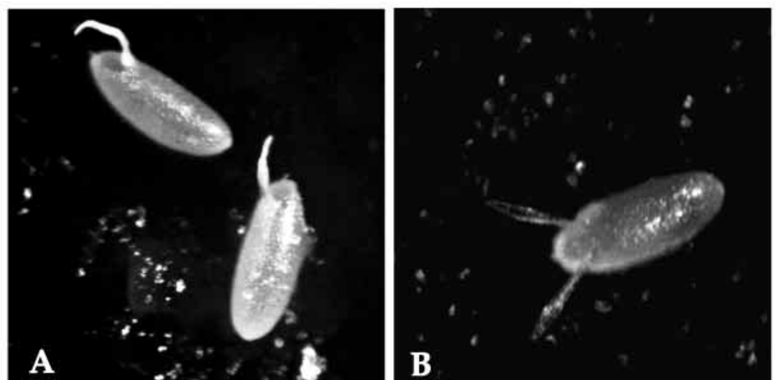

Fig. 2. *Drosophila* embryos showing only one fused dorsal appendage following Tat expression (A); wild-type *Drosophila* embryo (B).

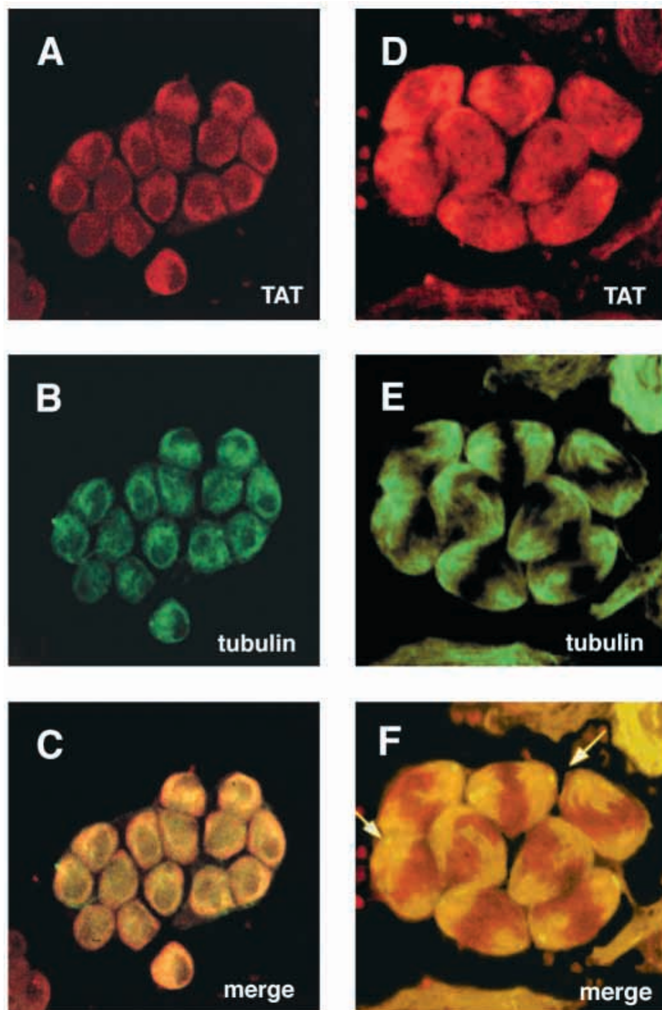

Fig. 3. In vivo interaction between Tat and microtubules. (A-C) *Drosophila* spermatocytes at late S phase double-labelled with anti-Tat (A) and with anti-α-tubulin (B); merged image (C) reveals that Tat and α-tubulin colocalize. (D-F) *Drosophila* spermatocytes at anaphase double-labelled with anti-Tat (D) and with anti-α-tubulin (E); merged image (F) reveals the colocalization of Tat with both α-tubulin and centrosome (arrowhead).

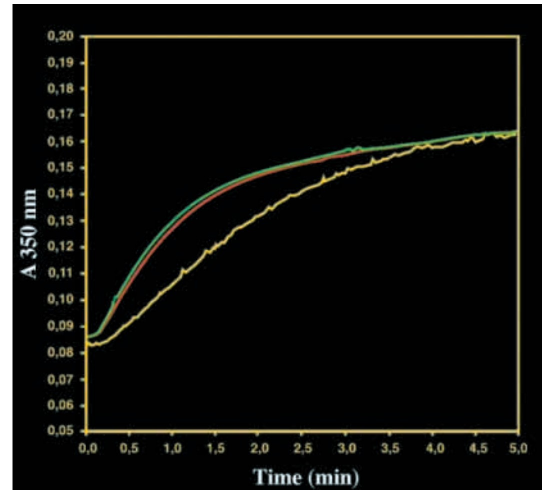

Fig. 4. In vitro effect of Tat on tubulin polymerization process. Upper curves, tubulin polymerization rate in presence of GTP at 37°C (red curve) and in presence of aprotinin and GTP at 37°C (green curve). Lower curve, tubulin polymerization rate in presence of Tat and GTP at 37°C.

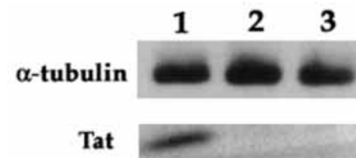

Fig. 5. Tat co-immunoprecipitation with α-tubulin. Protein extracts immunoprecipitated with anti-α-tubulin and immunoblotted with anti-α-tubulin (top) and with anti-Tat (bottom) antibodies. Lane 1, protein extracts from Tat-transgenic embryos following heat-shock treatment. Lane 2, protein extracts from Tat-transgenic embryos without heat-shock treatment. Lane 3, protein extracts from non-transgenic embryos.

associated proteins (MAPs) (Nogales et al., 1998). Recently, Chau et al. reported that the tubulin amino acid sequence found between domains II and III, contains the binding site for the MAP-Tau (Chau et al., 1998). On the basis of these data, we performed experiments to test whether domains of *Drosophila* α-tubulin interact with Tat and, if so, which. For this purpose, α4-tubulin DNA coding for domain I (amino acids 1-215) and for domains II and III (amino acids 216-462) was amplified by PCR and each α-tubulin fragment was cloned into the pC169 vector (Longo et al., 1995) that contained a sequence coding for the λ cI DNA-binding domain (cI*) carrying a mutation that prevents its binding to the operator. The recombinant plasmids were used to transform *E. coli* cells containing the cI-Tat fusion cloned in the pC168 low copy number compatible plasmid (Longo et al., 1995). The transformants were tested for λ immunity. If the interaction between the two proteins occurs, the functional chimeric repressor (cI-Tat) should be titrated out by the heterodimerizing cI*-Tub fusion protein and should become inactive, making the transformed cells sensitive to λ infection. The results show that *E. coli* cells transformed with Tat and plasmid carrying the α-tubulin domain I, are immune to λ phage, whereas *E. coli* cells co-transformed with Tat and plasmid carrying the α-tubulin domain II+III are sensitive to λ infection. Thus, we can conclude that Tat specifically interacts with the tubulin domains II+III but is unable to form dimers with tubulin domain I alone.

On the whole, these results demonstrate that: (1) Tat and tubulin interact with each other; and (2) the interaction specifically involves the MAPs-binding domain of tubulin and strongly suggests that the Tat-induced delay in tubulin polymerization depends on competition between Tat and MAPs in binding to tubulin.

Microtubule-dependent cytoplasmic streaming is prematurely blocked by Tat

To ascertain whether the delaying effect of Tat on microtubule polymerization occurs in vivo and to test the eventual consequences, we used *Drosophila* oocytes as the experimental model. During *Drosophila* gametogenesis, female gametes develop as syncitia connected by large cytoplasmic bridges called ring canals, which allow the flow of nutrients between

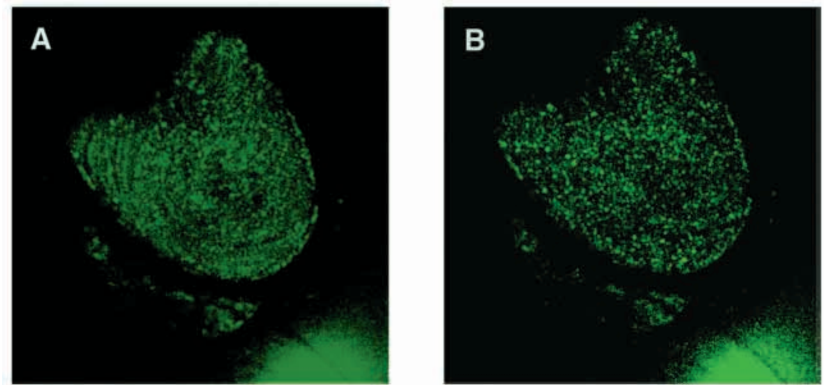

Fig. 6. Confocal images of microtubule-dependent streaming in *Drosophila* oocyte before (A) and after (B) Tat expression.

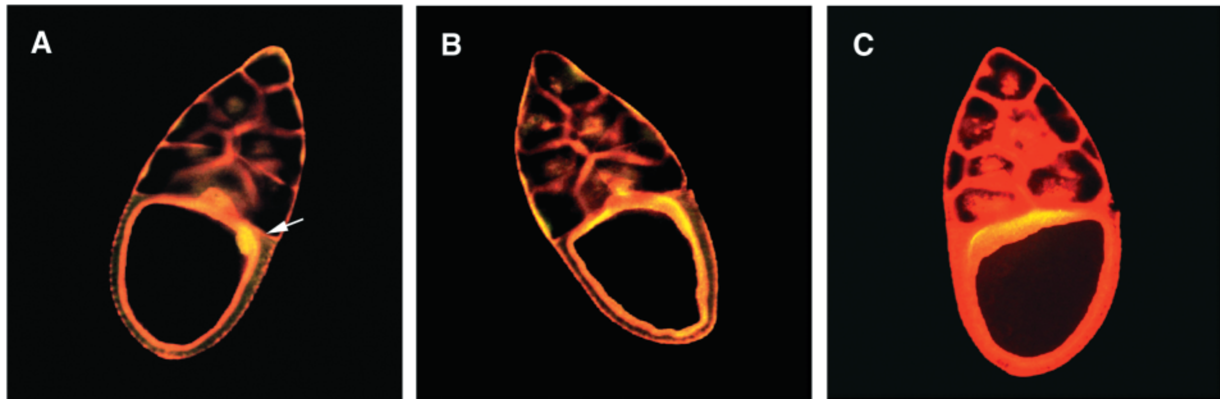

Fig. 7. Distribution pattern of Grk protein in egg chambers from Tat transgenic females. Confocal images of indirect immunofluorescent staining of egg chambers with anti-Grk antibody using a Cy3-conjugated anti-rat secondary antibody. The staining of the Grk protein is in green. F-actin is visualized with rhodamine-conjugated phalloidin in red; regions where labels overlap are yellow. All egg chambers are at stage 10 of oogenesis. (A) Egg chamber from a female not expressing Tat. The Grk protein is normally localized to the anterior-dorsal cortex of oocyte. (B,C) Egg chambers from females after Tat induction. Grk is localized around the entire circumference (B) or at the anterior margin (C).

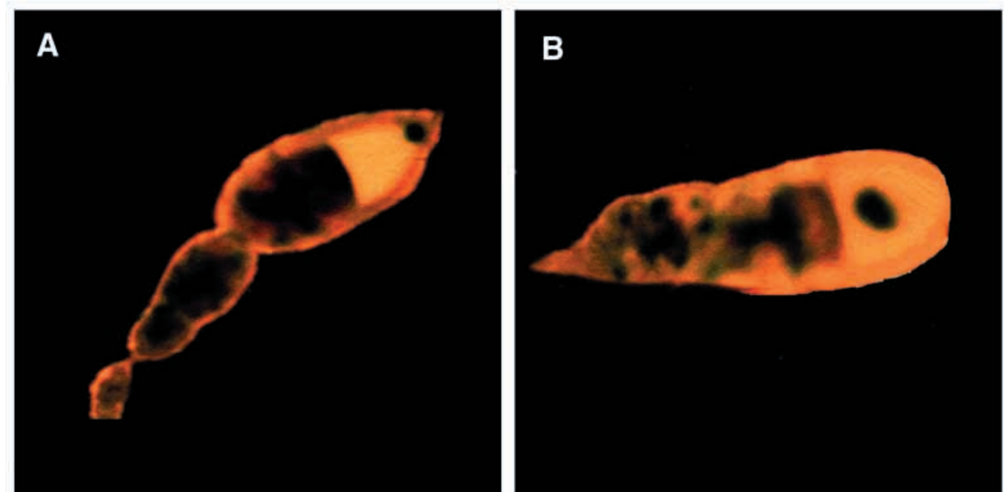

Fig. 8. Kinesin:β-gal localization in oocytes after heat shock treatment from both wild-type and Tat transgenic females. Both panels show X-gal staining of stage 10 egg chambers. (A) Egg chamber from a wild-type female not expressing Tat subjected to heat-shock. Kin:β-gal is normally localized at the posterior. (B) Egg chamber from a female after Tat induction. Kin:β-gal is abnormally localized in the middle of the oocyte.

cells in a syncitium (Robinson and Cooley, 1996). This transport is essential for the development of normal oocytes. The cytoskeleton plays an integral role in cytoplasm transport as shown by the fact that disruption of the cytoskeleton by mutation or by drugs, such as colchicine, causes defective transport (Theurkauf et al., 1993; Koch and Spitzer, 1983). During stages 10b-13 of oogenesis the molecules are distributed in the ooplasm by cytoplasmic streaming generated by microtubules to avoid the formation of the anterior-posterior particle-gradient and allow the binding of particles to localized specific anchors (Theurkauf, 1994a; Theurkauf, 1994b; Clarck et al., 1997; Glotzer et al., 1997).

To test whether Tat affects microtubule-mediated transport, we examined bulk cytoplasmic movements inside living egg chambers after heat-shock both in oocytes expressing Tat and in control oocytes. In these experiments, autofluorescent yolk granules within the ooplasm were followed with time-lapse laser scanning confocal microscopy (Theurkauf, 1994b). In all oocytes that expressed Tat (57 oocytes examined from different experiments), the cytoplasmic streaming gradually decreased to terminate immediately, or at most 1 hour, after Tat expression (Fig. 6A,B), whereas the cytoplasmic flow observed in control oocytes was not affected by heat shock and normally terminated in 2 hours and 30 minutes.

Therefore, we can conclude that Tat interacts with microtubules in vivo and that the consequence of this interaction produces a premature stop of the microtubule-dependent cytoplasmic streaming.

Tat depolarizes *Drosophila* oocytes

Egg polarization depends on the correct localization of the determinants of the antero-posterior and dorso-ventral axes which, in turn, depend on the microtubule cytoskeleton organization (Nilson and Schüpbach, 1999). During stages 8 through 10, microtubules associate preferentially with the anterior cortex of the oocyte, so that a broad anterior to posterior cortical gradient is formed at stage 9 (Theurkauf, 1994b).

To verify whether Tat can affect cytoskeletal functions that mediate axis specification, we tested the position, after the expression of Tat in oocytes, of the TGF-α-like protein Gurken, which is the basic determinant of the dorsal-ventral axis (Neuman-Silberger and Schüpbach, 1996) and that of the plus-end-directed microtubule motor protein Kinesin, which mirrors antero-posterior polarity of the *Drosophila* oocyte (Clarck et al., 1994; Clarck at al., 1997).

In *Drosophila* wild-type stage 9-10a egg chambers, the Gurken protein is spatially localized on the dorsal-anterior corner of oocytes (Neuman-Silberger and Schupbach, 1996). After Tat induction by heat shock treatment, the Gurken protein (Grk), detected by specific anti-Grk antibody (Neuman-Silberger and Schupbach, 1996), was mislocalized in oocytes (Fig. 7). It appears, in fact, to be distributed either along the anterior border of the oocyte, or along the anterior and dorsal border (Fig. 7B,C), whereas the heat shock treatment in non-transgenic lines did not affect the localization of the Gurken protein (Fig. 7A).

Kinesin is normally localized in the posterior of the oocytes during stages 8 and 9 of oogenesis. We examined the localization of Kinesin in egg chambers from *Drosophila* females transgenic both for kinesin:βgal fusion and for hsp:Tat construct following heat shock treatment. As shown in Fig. 8 by β-galactosidase staining, the expression of Tat resulted in the mislocalization of Kinesin to the middle of the oocyte (Fig. 8B). By contrast, in the oocyte in which Tat was not expressed, kinesin was localized normally (Fig. 8A).

Therefore, the interaction of Tat with microtubules determines the mislocalization of axis determinants. Thus, these results account for the mutated phenotype occurring in embryos after Tat induction.

DISCUSSION

The expression of the HIV-Tat protein during *Drosophila* oogenesis led to the production of embryos with only one dorsal appendage. Inspection of the mutated phenotype allowed the identification of the *Drosophila* gene product with which Tat interacts, producing mutated embryos. This Tat-interacting product is the tubulin. We have shown, in fact, that Tat specifically binds to tubulin. This binding affects microtubule polymerization by delaying the tubulin polymerization rate and, as a result, depolarization of oocytes occurs.

The role of Tat in the microtubule polymerization process

Here we have shown that when Tat is expressed in *Drosophila* oocytes at stage 10b-13, oocyte cytoplasmic streaming is prematurely blocked. A similar effect has been observed (Theurkauf, 1994b) by treating oocytes with colchicine, a drug that inhibits microtubule polymerization. Thus, Tat exhibits the same effect as colchicine but via a different mechanism. Colchicine, in fact, inhibits the polymerization of microtubules by binding to tubulin monomers during the nucleation process and is ineffective on polymerized tubulin. By contrast, Tat binds to already polymerized tubulin as shown by the in vivo experiments (Figs 3, 4). This binding occurs through the tubulin domain needed for tubulin-MAPs binding. MAPs contribute to microtubule stabilization by inhibiting tubulin dissociation at the microtubule ends (Drewes et al., 1998); therefore, we suggest that the polymerization delaying effect caused by Tat depends on the Tat-MAPs competition at the tubulin-MAPs binding site. Interestingly, the tubulin:Tat relative concentration in the in vitro experiment to measure the tubulin polymerization reaction rate, is 100:1. This condition is very similar to that present in HIV-infected cells, where the concentration of Tat is certainly lower compared with the concentration of tubulin. It is known that many human neurodegenerative conditions involve a reorganization of the neuronal cytoskeleton, which seems due to the loss of MAP-tubulin binding (Chau et al., 1998; Drewes et al., 1998). Therefore, besides the mechanisms already described, the Tat-tubulin association makes it possible for Tat to be involved in the pathogenesis of the AIDS-associated neurologic disorders, destabilizing the MTs through competition with MAPs.

In addition, we have shown that Tat colocalizes with tubulin throughout the cell cycle, including cells at phase S and anaphase (Fig. 3). Thus, the association of Tat with microtubules appears to be cell-cycle independent. This association seems to keep Tat far from receptors to which it may associate when secreted, and far from nuclear DNA to which Tat can associate via transcriptional complex of cellular genes and thus affect normal cell functions. Microtubules, by capturing Tat, may control both the translocation of Tat into the nucleus and the secretion of Tat from the cells (Battaglia et al., 1997). However, this Tat-microtubule interaction affects cell polarization and may result in further damage to the cell.

The role of Tat in oocyte polarization

The expression of Tat during *Drosophila* oogenesis results in embryos that present only one dorsal appendage. This mutated phenoype in *Drosophila* arises from dislocation of the dorso-ventral axis and is caused by mutations affecting the *spindle* genes, which are involved in patterning, and in DNA repair in

mitosis and meiosis. One of the *spindle* mutations affects the *gurken* gene expression by drastically reducing *gurken* mRNA translation but seems not to influence the microtubule polymerization process (Gonzalez-Reyes et al., 1997; Ghabrial, et al., 1998). On the contrary, we observe that, after Tat expression, Gurken is still produced in the egg, but it is abnormally localized. Thus, the mechanism by which Tat causes dorso-ventral axis mislocalization differs from that of *spindle* mutation and appears to depend on microtubule delayed polymerization. This result raises the possibility that the interaction of Tat with microtubules induces defects in mitotic and/or meiotic spindle formation that may result in chromosome aneuploidies.

We are grateful to J. Karn and MCR AIDS reagent project UK for giving us the anti-Tat monoclonal antibody, G. Gargiulo and F. Graziani for *Drosophila* strains and plasmid used in this work, T. Scüpbach for anti-Grk antibody, and N. Pietravalle for photograph composition. We also thank G. Callaini for helpful suggestions and discussion. This research was supported by grants from the Italian Heath Ministry (AIDS project) to P.A.B. and by a contribution of the Istituto Pasteur-Fondazione Cenci Bolognetti Università di Roma La Sapienza to F.G.

REFERENCES

Battaglia, P. A., Regoli, E. and Gigliani F. (1997). Measurement of the range of HIV-LTR transactivating activity of HIV-TAT in vitro. *Int. J. Oncology*. **11**, 1007-1011.

Benelli, R., Barbero, A., Ferrini, S., Scapini, P., Cassatella, M., Bussolino, F., Tacchetti, C., Noonan, D. M. and Albini, A. (2000). Human immunodeficiency virus transactivator protein (Tat) stimulates chemotaxis, calcium mobilization, and activation of human polymorphonuclear leukocytes: implications for Tat-mediated pathogenesis. *J. Infect. Dis*. **182**, 1643-1651.

Bukrinskaya, A., Brichacek, B., Mann, A. and Stevenson, M. (1998). Establishment of a functional human immunodeficiency virus type 1 (HIV-1) reverse transcription complex involves the cytoskeleton. *J. Exp. Med.* **188**, 2113-2125.

Cenacchi, G., Guiducci, G., Pasquinelli, G., Re, M. C., Ramazzotti, E., Furlini, G., Malorni, W., DeLuca, M. and Martinelli, G. N. (1996). Early ultrastructural changes of human keratinocytes after HIV-1 contact: an in vitro study. *Eur. J. Dermatol*. **6**, 213-218.

Chang, H. K., Gallo, R. C. and Ensoli, B. (1995). Regulation of cellular gene expression and function by tha human immunodeficiency virus type-1 Tat protein. *J. Biomed. Sci*. **2**, 189-202.

Chau, M. F., Radeke, M. J., de Inés, C., Barasoain, I., Kohlstaedt, L. A. and Feinstein, S. C. (1998). The microtubule-associated protein tau cross-links to two distinct sites on each α and β tubulin monomer via separate domains. *Biochemistry* **37**, 17692-17703.

Clarck I., Giniger, E., Ruhola-Baker, H., Jan, L. Y. and Jan Y. N. (1994). Transient posterior localization of a kinesin fusion protein reflects anteroposterior polarity of the *Drosophila* oocyte. *Curr. Biol*. **4**, 289-300.

Clarck, I. E., Jan, L. Y. and Jan, Y. N. (1997). Reciprocal localization of Noad and kinesin fusion proteins indicates microtubule polarity in the *Drosophila* oocyte, epithelium, neuron and muscle. *Development* **124**, 461-470.

Conant, K., Garzino-Demo, A., Nath, A., McArthur, J. C., Halliday, W., Power, C., Gallo, R. C. and Major, E. O. (1998). Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia. *Proc. Natl. Acad. Sci. USA* **95**, 3117-3121.

Cupp, C., Taylor, J. P., Khalili, K. and Amini, S. (1993) Evidence for stimulation of the transforming growth factor beta 1 promoter by HIV-1 Tat in cells derived from CNS. *Oncogene* **8**, 2231-2236.

Delezay, O. , Yahi. N., Tamalet, C., Baghdiguian, S., Boudier, J. A. and Fantini, J. (1997). Direct effect of type 1 human immunodeficiency virus (HIV-1) on intestinal epithelial cell differentiation: relashionship to HIV-1 enteropathy. *Virology* **238**, 231-242.

Delli Bovi, P., Donti, E., Knowles, D. M., Fruedman-Kien, A., Luciw, P. A., Dina, D., Della-Favera, R. and Basilico, C. (1986). Presence of chromosomal abnormalities and lack of AIDS retrovirus DNA sequences in AIDS-associated Kaposi's sarcoma. *Cancer Res.* **46**, 6333-6338.

Drewes, G., Ebneth, A. and Mandelkow, E. (1998). MAPs, MARKs and microtubules dynamics. *Trends Biochem. Sci.* **23**, 307-311.

Ensoli, B., Monini, B. and Sgadari, C. (1999). Pathogenesis and cell biology in Kaposi's sarcoma. In *HIV and the New Viruses*. 2nd edn (ed. A. Dalgleish and R. Weiss), pp. 385-413. Academic Press, San Diego, CA.

Feany, M. B. and Bender, W. W. (2000). A *Drosophila* model of Parkinson's disease. *Nature* **404**, 394-398.

Fortini, M. E. and Bononi, N. M. (2000). Modeling human neurodegenerative diseases in *Drosophila*. *Trends Genet.* **16**, 161-167.

Garcia-Bellido, A., Morata, G. and Ripoll, P. (1973). Developmental compartimentalization of the wing disk of *Drosophila*. *Nature New Biol*. **245**, 251-253.

Ghabrial, A., Ray, R. P. and Schüpbach, T. (1998). *okra* and *spindle-B* encode components of the RAD52 DNA repair pathway and affect meiosis and patterning in *Drosophila* oogenesis. *Genes Dev*. **12**, 2711-2723.

Glotzer, J. B., Saffrich, R., Glozer, M. and Ephrusssi, A. (1997). Cytoplasmic flows localize injected oskar RNA in *Drosophila* oocytes. *Curr. Biol.* **7**, 326-337.

Goldstein, G. (1996). HIV-1 Tat protein has a potential AIDS vaccine. *Nat. Med*. **2**, 960-964

Gonzalez-Reyes, A., Elliot, H. and St Johnston, D. (1997). Oocyte determination and origin of polarity in Drosophila: the role of the spindle genes. *Development* **124**, 4927-4937.

Jacobson, S., Henriksen, S. J., Prospero-Garcia, O. Phillips, T. R., Elder, J. H., Young, W. G., Bloom, F. E. and Fox, H. S. (1997). Cortical neuronal cytoskeletal changes associated with FIV infection. *J. Neurovirol*. **3**, 283-289.

Karn, J. (1999). Tackling Tat. *J. Mol. Biol*. **293**, 235-254.

Koch, E. A. and Spitzer, R. H. (1983). Multiple effects of colchicine on oogenesis in *Drosophila*: induced sterility and switch of potential oocyte to nurse-cell developmental pathway. *Cell Tissue Res*. **228**, 21-32.

Lawrence, P. A. (1973). A clonal analysis of segment development in *Oncopeltus* (Hemiptera). *J. Embryol. Exp. Morph*. **30**, 681-699.

Longo, F., Marchetti, M. A., Castagnoli, L., Battaglia, P. A. and Gigliani, F. (1995). A novel approach to protein-protein interaction: complex formation between the p53 tumor suppressor and the HIV Tat proteins. *Biochem. Biophys. Res. Comm*. **206**, 326-334.

Malorni, W., Guiducci, G., Pasquinelli, G., Rivabene, R., Re, M. C., Ramazzotti, E., DeLuca, M, LaPlaca, M. and Cenacchi, G. (1997). HIV-type 1 induces specific cytoskeleton alterations in human epithelial cells in culture. *Eur. J. Dermatol*. **7**, 263-269.

Mitchison, T. and Kirschner, M. W. (1984). Microtubule assembly nucleated by isolate centrosome. *Nature* **312**, 232-237.

Mitola, S., Soldi, R., Zanon, I., Barra, L., Gutierrez, M. I., Berkhout, B., Giacca, M., Bussolino, F. (2000). Identification of specific molecular structures of human immunodeficiency virus type 1 Tat relevant for its biological effects on vascular endothelial cells. *J. Virol*. **74**, 344-353.

Moore, M. S., DeZazzo, J., Luk, A. Y., Tully, T., Singh, C. M. and Heberlain, U. (1998). Ethanol intoxication in *Drosophila* and pharmacological evidence for regulation by the cAMP signaling pathway. *Cell* **93**, 997-1007.

Neuman-Silberger, F. S. and Schüpbach, T. (1996). The *Drosophila* TGF-α like protein Gurken: expression and cellular localization during *Drosophila* oogenesis. *Mech. Dev*. **59**, 105-113.

Nilson L. A. and Schüpbach T. (1999). EGF receptor signalling in *Drosophila* oogenesis. *Curr. Top. Dev. Biol*. **44**, 203-243.

Nogales, E., Wolf, S. G. and Downing, K. H. (1998). Structure of the αβ tubulin dimer by electron crystallography. *Nature* **391**, 199-203.

Potter, C. J., Turenchalk, G. S. and Xu, T. (2000). *Drosophila* in cancer research. *Trends Genet.* **16**, 33-39.

Robinson, D. N. and Cooley, L. (1996). Stable intercellular bridges in development: the cytoskeleton lining the tunnel. *Trends Cell Biol*. **6**, 474-479

Rubartelli, A., Poggi, A., Sitia, R. and Zocchi, M. R. (1998). HIV-1 Tat: a polypeptide for all season. *Immunol. Today* **19**, 543-545.

Spradling, A. C. (1986). P element-mediated transformation. In *Drosophila: A Practical Approach* (ed. D. B. Roberts), pp. 175-197. IRL press, Oxford, UK.

Theurkauf, W. E. (1994a). Immunofluorescence analysis of the cytoskeleton during oogenesis and early embryogenesis. In *Methods in Cell Biology* (ed. S. B. Goldstein and E. A. Fryberg), pp. 489-505. Academic Press.

Theurkauf, W. E. (1994b). Premature microtubule-dependent cytoplasmic streaming in *cappucino* and *spire* mutant oocytes. *Science* **265**, 2093-2096.

Theurkauf, W. E., Baum, H., Bo, J. and Wensink, P. C. (1986). Tissue-specific and constitutive α-tubulin genes of *Drosophila melanogaster* code for structurally distinct proteins. *Proc. Natl. Acad. Sci. USA* **83**, 8477-8481.

Theurkauf, W. E., Alberts, B. M., Jan, Y. N. and Jongens, T. A. (1993). A central role for microtubules in the differentiation of *Drosophila* oocytes. *Development* **118**, 1169-1180.

Warrick, J. M., Paulson, H. L., Gray-Board, G. L., Bui, Q. T., Fischbeck, K. H., Pittman, R. N. and Bonini, N. M. (1998). Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. *Cell* **93**, 939-949.

Watts, N. R., Sackett, D. L., Ward, R. D., Miller, M. W., Wingfield, P. T., Stahl, S. S. and Steven, A. C. (2000). HIV-1 Rev depolymerizes microtubules to form stable bilayered rings. *J. Cell. Biol*. **150**, 349-360.

Wieschaus, E. and Nüsslein-Volhard, C. (1986). Looking at embryos. In *Drosophila: A Pratical Approach* (ed. D. B. Roberts), pp. 199-227, IRL press, Oxford, UK.