Mitotic Infidelity and Centrosome Duplication Errors in Cells Overexpressing Tripeptidyl-Peptidase II

Vaia Stavropoulou,1 Jianjun Xie,1 Marie Henriksson,1 Birgitta Tomkinson,2 Stefan Imreh,1 and Maria G. Masucci1

1Microbiology and Tumor Biology Centre, Karolinska Institutet, Stockholm, Sweden and 2Department of Biochemistry, Biomedical Centre, Uppsala University, Uppsala, Sweden

Abstract

The oligopeptidase tripeptidyl-peptidase II (TPP II) is up-regulated Burkitt’s lymphoma (BL) cells that overexpress the c-myc proto-oncogene and is required for their growth and survival. Here we show that overexpression of TPP II induces accelerated growth and resistance to apoptosis in human embryonic kidney 293 cells. This correlates with the appearance of multiple chromosomal aberrations, numerical and structural centrosome abnormalities, and multipolar cell divisions. Similar mitotic aberrations were also observed in a panel of BL lines and were suppressed, in parallel with TPP II down-regulation, upon reversion of BL-like characteristics in EBV-immortalized B lymphocytes carrying a tetracycline-regulated c-myc. Functional TPP II knockdown by small interfering RNA expression in BL cells caused the appearance of giant polynucleated cells that failed to complete cell division. Collectively, these data point to a role of TPP II in the regulation of centrosome homeostasis and mitotic fidelity suggesting that this enzyme may be a critical player in the induction and/or maintenance of genetic instability in malignant cells. (Cancer Res 2005; 65(4): 1361-8)

Introduction

Tripeptidyl-peptidase II (TPP II) is an evolutionary conserved serine peptidase of the subtilisin family that removes tripeptides from the free NH2 terminus of oligopeptides (1–4). The 138-kDa TPP II polypeptide forms large oligomeric complexes of >1,000 kDa that are detected in the cytoplasm of all cell types. In addition, a membrane-associated TPP II isoform is present in most cells (1, 2, 5, 6).

The contribution of TPP II to cellular proteolysis is poorly understood. The finding that TPP II is up-regulated in cells adapted to grow in the presence of toxic doses of proteasome inhibitors (5, 7) has led to the suggestion that TPP II may facilitate the activity of the proteasome by accelerating the production of free amino acids from longer precursors (8, 9). This possibility was recently substantiated by the finding that TPP II is the only cytosolic aminopeptidase that can recognize peptides longer than 15 amino acids, a preferred length of proteasomal products (10). In addition, TPP II seems to be involved in the processing of certain cellular substrates as it is the main cholecystokinin-inactivating enzyme in rat brain (6) and regulates apoptotic responses by promoting the maturation of procaspase-1 in macrophages infected with the Shigella flexneri (11). Recent evidence suggests that TPP II may also participate in the regulation of immune responses by producing a specific subset of antigenic peptides in cells with impaired proteasome activity (10, 12, 13). Some of these functions could be dependent on the endopeptidase activity of TPP II (5, 12), which may allow the generation of longer peptides from intact proteins or polypeptide precursors.

We have previously shown that TPP II is highly expressed in Burkitt’s lymphoma (BL) that carry a chromosomal translocation leading to deregulation of the c-myc oncogene (14). The contribution of c-Myc to TPP II overexpression was confirmed by the finding that the enzyme is up-regulated in parallel with the switch to a BL-like phenotype in an EBV transformed lymphoblastoid cell line (CLL) carrying a tetracycline-inducible c-myc gene. Treatment with an inhibitor of TPP II induced apoptosis in BL and BL-like cells suggesting that the enzyme may control processes that are intimately associated with the growth and survival of this tumor. A similar involvement of TPP II in the regulation of tumor cell growth was recently suggested by the finding that up-regulation of TPP II in proteasome inhibitors adapted EL4 cells correlated with enhanced tumorigenicity in vivo and with up-regulation of members of the IAP family of antiapoptotic proteins (15).

In addition to chromosomal translocations, Burkitt’s lymphoma cells frequently display signs of mitotic infidelity with gains and losses of whole chromosomes (16). Such chromosomal aberrations often arise from abnormal multipolar cell divisions (17) following centrosome duplication errors and the generation of supernumerary spindle poles (18, 19). Centrosomes are the major microtubule-organizing center in mammalian cells and, to organize a bipolar mitotic spindle, the centrosome duplicates once during mitosis. Initiation of centrosome duplication is dependent on proteolytic processes mediated by the ubiquitin/proteasome system (20). Thus, deregulation of intracellular proteolysis could have far-reaching consequences in the establishment of malignant transformation and tumor progression.

We have addressed the role of TPP II in the regulation of cell growth by investigating the effects of overexpression and functional knockdown in cells transfected with TPP II-expressing plasmids or infected with a lentivirus expressing TPP II specific small interfering RNAs. Our data point to a role of TPP II in the generation of cells with altered centrosome homeostasis and mitotic fidelity, suggesting that this enzyme may be a critical player in the c-Myc induced deregulation of the cell cycle in malignant cells.

Materials and Methods

Cell Lines. The human embryonic kidney (HEK) 293 cells line was purchased from American Type Culture Collection (LGC Prochem AB, Bucås, Sweden). Stably transfected sublines expressing the murine TPP II (4) were grown in DMEM supplemented with 10% heat-inactivated FCS (Life Technologies, Ltd., Grand Island, NY), 100 units/mL penicillin and 100 µg/mL streptomycin (complete medium) containing 400 µg/mL of Geneticin (G418, Life Technologies). EBV-transformed LCLs were established by

Request for reprints: Maria G. Masucci, MTC Karolinska Institutet, Box 280, S-171 77 Stockholm, Sweden. Phone: 46-8-32486755; Fax: 46-8-331399; E-mail: Maria.Masucci@mrtc.ki.se.
infection of B lymphocytes with the B95.8 strain of EBV (21). The EBV-
positive BL lines Namalwa and Raji (22) and EBV-negative lines Akata (23) and
BL-A1 (24) were established from tumor biopsies. The EREB5-5 LCL
expressing an estrogen-regulated EBNA-2 (25) was maintained in RPMI 1640
complete medium supplemented with 1 μM/L β-estradiol (Sigma, St.
Louis, MO). The A1 cell line was obtained by transfection with a constitutive
m-cyc- plasmid (26). The P493 cell line carries an estrogen regulated EBNA-2 and
a tetrasaccylated m-cyc (27). P493-BL was cultured in complete medium
alone, whereas P493-LCL was maintained in medium containing 0.5
μg/mL tetracycline and 1 μM/L of β-estradiol.

**Western Blotting.** Cells (2 × 10⁶) were lysed in NP40 lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP-40, and 1 mmol/L
phenylmethylsulfonyl fluoride], centrifuged at 14,000 rpm for 15 minutes
and the protein concentration of the supernatants was determined by protein
assay reagent; bichinonic acid protein assay (Pierce, Rockford, IL). Twenty micrograms of cell lysates were mixed with equal
volume of SDS-PAGE loading buffer, separated in an 8% polyacrylamide gel, and
electrobotted to Protran nitrocellulose membranes (Schleicher and
Schuell, Keene, NH). The blots were probed with a chicken antibody specific
for TIP II (Immunosystem, Uppsala, Sweden), and developed by enhanced
chemiluminescence (Amersham-Pharmacia Biotech, Uppsala, Sweden).
Densitometric analysis was done using the Image Quant software
(Molecular Dynamics, Sunnyvale, CA).

**Enzyme Assays.** Cells (2 × 10⁶) were lysed in 100 μL of 50 mmol/L Tris-
buffer (pH 7.5), containing 1% Triton X-100. After centrifugation for 30
minutes at 14,000 rpm and the supernatant was diluted 10-fold with 100
mmol/L potassium phosphate buffer (pH 7.5), containing 30% (v/v) glycerol
and 1 mmol/L DTT and protein concentration was determined by bichinonic acid protein assay. Fluorogenic substrates (100 μmol/L)
detecting TIP II activity (AAM-AMC) were incubated for 45 minutes at 37°C
with 1 μg of cytosolic extracts in buffer containing 50 mmol/L Tris-HCl (pH
7.5), 5 mmol/L MgCl₂, and 1 mmol/L DTT in a final volume of 100 μL. The
fluorescence emission was determined by fluorometer (Perkin-Elmer,
Beaconsfield, United Kingdom) with excitation at 380 nm and emission at
460 nm and is presented as arbitrary units.

**Proliferation Assays and Growth Curves.** Cells (5 × 10³) were distributed in triplicates wells of 96-well U-bottomed microtiter plates in
DMEM containing the indicated amounts of FCS. After culture for 48 hours, the cells were pulsed with 0.5 μCi per well [³H]thymidine for 6 hours and
then harvested onto glass filters. Incorporated radioactivity was counted on a
Wallac 1450 Microbeta liquid scintillation counter (Wallac, Finland). Cells
(4 × 10⁴) from exponentially growing cultures were plated in 35 × 10 mm cell
culture dishes with 2-mm grids (Nalge Nunc International, Rochester,
NY) in 2 mL of DMEM supplemented with 10% FCS. Half of the medium
was replaced every other day. The cell number was scored visually by
number of live cells was counted by trypan blue dye exclusion.

**Mitic Index and Chromosome Counts.** Cells (2 × 10⁶) were harvested by trypsinization, washed twice in PBS and resuspended in
75 mmol/L KCl for 5 minutes at room temperature. The cell pellets were
then fixed by incubation for 5 minutes in 5% glacial acetic acid and 3% methanol
and 5 minutes in freshly prepared fixative solution (3:1, methanol and
acetic acid), resuspended in 0.5 mL of fixative solution and dropped onto
a clean, wet slide. After air-drying the slides were stained with Giemsa
(Pierce, Rockford, IL). Triplicate slides were prepared for each cell line and
10 fields containing 70 to 100 cells were scored from each slide. The mitotic
index (MI) corresponds to the % cells in mitosis. Chromosome preparations
were made by culturing semiconfluent cells with 50 ng/mL colcemid (Invitrogen AB Life Technologies, Lidingö, Sweden) for 1 hour at 37°C.
Pictures of ~100 cells from each clone were taken with a cooled CCD
camera (Hamamatsu, Osaka, Japan).

**Immunofluorescence and Flow Cytometry.** Cells (3 × 10⁶) were
washed twice in PBS and 100 μL of cell suspension was used for cytospin
preparations. The cells were fixed in 4% paraformaldehyde in PBS and
permeabilized with 0.2% Triton-X 100 in PBS for 10 minutes at room
temperature. For detection of centrosome the slides were stained with a
mouse monoclonal anti γ-tubulin antibody (GTU-88, Sigma-Aldrich, St.
Louis, MO) at a 1:300 dilution followed by Alexa fluor 594 labeled goat
anti-mouse antibody (Molecular Probes, Eugene, OR) at a 1:1,000 dilution.
Pericentrin and γ-tubulin containing was done by sequential incubation with a
rat monoclonal anti-6-tubulin antibody (Serotec, Oxford, United Kingdom)
at 1:50 dilution and a rabbit anti-pericentrin serum (Covance/Babeo,
Berkeley, CA) at a 1:50 dilution. Cells were counterstained with 4′,6-
diamidino-2-phenylindole (Vector, Burlingame, CA). Fluorescence was
analyzed using a CCD camera equipped LEITZ-BMRB fluorescence
microscope (Leica, Wetzlar, Germany) and images were analyzed using the
Adobe’s Photoshop software. Flow cytometric analysis was done using a
FACSort flow cytometer (Becton Dickinson, Sunnyvale, CA) and CELL-
QUEST software. For cell cycle analysis the cells fixed with 70% ice-cold
ethanol and incubated with a propidium iodine (5 mg/mL, Sigma) solution
containing 10 mg/mL sodium citrate, 0.3% o-phenylenediamine and
0.1 mg/mL RNAase.

**Production of Lentiviruses and Infections.** The oligonucleotides TIP II-1 forward 5′-CCGGTGTCGGATGTGAAATCATGTCATGGAGTTATCCATCATGCGCCACCTTTTTG-3′ and TIP II-1 reverse 5′-AATT-
CCAAAAAGATGGGAATGAAATACGTCATGTCAGTTATCCATCATGCGCCACCTTTTTG-3′were annealed and cloned into the
into the AgeI and EcoRI sites of the plKO.puro.1 plasmid containing the
human U6 promoter (28). Lentivirus stocks were produced in 293T cells
cotransfected with the plasmids lent-VSV-G, Lent-PACK pLKO.puro.1
plasmid containing the RNAi cassette at a 1:2:3 ratio using LipofectAMINE
2000 (Invitrogen, San Diego, CA). Control lentivirus was produced by using the baculovirus vector plKO.puro.1. Namalwa cells were plated at a density of 0.5 × 10⁶ cells per well in 6-well plates and infected with 500 μL of virus
stock for 2 hours at 37°C. Transduced cells were selected in medium
containing 5 μg/mL puromycin (Sigma) for 2 weeks before phenotypic and
functional analysis.

**Results**

**Effect of TIP II on Cell Growth and Apoptosis.** The contribution of TIP II to cell proliferation and sensitivity to apoptosis, was investigated in HEK-293 cells and transfected
sublines. Three TIP II transfected clones (TIP II-5, TIP II-17, TIP II-23) expressed >10-fold higher levels of TIP II compared with the
untransfected parental cells and three empty vector transfected
clones (vector-1, vector-3, and vector-10; Fig. 1A) expressed >10-fold higher levels of TIP II compared with the
untransfected parental cells and three empty vector transfected
clones (vector-1, vector-3, and vector-10; Fig. 1A). Overexpression of TIP II resulted in 3- to 5-fold increase of enzymatic activity in
cytosolic extracts as measured by cleavage of the fluorogenic
substrate AAF-AMC (Fig. 1B), whereas proteasome activity,
assayed by cleavage of the chromotrypsin-like substrate Suc-
LLVY-AMC, was not affected (data not shown).

The growth of control cells was compared with TIP II
overexpressing cells by monitoring [³H]thymidine incorporation and the increase in cell number in cultures kept under standard
settings. TIP II overexpressing cells grew faster compared with vector-transfected (Fig. 1C) and untrans-
fected cells (data not shown). The enhanced growth potential of the TIP II transfecants was further confirmed in [³H]thymidine
incorporation assays where the cells were grown for 48 hours in
medium containing decreasing amount of FCS. As illustrated in
Fig. 1D where the TIP II-17 was compared with the control vector-
1, TIP II overexpression was accompanied by 2- to 3-fold higher
levels of [³H]thymidine incorporation. Similar results were obtained with
the TIP II-23 and TIP II-5 clones (data not shown).

The growth advantage of the TIP II overexpressing clones could...
not be ascribed to increased expression of c-Myc (data not shown) and was also maintained under conditions of serum deprivation.

The proliferative capacity of the TPP II overexpressing and control cell lines was further assessed by measuring their MI (i.e., the percentage of cells undergoing cell division at any given time in unsynchronized cultures). The MI of the three vector transfected clones was 3.1, 3.1, and 3.3, whereas a reproducibly higher MI (4.5, 4.7, and 4.9) was recorded in the three TPP II expressing clones. Calculation of the \( P \)s using the two-tailed Student’s \( t \) test showed that the differences between individual TPP II and control clones were either significant (\( P < 0.05 \)) or very significant (\( P < 0.001 \)).

TPP II Overexpression Is Associated with Chromosomal Instability and Mitotic Aberrations in HEK-293 Cells. Because TPP II overexpression correlated with enhanced DNA synthesis and faster cell proliferation we investigated whether other variables linked to cell division might also be affected. Chromosome counts in TPP II and vector transfected cells revealed that the mean chromosome number was higher in the TPP II expressing clones (63.9, 64.6, and 65 for TPP II-17, TPP II-23, and TPP II-5, respectively) compared with vector transfected and parental cells (59.1 and 56.7, respectively). Furthermore, the TPP II overexpressing clones showed a higher degree of aneuploidy with chromosome

![Figure 1](image1.png)

**Figure 1.** Expression of TPP II confers enhanced growth capacity to transfected HEK-293 cells. The proliferative capacity of HEK-293, vector-transfected, and TPP II-transfected sublines was compared under standard and stress conditions. A, Western blot analysis of the expression of TPP II in parental and transfected cells. Position of molecular weight markers. B, TPP II activity was measured by hydrolysis of the fluorogenic substrates AAF-AMC. One representative experiment out of three. C, cells were plated into cell culture dishes with 2-mm square grids and the number of cells was counted in 20 squares per plate. One representative experiment out of three. Similar growth curves were obtained for the three vector-transfected clones and parental HEK-293 cells. D, \([\text{H}]\)thymidine incorporation was measured after culture for 48 hours in medium containing the indicated amount of FCS. Points, mean of three experiments; bars, SD.

![Figure 2](image2.png)

**Figure 2.** TPP II expression correlates with increased aneuploidy in HEK-293 cells. Chromosome numbers were counted in metaphases of colcemid-treated cells. Pictures of 100 metaphases were taken for each clone.

<table>
<thead>
<tr>
<th>% Cell no. *</th>
<th>Vector 1</th>
<th>TPP II-17</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2 Centrosomes</td>
<td>73.2 ± 6.8</td>
<td>60.6 ± 7.4</td>
</tr>
<tr>
<td>≥3 Centrosomes</td>
<td>1.2 ± 0.8</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>Enlarged centrosome (^\dagger)</td>
<td>9.6 ± 3.9</td>
<td>23.3 ± 4.4</td>
</tr>
<tr>
<td>Other (^\dagger)</td>
<td>14.8 ± 1.2</td>
<td>11.5 ± 4.2</td>
</tr>
</tbody>
</table>

\(^\dagger\)Centrosomes were visualized by immunofluorescence staining for γ-tubulin and centrosome numbers were scored in at least 300 cells for each cell line.

\(^\dagger\)The appearance of the enlarged centrosome structures is shown in Fig 3B.

\(^\dagger\)Cells where centrosomes could not be unequivocally identified.
numbers ranging from 34 to 133 in individual cells whereas a much narrower spread (42-71 chromosomes) was detected in vector-transfected (Fig. 2) and untransfected cells (data not shown).

The wider spread of chromosome numbers suggests that the TPP II overexpressing cells may be more prone to mitotic disturbances. Because chromosomal instability with gains or losses of whole chromosomes can arise from centrosome abnormalities (19), centrosome number, and centrosome morphology were examined by staining for γ-tubulin, a marker of pericentriolar material. Numerical and structural centrosome abnormalities were detected in the TPP II overexpressing cells (Table 1; Fig. 3). Approximately 23% of the cells in the TPP II-17 clone contained large γ-tubulin stained bodies that seemed to be centrosome conglomerates (Fig. 3).

This correlated with the presence of multipolar mitosis with more than two γ-tubulin positive spindle poles.

**TPP II Up-regulation Correlates with Centrosome Duplication Errors and Mitotic Infidelity in c-Myc Overexpressing Cells.** The surprising finding that overexpression of TPP II may be associated with genetic instability in transfected cells prompted us to investigate whether similar defects may occur in cells that spontaneously express high levels of this protease. TPP II is up-regulated in BL and BL-like cells in parallel with deregulated expression of c-myc (14); we asked therefore whether these cells show any sign of mitotic infidelity. Numerical and structural centrosome abnormalities (Fig. 3) were detected by immunofluorescence staining for γ-tubulin in 18% to 24% of the cells from the BL.
To assess whether centrosome and spindle abnormalities are connected between these defects and the overexpression of TPP II. In the final set of experiments, we wished to assess the contribution of TPP II to the regulation of cell division in BL cells. Aberrant Mitosis in Cells Overexpressing TPP II

Aberrant Mitosis in Cells Overexpressing TPP II

**Table 2. Centrosome numbers in LCL and BL cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Cell no.*</th>
<th>≤2 Centrosomes</th>
<th>≥3 Centrosomes</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>VS-B1</td>
<td>LCL</td>
<td>102</td>
<td>7</td>
<td>9</td>
<td>6.4</td>
</tr>
<tr>
<td>SS-B1</td>
<td>LCL</td>
<td>101</td>
<td>5</td>
<td>6</td>
<td>4.7</td>
</tr>
<tr>
<td>MU-B1</td>
<td>LCL</td>
<td>101</td>
<td>9</td>
<td>8</td>
<td>8.1</td>
</tr>
<tr>
<td>VM-B1</td>
<td>LCL</td>
<td>102</td>
<td>6</td>
<td>5</td>
<td>5.3</td>
</tr>
<tr>
<td>ER/EB</td>
<td>LCL-like</td>
<td>100</td>
<td>9</td>
<td>8</td>
<td>8.2</td>
</tr>
<tr>
<td>Namalwa</td>
<td>BL</td>
<td>102</td>
<td>30</td>
<td>22</td>
<td>22.7</td>
</tr>
<tr>
<td>Raji</td>
<td>BL</td>
<td>100</td>
<td>22</td>
<td>18</td>
<td>18.0</td>
</tr>
<tr>
<td>BL-41</td>
<td>BL</td>
<td>100</td>
<td>21</td>
<td>17</td>
<td>17.3</td>
</tr>
<tr>
<td>Akata</td>
<td>BL</td>
<td>95</td>
<td>30</td>
<td>24</td>
<td>24.0</td>
</tr>
<tr>
<td>AI</td>
<td>BL-like</td>
<td>97</td>
<td>21</td>
<td>17</td>
<td>17.7</td>
</tr>
</tbody>
</table>

*Centrosomes were visualized by immunofluorescence staining for γ-tubulin. One representative experiment out of three performed with each cell line is shown.

Discussion

Studies involving mouse and human tumor cell lines adapted to grow in the presence of toxic doses of proteasome inhibitors and BL lines that spontaneously express high levels of TPP II have suggested the intriguing possibility that this cytosolic peptidase may be involved in the regulation of cell survival and growth under HEK293T cells (data not shown) and were therefore used either alone or in combination for lentivirus production. TPP II RNAi lentivirus transduced BL cells were selected for 2 weeks in puromycin containing medium before functional and phenotypic analysis. Control experiments done by infecting BL cells with a green fluorescent protein encoding lentivirus showed virtually 100% transduction efficiency under the chosen condition of infection and selection (data not shown). Representative experiments where Namalwa cells were transduced with the TPP II RNAI and control lentiviruses are shown in Fig. 5. Western blot analysis showed a >10-fold reduction in the expression of endogenous TPP II in cells infected with the TPP II RNAi lentivirus compared with control lentiviruses transduced and uninfected cells, whereas c-Myc expression was not affected (Fig. 5A). Down-regulation of TPP II correlated with decreased enzymatic activity as assessed by cleavage of AAF-AMC (Fig. 5B). TPP II knockdown was accompanied by dramatic changes in cell morphology and growth characteristics. Giant cells were readily detected on examination of the cultures under inverted light microscope (Fig. 5C) and multiple nuclei were detected in ~10% of the cells by 4′,6-diamidino-2-phenylindole staining and fluorescence microscopy (Fig. 5D). A similar percentage of cells with a more than diploid DNA content was detected by propidium iodine staining and fluorescence-activated cell sorting analysis (Fig. 5D). In addition, down-regulation of TPP II correlated with significant slowdown of cell proliferation (Fig. 5E). This slowdown was reproducibly observed in the BL lines Namalwa, Raji, and BL28 during a time window of 2 to 5 weeks post-infection, after which the proliferation rate slowly returned to the levels observed in control cells. This reversion of growth phenotype correlated with progressive increase of TPP II expression in spite of continuous puromycin selection suggesting that the revertants have a selective advantage in culture.

**Table 3. Characteristics of the mitotic spindle in LCL and BL cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Spindle*</th>
<th>% Disorganized spindle</th>
</tr>
</thead>
<tbody>
<tr>
<td>VS-B1</td>
<td>LCL</td>
<td>Normal</td>
<td>Disorganized†</td>
</tr>
<tr>
<td>SS-B1</td>
<td>LCL</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>VM-B1</td>
<td>LCL</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Namalwa</td>
<td>BL</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>Akata</td>
<td>BL</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>BL-41</td>
<td>BL</td>
<td>64</td>
<td>33</td>
</tr>
<tr>
<td>Raji</td>
<td>BL</td>
<td>32</td>
<td>16</td>
</tr>
</tbody>
</table>

*The mitotic spindle was visualized by immunofluorescence for α-tubulin (microtubules) and pericentrin (pericentriolar material). Table 3; Fig. 3F). Approximately 40% of the mitotic cells in the Namalwa, Akata, BL 41, and Raji cell lines showed a gravely abnormal spindle apparatus, which seemed as a star-shaped clump with very short microtubules often departing from a central conglomerate of centrosome-like material, whereas the majority of mitotic LCL cells exhibited a normal bipolar spindle (Fig. 3C and E).

**HEK293T cells**

HEK293T cells were used either alone or in combination for lentivirus production. TPP II RNAi lentivirus transduced BL cells were selected for 2 weeks in puromycin containing medium before functional and phenotypic analysis. Control experiments done by infecting BL cells with a green fluorescent protein encoding lentivirus showed virtually 100% transduction efficiency under the chosen condition of infection and selection (data not shown). Representative experiments where Namalwa cells were transduced with the TPP II RNAI and control lentiviruses are shown in Fig. 5. Western blot analysis showed a >10-fold reduction in the expression of endogenous TPP II in cells infected with the TPP II RNAi lentivirus compared with control lentiviruses transduced and uninfected cells, whereas c-Myc expression was not affected (Fig. 5A). Down-regulation of TPP II correlated with decreased enzymatic activity as assessed by cleavage of AAF-AMC (Fig. 5B). TPP II knockdown was accompanied by dramatic changes in cell morphology and growth characteristics. Giant cells were readily detected on examination of the cultures under inverted light microscope (Fig. 5C) and multiple nuclei were detected in ~10% of the cells by 4′,6-diamidino-2-phenylindole staining and fluorescence microscopy (Fig. 5D). A similar percentage of cells with a more than diploid DNA content was detected by propidium iodine staining and fluorescence-activated cell sorting analysis (Fig. 5D). In addition, down-regulation of TPP II correlated with significant slowdown of cell proliferation (Fig. 5E). This slowdown was reproducibly observed in the BL lines Namalwa, Raji, and BL28 during a time window of 2 to 5 weeks post-infection, after which the proliferation rate slowly returned to the levels observed in control cells. This reversion of growth phenotype correlated with progressive increase of TPP II expression in spite of continuous puromycin selection suggesting that the revertants have a selective advantage in culture.
stress conditions (7, 8, 14). This was attributed to the capacity of TPP II to sustain proteolysis under conditions of impaired proteasome activity, which may selectively alter the turnover of certain substrates, including some inhibitors of apoptosis (15). We have here shown that overexpression of TPP II in transfected HEK-293 cells and in BL-like cells that up-regulate TPP II in response to overexpression of c-myc correlates with accelerated cell proliferation and the appearance of numerical and structural centrosome abnormalities, multipolar mitosis, and genetic instability. TPP II itself may play an important role in the regulation of cell division since functional knockdown by RNA interference resulted in significant slowdown of cell growth and the appearance of giant multinucleated cells with aneuploid DNA content, which is consistent with their incapacity to progress through mitosis. The critical involvement of TPP II in this process and its consequent requirement for the maintenance of cell viability is further supported by the observation that we could never achieve >90% reduction in TPP II expression. Furthermore, revertants expressing high levels of the enzyme under conditions of continuous selection seemed to have a strong growth advantage in vitro. It should be stressed that failure to generate stable knockdowns is not due to the particular cell type used in our experiments since stable knockdowns could be produced in the same BL lines by targeting components of the ubiquitin-proteasome system such as certain isopeptidases (data not shown).

The most surprising aspect of our findings is the correlation between TPP II overexpression and disturbance of mitosis, including the accumulation of numerical and structural centrosome abnormalities and the formation of multipolar spindles. This is likely to be the primary mechanism underlying the enhanced aneuploidy observed in the HEK-293 transfectants and could also play a role in the mitotic infidelity with loss and gain of whole chromosomes that frequently occurs in BLs (29). Two possible scenarios may be considered. TPP II may be involved in the turnover of one or more regulators of cell division. Many of these proteins are known substrates of the ubiquitin-proteasome system and TPP II was shown to interact with this proteolytic machinery, probably by acting downstream of the proteasome and facilitating the flow-through of substrates (5, 7, 8, 15). Thus, whereas lacking the exquisite substrate specificity of ubiquitin-dependent proteolysis, TPP II may still preferentially affect the turnover of certain substrates and favor the selection of cells with distinct growth advantages. However, TPP II may also act independently of the proteasome because overexpression of TPP II did not affect proteasome activity in transfected HEK-293 cells and, in contrast to the reported effect in EL4 cells (8), it did not protect the transfectants from intoxication with various types of proteasome inhibitors (data not shown). The possibility that TPP II may by an independent player in proteolysis is in line with its multimeric structure (5) and potential association-dependent regulation of enzyme activity (30). This structural complexity suggests that TPP II may be capable of sophisticated functions, including endopeptidase activity and substrate recognition. TPP II is exclusively localized in the cytosol and could therefore gain access to nuclear regulator of the cell cycle only after disappearance of the nuclear membrane during mitosis. The peculiar phenotype of TPP II overexpressing cells where multiple centrosomes and spindle abnormalities are accompanied by accelerated cell proliferation, and the appearance of multinucleated cells following TPP II knockdown, suggest that TPP II may be involved in processes that connect centrosome duplication with late events leading to the separation of daughter cells after mitosis. Indeed, recent evidence confirms that the centrosome plays a direct role in the completion of cytokines via relocation of the mother centriole to the intercellular bridge close to the midbody (31). Whereas the molecular events triggered by this relocation remain unknown, time lapse videomicroscopy studies have shown that cell separation starts only when the centriole has moved away from the bridge, whereas removal of the centrosome results in defects of cytokinesis and polyploidy (reviewed in ref. 32).

Although a direct role of TPP II in the regulation of cell division seems likely, we cannot formally exclude that TPP II may indirectly promote the accumulation of genetically altered cells by affecting...
their capacity to undergo apoptosis. Overexpression of c-Myc was shown to be associated with DNA damage (33) and the rapid induction of numerical and structural chromosomal aberration in normal fibroblasts and other cell types (34–36). Furthermore, numerical and structural centrosome defects are often observed in cells expressing viral oncogenes, including HPV-16 E7 and adenovirus E1A and E1B proteins (17). Thus, adenovirus-transformed HEK-293 cells and BL lines share the expression of viral or cellular oncogenes that may promote mitotic infidelity. The genetically altered cells generated as a consequence of aberrant mitosis may not survive unless apoptosis is also blocked. A possible involvement of TPP II in the regulation of apoptosis was suggested by the finding that TPP II overexpressing EL-4 cells express high levels of the inhibitor of apoptosis protein c-IAP-1 and fail to degrade c-IAP-1 and XIAP after treatment with etoposide (15). Interestingly, another member of the IAP family, survivin, was shown to play a double role in the regulation of cell survival and cell division (37). In addition to spontaneous apoptosis, antisense suppression of survivin was shown to produce a phenotype of aberrant mitotic progression with supranumerary centrosomes, formation of multimeric mitotic spindles, failure of cytokinesis, and generation of multinucleated cells (38) that bears striking similarity to that of the functional TPP II knockdown observed in our experiments. It should be stressed, however, the mechanism underlying that stabilization of IAPs following TPP II overexpression remains unclear and a direct role of the enzyme in the turnover of these antiapoptotic proteins seems unlikely since their level of expression was not affected following TPP II knockdown in BL cells (data not shown).

Our results highlight an unexpected role of TPP II in the regulation of cell division. By acting in concert with cellular or viral oncogenes, TPP II seems to play an important role in promoting the survival of cells with numerical and structural chromosomal aberration, a hallmark of malignant transformation and progression. These findings warrant further investigation on the potential therapeutic use of TPP II inhibitors to target genetic instability, tumor progression and the development of drug resistance in various types of malignancies.

Acknowledgments

Received 6/15/2004; revised 11/9/2004; accepted 12/7/2004.

Grant support: Swedish Cancer Society, Swedish Foundation for Strategic Research, and Karolinska Institute, Stockholm Sweden.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. S.A. Stewart and L. Naldini (University of Torino, Italy) for the kind gifts of the lentiviral plasmids and all the colleagues who have helped us to critically evaluate and discuss our data.
References


Mitotic Infidelity and Centrosome Duplication Errors in Cells Overexpressing Tripeptidyl-Peptidase II

Vaia Stavropoulou, Jianjun Xie, Marie Henriksson, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/4/1361

Cited articles
This article cites 35 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/4/1361.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
/content/65/4/1361.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.