0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(11):4991–4998

doi: 10.1210/en.2003-0305

Overexpressed Pituitary Tumor-Transforming Gene Causes Aneuploidy in Live Human Cells

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The mammalian securin, pituitary tumor-transforming gene (PTTG), is overexpressed in several tumors and transforms cells *in vitro* and *in vivo*. To test the hypothesis that PTTG overexpression causes aneuploidy, enhanced green fluorescent protein (EGFP)-tagged PTTG (PTTG-EGFP) was expressed in human H1299 cancer cells (with undetectable endogenous PTTG expression) and mitosis of individual live cells observed. Untransfected cells and cells expressing EGFP alone exhibited appropriate mitosis. PTTG-EGFP markedly prolonged prophase and metaphase, indicating that PTTG blocks progression of mitosis to anaphase. In cells that underwent apparently normal mitosis (35 of 65 cells), PTTG-EGFP was degraded about 1 min before anaphase onset. Cells that failed to degrade PTTG-EGFP exhibited asymmetrical cytokinesis without chromosome segregation (18 of 65 cells) or chromosome decondensation without cytokinesis (9 of 65 cells), resulting in appearance of a macronucleus. Fifty-one of 55 cells expressing a nondegradable mutant PTTG exhibited asymmetrical cytokinesis without chromosome segregation, and some (4 of 55) decondensed chromosomes, both resulting in macronuclear formation. During this abnormal cytokinesis, all chromosomes and spindles and both centrosomes moved to one daughter cell, suggesting potential chaos in the subsequent mitosis. In conclusion, failure of PTTG degradation or enhanced PTTG accumulation, as a consequence of overexpression, inhibits mitosis progression and chromosome segregation but does not directly affect cytokinesis, resulting in aneuploidy. These results demonstrate that PTTG induces aneuploidy in single, live, human cancer cells. (*Endocrinology* 144: 4991–4998, 2003)

ANEUPLOIDY, DEFINED AS an abnormal number of chromosomes or chromosome segments, is a ubiquitous feature of human solid tumors, causes genetic instability, and also promotes further aneuploidy (1). Multiple mechanisms are involved in aneuploidy, including oncogenes, inappropriate cyclin expression, telomere defects, and mutations of tumor suppressor genes (2–7). Here we demonstrate that the oncogene PTTG (pituitary tumor-transforming gene) causes aneuploidy in human cancer cells by disrupting mitosis.

PTTG was isolated from pituitary tumor cells and PTTG overexpression demonstrated in a variety of tumors, including those arising from pituitary, colon, and thyroid tissues (8–12). PTTG transforms cells *in vitro* and *in vivo*; and, because PTTG mutations have not been identified, PTTG tumor abundance is likely due to overexpression (9). PTTG is a mammalian securin, localizes to the interphase nucleus and mitotic spindles, and binds to and inhibits separin (or separase). Separin cleaves cohesin, which binds sister chromatids. At the end of metaphase, PTTG is degraded, thus allowing separation of sister chromatids (13–16) (Fig. 1). PTTG is not absolutely required for cell and animal viability, and an additional mechanism for regulating chromatid separation has recently been suggested by *in vitro* and *in vivo* observations (17–19). Mechanisms for the role of PTTG in tumorigenesis are not clear, and it has been proposed that PTTG induces aneuploidy by inhibiting chromosome segregation (12, 13) (Fig. 1).

To test the hypothesis that PTTG induces aneuploidy, we observed mitosis of single, live human and animal cells expressing enhanced green fluorescent protein (EGFP)-tagged PTTG and found that PTTG indeed induces aneuploidy in cancer cells by causing several abnormal mitotic features. Failure of PTTG degradation was critically involved in these mitotic disturbances. In primary fibroblasts, however, PTTG-EGFP expression invariably causes cell death. The results indicate that PTTG promotes aneuploidy in neoplastic cells.

Materials and Methods

Cells and plasmids

Human lung cancer H1299 cells, human choriocarcinoma JEG-3 cells, mouse pituitary AtT20 cells, and human and mouse primary fibroblasts were grown in DMEM with 10% FBS and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). PTTG-EGFP and p55CDC-EGFP were constructed by cloning PTTG or p55CDC (J. Weinstein, Amgen, Thousand Oaks, CA) into pEGFP-N3 (Clontech, Palo Alto, CA). EGFP-conjugated, nondegradable mutant PTTG (20) was made by site-directed mutagenesis. Nondegradable PTTG is mutated in both the KEN box (KEN to KAA from amino acid residue 9 to 11) and destruction box (RKALGTVNR to AKAAGTVNR from amino acid residue 61 to 69). All constructs were sequenced and verified. EGFP was at the C terminus of PTTG or p55CDC. Cells were studied (microscopy or Western blot) 18–24 h after transfection.

Immnuofluorescent staining

H1299 cells transfected with PTTG-EGFP, p55CDC-EGFP, or EGFP were trypsinized, washed with DMEM, and resuspended in hypotonic buffer (10 mM Tris, 10 mM NaCl, 5 mM $MgCl_2$, (pH 7.0) for 15 min, spun

Abbreviations: EGFP, Enhanced green fluorescent protein; NDF, neutral density filter; PTTG, pituitary tumor-transforming gene.

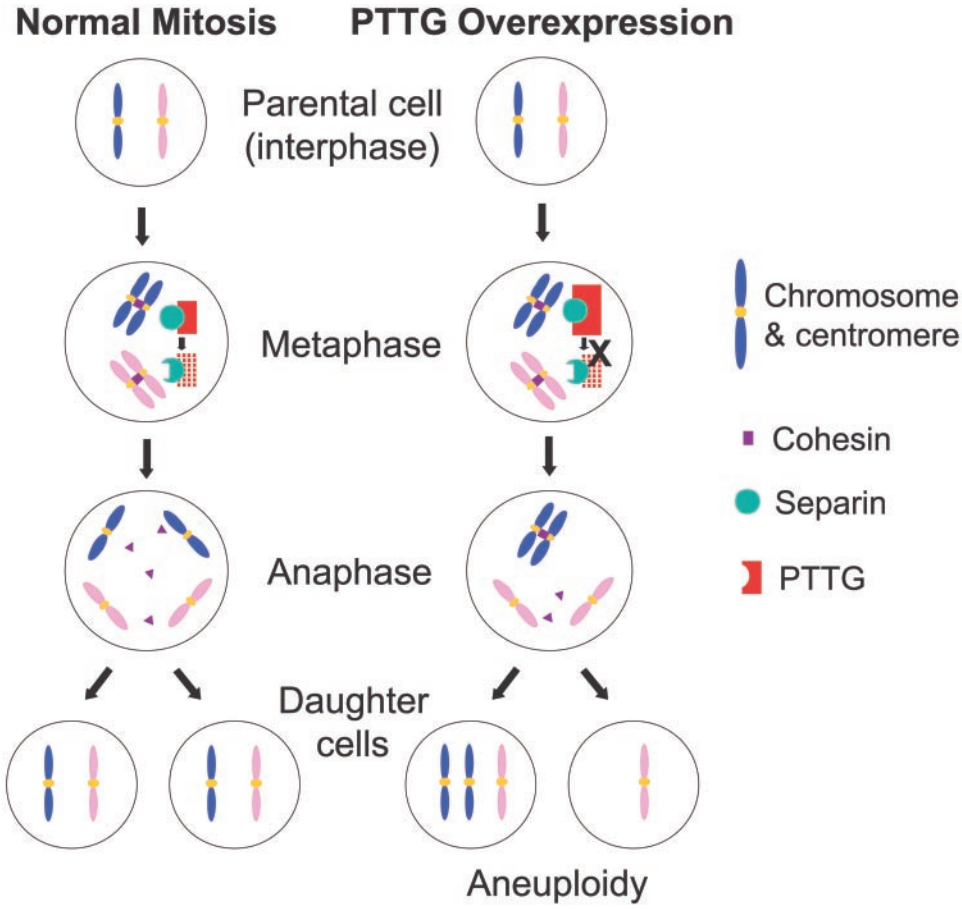

FIG. 1. Depiction of PTTG securin function. *Left*, Normal mitosis. PTTG is a mammalian securin, which maintains binding of sister chromatids. During mitosis, sister chromatids are bound with cohesins. PTTG inhibits separin, an enzyme that regulates cohesin degradation. At the end of metaphase, securin is degraded by an anaphase-promoting complex, releasing tonic separin inhibition and allowing degradation of cohesins. In this manner, sister chromatids are separated equally into daughter cells. *Right*, Abnormal mitosis that may occur in cells overexpressing PTTG. PTTG overexpression may dysregulate sister chromatid separation and results in aneuploidy (adapted from Ref. 12 with permission; see text for details).

onto a Nunc chamber slide at 1350 × *g* for 3 min, and immediately fixed with ice-cold ethanol and rehydrated. This procedure allows removal of cytosolic proteins and clearer visualization of chromosomally associated proteins (21). Cells were stained with human anticentromere serum (Dr. Robert Morris, RDL, Los Angeles, CA) and rhodamine-labeled antihuman IgG, counterstained with Hoechst 33342, and observed with appropriate filters. Staining of α-tubulin of cells grown on coverslips was performed as before (15). Cells were fixed in methanol for staining with antibodies to γ-tubulin and actin (Sigma, St. Louis, MO), and rhodamine-labeled second antibodies were used. For MAD2 staining, cells were fixed in methanol and acetone, and MAD2 antibody (BD Sciences, Franklin Lakes, NJ, 1:200) and rhodamine-labeled goat antimouse IgG were used. The 3F3/2 phosphoepitope was stained in cells fixed in PHEM (60 mM piperazine diethanesulfonic acid, 25 mM HEPES, 10 mM EGTA, 2 mM $MgSO_4$, pH 6.9) containing 0.5% Triton X-100, 2% paraformaldehyde, and 100 nM microcystin (Calbiochem, La Jolla, CA) with monoclonal 3F3/2 antibody (Gary J. Gorbsky, University of Oklahoma Health Sciences Center, 1:2000) and rhodamine-labeled goat antimouse IgG (22, 23).

Single, live-cell imaging

Observation of individual live cells over 48 h was performed by incubating cells in an FCS-2 closed perfusion system (Bioptechs, Butler, PA). Cells were perfused with CO_2-independent L15 medium (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin and saturated with ambient air. Perfusion chamber temperature was set to 37 C, and cells were grown in the perfusion system, for up to a week, until confluency. The perfusion chamber was placed on a Nikon inverted fluorescence microscope and observed with a ×40 extra-long working distance objective. Cells were observed from every few seconds to every several hours, depending on the speed of cell changes. Durations of mitosis phases were determined by counting the minutes between two sequential mitotic milestones. Phase-contrast and EGFP fluorescent images were taken simultaneously at frequencies ranging from every minute (*e.g.* during metaphase to anaphase transition) to every few hours (*e.g.* after telophase), with a CCD digital camera. Relative fluorescence intensity was objectively determined with the application of two neutral density filters (NDFs). Each NDF reduces incident light by 50%. High fluorescence was defined as a cell clearly visualized after application of two NDFs. Medium fluorescence was defined as a cell clearly visualized after application of one (but not two) NDFs. Low fluorescence was defined as a cell visualized only when neither NDF was applied.

Results

PTTG localizes to mitotic chromosomes

Endogenous PTTG levels are undetectable in H1299 cells (Fig. 2). PTTG-EGFP was reactive to antibodies against both PTTG and EGFP (Fig. 2), and subcellular PTTG-EGFP localization is similar to that of PTTG (15). We have shown that PTTG was localized to interphase nucleus and mitotic spindles (15). Because cohesin is localized to chromosomes (21), and separin and PTTG should be close to cohesin, PTTG may also localize to chromosomes. In previous experiments, mitotic spindle-associated PTTG was predominant, and it was not possible to ascertain whether PTTG also localizes to mitotic chromosomes (15). Cytosolic proteins were therefore removed, and significant PTTG-EGFP chromosomal localization was observed (Fig. 2). To confirm the specificity of PTTG localization, we also studied localization of EGFP-conjugated p55CDC, a regulator of mitosis that is localized to centromeres during metaphase. Unlike p55CDC, which

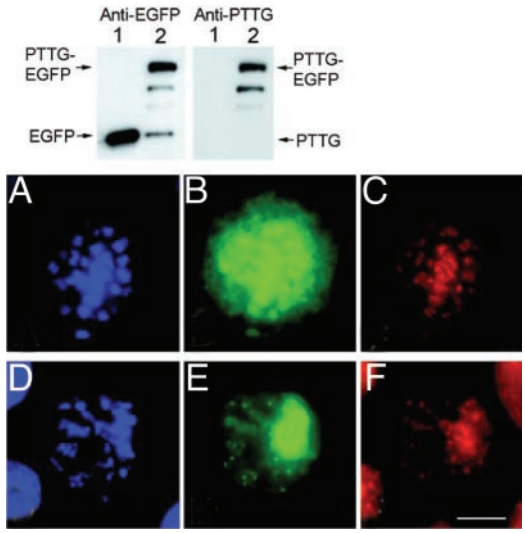

FIG. 2. PTTG-EGFP expression. H1299 cells transfected with EGFP or PTTG-EGFP were lysed in SDS-PAGE lysis buffer, 24 h after transfection, and equal amounts of cell lysates subjected to Western blotting. The membrane was first blotted with mouse anti-EGFP (Clontech) and antimouse peroxidase, washed in 0.3% NaN_3, reblotted with rabbit anti-PTTG (Zymed, South San Francisco, CA) and antirabbit peroxidase, and developed with enhanced chemiluminescence. Lane 1, Lysates from cells expressing EGFP alone; lane 2, lysates from cells expressing PTTG-EGFP. For chromosomal localization of PTTG-EGFP, H1299 cells transfected with PTTG-EGFP, p55CDC-EGFP, or EGFP were treated hypotonically and spun onto chamber slides, fixed and stained with human anticentromere and Hoechst 33342. EGFP alone did not associate with chromosomes. A–C, Cell expressing PTTG-EGFP; C–E, cell expressing p55CDC-EGFP; A and D, chromosome (*blue*); B, PTTG-EGFP (*green*); C and F, centromere (*red*); E, p55CDC-EGFP (*green*). *Bar,* 10 μm.

TABLE 1. Duration of each phase of mitosis of cells expressing PTTG-EGFP

	Pro	Meta	Ana	Telo
Control (n = 16)	15 ± 2	25 ± 3	4 ± 1	5 ± 0
EGFP (n = 28)	14 ± 1	16 ± 2	4 ± 0	6 ± 1
PTTG-EGFP				
Low (n = 11)	25 ± 3	26 ± 4	4 ± 0	5 ± 0
Medium (n = 10)	35 ± 12[a]	49 ± 16[a]	4 ± 0	5 ± 0
High (n = 3)	59 ± 7[a]	112 ± 31[a]	N/A	N/A

Eighteen hours after transfection, the duration (minutes) of prophase (Pro), metaphase (Meta), anaphase (Ana), and telophase (Telo) of single, live H1299 cells untransfected (Control), expressing EGFP only (EGFP), or PTTG-EGFP (PTTG-EGFP) was measured. See text for definitions of low, medium, and high PTTG-EGFP expression levels. N/A, Not applicable.

[a] $P < 0.01$, compared with control or EGFP.

colocalizes with centromeres (24), PTTG-EGFP distributed evenly on mitotic chromosomes. It is also evident that H1299 cell chromosomes harbor a single centromere (Fig. 2, C and F).

PTTG overexpression blocks mitosis progression

Prophase, metaphase, anaphase, and telophase durations were recorded in untransfected control cells, in cells expressing EGFP only, and in cells expressing PTTG-EGFP (Table 1). EGFP alone, or low levels of PTTG-EGFP, did not affect the duration of each phase, but medium or high PTTG-EGFP levels dramatically prolonged prophase and metaphase, indicating that PTTG blocks the progression of mitosis to anaphase. For some cells expressing PTTG-EGFP, no distinct anaphase or telophase was seen because of abnormal chromosome segregation and cytokinesis (see below).

To study the mechanism of the prophase and metaphase block, we immunofluorescently stained the mitosis checkpoint protein MAD2 (25, 26) and the phosphoepitope 3F3/2 (22, 23). MAD2 and 3F3/2 localize at the kinetochore end of microtubules. When sister chromatids are perfectly aligned on the equatorial plate, MAD2 and 3F3/2 leave the kinetochore and signal the start of anaphase. Because PTTG blocks the metaphase-anaphase transition, PTTG may interfere with the MAD2 and 3F3/2 dissociation. In metaphase, MAD2 staining was not seen on well-aligned chromosomes in cells expressing EGFP or high levels of PTTG-EGFP (Fig. 3). We did not find any differences in the 3F3/2 phosphoepitope localization in untransfected cells, in cells expressing EGFP only, or in cells expressing PTTG-EGFP (data not shown). These results suggest that PTTG does not interfere with MAD2 or 3F3/2 dissociation.

PTTG overexpression disrupts mitosis and causes aneuploidy

The destiny of mitosis (from G2, prophase or metaphase to the subsequent interphase) was observed in 50 untransfected cells, 38 cells expressing EGFP only, and 65 cells expressing PTTG-EGFP (Table 2). All but one untransfected cell, and all cells expressing EGFP alone, exhibited appropriate chromosome segregation and cytokinesis, resulting in two daughter cells sharing equal DNA content. EGFP expression levels did not affect the mitosis outcome of cells expressing EGFP alone (data not shown). An EGFP-conjugated inactive mutant PTTG (PM9), in which the SH3 binding domain is mutated, behaves similarly to EGFP (Table 2), suggesting that EGFP itself does not interfere with mitosis.

EGFP was stable during and after mitosis (Fig. 4A). In all PTTG-EGFP-expressing cells that underwent apparently normal mitosis (normal chromosome segregation, no chromosome decondensation, and normal cytokinesis), PTTG-EGFP was degraded about 1 min before the onset of

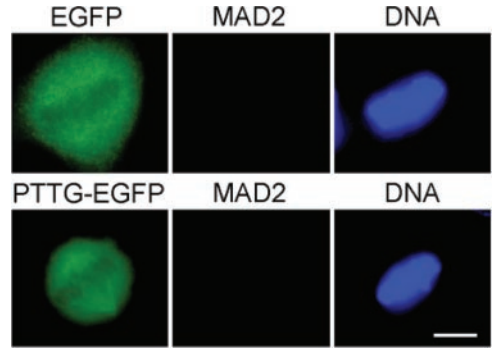

FIG. 3. MAD2 in mitotic cells expressing PTTG-EGFP. H1299 cells transfected with EGFP or PTTG-EGFP were fixed and stained for MAD2 (*red*). EGFP or PTTG-EGFP fluorescence was visualized directly (*green*), and chromosomes were visualized by Hoechst 33342 (*blue*). The cells are in metaphase. *Bar*, 10 μm.

TABLE 2. Mitosis of H1299 cells expressing PTTG-EGFP

	n (%)	EGFP degradation	Chromosome segregation	Chromosome decondensation	Cytokinesis	Daughter cell nucleus	Percent aneuploidy
Control (50 cells)	49 (98)	N/A	Yes	No	Yes	Normal	2
	1 (2)	N/A	No	No	Yes	Macro	
EGFP (38 cells)	38 (100)	No	Yes	No	Yes	Normal	0
PTTG-EGFP low (34 cells)	28 (82)	Yes	Yes	No	Yes	Normal	18
	2 (6)	No	No	No	Yes	Macro	
	2 (6)	No	No	Y (M)	No	Macro	
	1 (3)	Yes	Y/N (1[a])	No	Y/N	Double	
	1 (3)	No	H/N (1[a])	No	Y/N	Double	
PTTG-EGFP medium (19 cells)	7 (37)	Yes	Yes	No	Yes	Normal	63
	8 (42)	No	No	No	Yes	Macro	
	3 (16)	No	No	Y (M)	No	Macro	
	1 (5)	No	Y/N (multiple[a])	Y (M)	Y/N	Micro	
PTTG-EGFP high (12 cells)	8 (67)	No	No	No	Yes	Macro	100
	3 (25)	No	No	Yes (M)	No	Macro	
	1 (8)	No	No	Yes (P)	No	Macro	
DM-PTTG-EGFP (55 cells)	51 (92)	No	No	No	Yes	Macro	100
	4 (8)	No	No	Yes (M)	No	Macro	
PM9-EGFP (9 cells)	9 (100)	Yes	Yes	No	Yes	Normal	0

Mitosis of single, live H1299 cells untransfected (Control), expressing EGFP only (EGFP), PTTG-EGFP, nondegradable mutant PTTG-EGFP (DM-PTTG-EGFP), or an inactive PTTG-EGFP (PM9-EGFP) was observed for presence (yes) or absence (no) of PTTG degradation, chromosome segregation and condensation, and cytokinesis. Y/N, Incomplete chromosome segregation or cytokinesis; M or P, chromosome decondensation at metaphase or prophase; Macro and micro, macronucleus and micronucleus, respectively.

[a] Number of anaphase bridges.

anaphase, as demonstrated by the disappearance of EGFP fluorescence (Table 2 and Fig. 4B), consistent with the securin function of PTTG. An anaphase bridge was infrequently observed (2 of 65 cells) and persisted for more than 1 h, resulting in aborted cytokinesis and a "daughter" cell with two nuclei (Table 2 and Fig. 4C). Absence of PTTG degradation is a prerequisite for chromosome nonsegregation (Table 2); cytokinesis, however, occurred independently of chromosome segregation (Fig. 5A and Table 2). In this asymmetrical cytokinesis, metaphase chromosomes moved closer to one cell pole, accompanied by cell elongation and appearance of a cell midline furrow. Complete nonsegregation during cytokinesis resulted in one daughter cell containing all chromosomes, turning into a cell harboring a macronucleus, and the other nonviable cell devoid of a nucleus (Fig. 5A). The asymmetrical cytokinesis without chromosome segregation is the most commonly observed abnormal mitosis (Table 2). Chromosome segregation was sometimes incomplete, with multiple anaphase bridges and the appearance of several micronuclei (Fig. 5B). To demonstrate that the metaphase cells previously underwent a normal interphase, a PTTG-EGFP-expressing cell was shown to progress from interphase, mitosis, to interphase again but doubled its nuclear size because of incomplete PTTG-EGFP degradation and consequent chromosome nonsegregation (Fig. 5C). In a few cells, chromosomes were decondensed after extended prophase or metaphase, and no cytokinesis was observed, resulting in a cell containing a macronucleus (Table 2). Chromosome decondensation mostly occurred at metaphase and occasionally at prophase. In both cases, PTTG-EGFP degraded continuously, but complete degradation was achieved only after chromosome decondensation.

A nondegradable mutant PTTG invariably causes aneuploidy

Because failure of PTTG degradation results with chromosome nonsegregation, an EGFP-tagged nondegradable mutant PTTG (DM-PTTG-EGFP) (21) was expressed in H1299 cells and chromosome segregation and cytokinesis of individual live cells observed (Table 2 and Fig. 6A). Similar to the wild-type PTTG, DM-PTTG localizes to mitotic spindles (Fig. 6B) and chromosomes (data not shown). All 55 cells observed expressing nondegradable PTTG exhibited abnormal mitosis, irrespective of expression levels, and none of the cells degraded mutant PTTG-EGFP or segregated chromosomes. Most (51 of 55) cells underwent asymmetrical cytokinesis, whereas some cells decondensed chromosomes; macronuclei ensued in both cases. During asymmetrical cytokinesis, chromosomes and mitotic spindles were closely attached and moved to one cell pole (Fig. 6B). The cytokinesis furrow occurred roughly at the position of the distal centrosome (Fig. 6B, cells b and c). Both centrosomes were retained in the daughter cell containing chromosomes (Fig. 6, B and C, cells d and e). Actin was concentrated between the two daughter cells (Fig. 6C, cell f), suggesting a normal actomyosin mechanism in the cytokinesis.

Macronuclei, micronuclei, and multiple nuclei are signs of aneuploidy. Frequency of aneuploidy in daughter cells increased with PTTG-EGFP expression levels (Table 2) and only occurred in 0–2% of daughter cells if parent cells were untransfected or expressed EGFP alone. However, this rate increased to 18% or 63% if parent cells expressed low or medium levels of PTTG-EGFP, respectively. All daughter cells derived from parent cells expressing high levels of PTTG-EGFP were aneuploid.

H1299 is a human lung carcinoma line that does not ex-

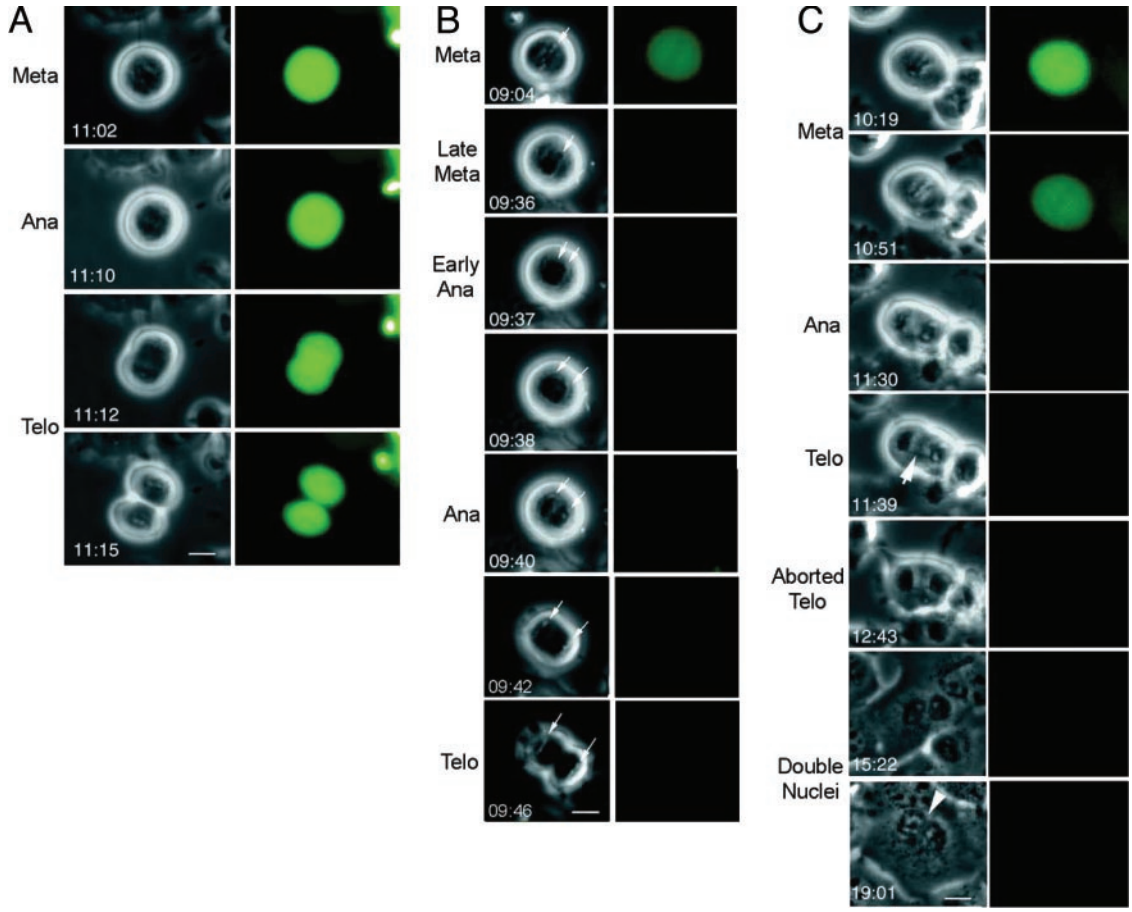

FIG. 4. PTTG-EGFP degradation and anaphase bridge in PTTG-EGFP-expressing cells. Single, live H1299 cells expressing EGFP alone (A) or PTTG-EGFP (B and C) were continuously observed and representative images shown. Both phase contrast (*bright field*) and EGFP or PTTG-EGFP images (*green*) are shown. A, EGFP remained intact throughout mitosis; B, PTTG-EGFP degradation before anaphase onset; C, persistent anaphase bridge resulting in aborted cytokinesis. Phases in mitosis are shown *on the left* of images. Meta, Metaphase; ana, anaphase; telo, telophase. *Thin arrow*, Chromosomes; *thick arrow*, anaphase bridge; *arrowhead*, double nuclei. Time when image was taken is shown for each frame. *Bar*, 10 μm.

press p53. We initially attempted to express PTTG-EGFP in diploid primary fibroblasts, but virtually all cells died of apoptosis as a result of overexpression. In human choriocarcinoma JEG-3 cells that express p53, PTTG-EGFP and degradation-deficient PTTG-EGFP caused aneuploidy similar to that in H1299 cells, although significant apoptosis does occur (55% for PTTG-EGFP and 12% for degradation-deficient PTTG-EGFP) (Table 3). AtT20 murine pituicytes also undergo apoptosis after transfection with PTTG-EGFP and degradation-deficient PTTG-EGFP; but in the few cells expressing degradation-deficient PTTG-EGFP that survived, similar chromosomal abnormalities were observed (Table 3). These results confirm that PTTG overexpression causes aneuploidy and are consistent with our previous findings that PTTG causes p53-dependent apoptosis (16). Like most cancer cell lines, H1299, JEG-3, and AtT20 cells are already aneuploid (data not shown), but they are still suitable in revealing additional, PTTG-induced aneuploidy that are mostly wholesale doubling of chromosome numbers.

Discussion

These results demonstrate that PTTG causes aneuploidy in single, live human cancer cells by disrupting mitosis. Several mechanisms have been proposed to account for the PTTG role in tumorigenesis (12). PTTG and fibroblast growth factor form a positive paracrine feedback cycle, which may contribute to PTTG-induced cell transformation *in vivo* (9, 10). PTTG directly stimulates c-myc transcription (27); but unlike most other transforming genes, PTTG does not promote cell proliferation (8). Based on the PTTG securin function, we and others (12, 13) have proposed that PTTG may disturb mitosis; and we now demonstrate, in this study, that PTTG indeed disrupts mitosis and causes aneuploidy. Aneuploidy is well documented in pituitary tumors and colon cancers, where PTTG is overexpressed (10–12, 28), suggesting that PTTG-induced aneuploidy may play a role in tumorigenesis. Aneuploidy can be caused by abnormal DNA synthesis during the S phase or G2/M because of oncogene expression (2, 3) or by mitosis disruption because of mutations of tumor suppressor genes (6, 7). Because most oncogenes stimulate cell proliferation and presumably cause abnormal DNA synthesis, PTTG is unique in that, unlike other oncogenes, it disrupts mitosis but does not directly increase DNA synthesis. It seems that PTTG-induced aneuploidy is only evident in neoplastic cells, whereas PTTG causes apoptosis in nontransformed cells *in vitro*; thus, aneuploidy may not necessarily be

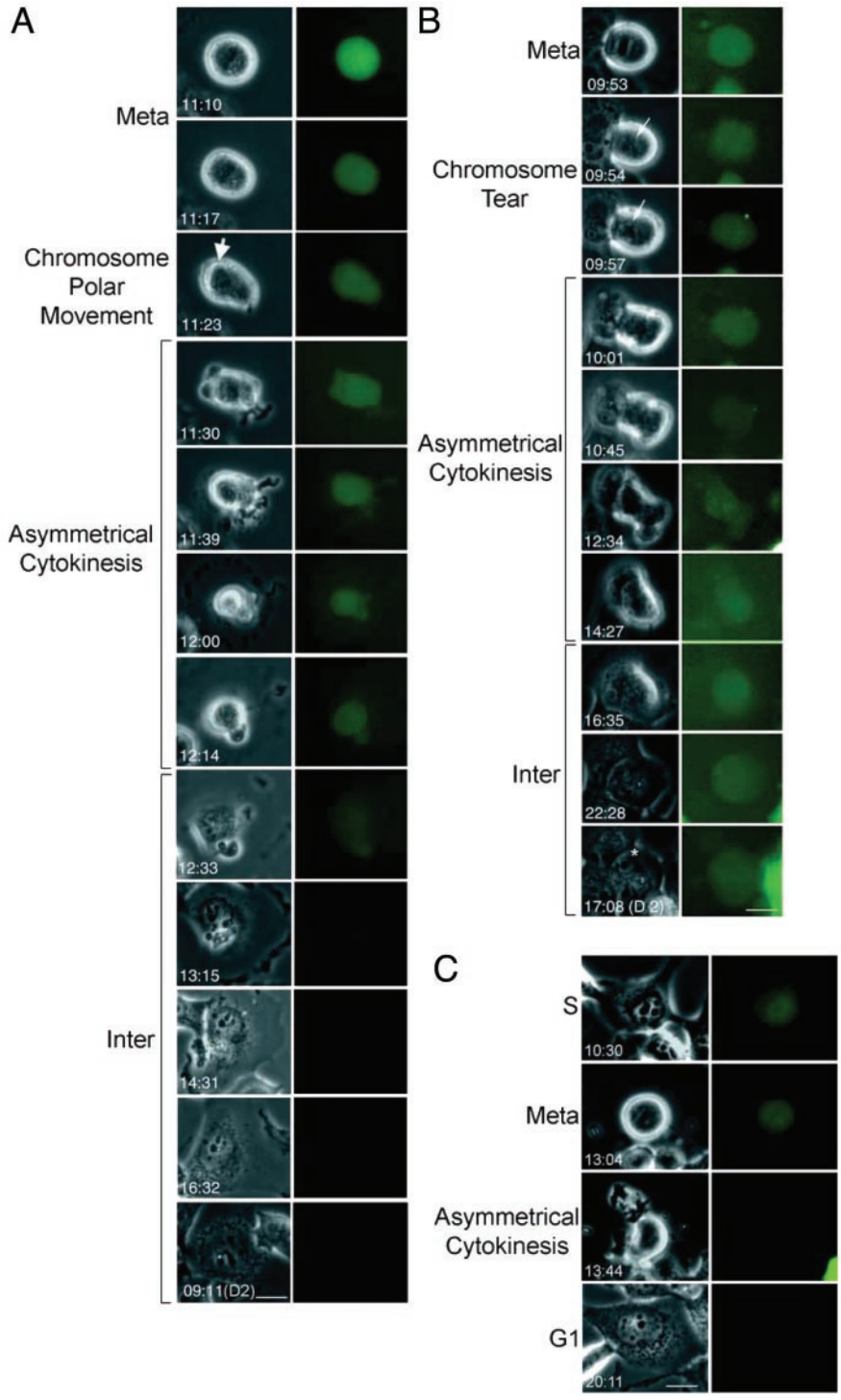

FIG. 5. Chromosome nonsegregation and aneuploidy resulting from failure of PTTG-EGFP degradation. Single, live H1299 cells expressing PTTG-EGFP were continuously observed and representative images shown. Both phase contrast (*bright field*) and PTTG-EGFP images (*green*) are shown. A, Complete absence of chromosome segregation with completed cytokinesis; B, incomplete chromosome segregation with aborted cytokinesis; C, a cell that doubles nuclear size as a result of chromosome nonsegregation. Phases in mitosis are shown *on the left* of images. Inter, Interphase. *Thick arrow*, Nonsegregated chromosomes; *thin arrow*, torn chromosomes; *asterisk*, a micronucleus; D2, second day of observation. *Bar*, 10 μm.

a direct mechanism for the transforming activity of PTTG. Although PTTG-induced aneuploidy may play a role in tumor cell genetic instability, it is apparent that neoplastic cells have variable phenotypic responses to PTTG overexpression.

In neoplastic cells, PTTG disrupts mitosis in several ways, most commonly by cytokinesis without chromosome segregation. In contrast to a recent report that cohesin degradation is required for cytokinesis (29), the results shown here indicate that cytokinesis occurs independently of chromosome segregation in H1299 cells, in agreement with a recent report (30). Cytokinesis regulation is not completely understood; but generally, proteolysis of cyclin-dependent kinases triggers cytokinesis initiation, and the position of mitotic spindles determines localization of the cleavage furrow (31). The data shown here, however, suggest that movement of chromosomes, mitotic apparatus, and astral spindles seem to trigger cytokinesis and specify furrow position. Further studies are required to address molecular mechanisms account-

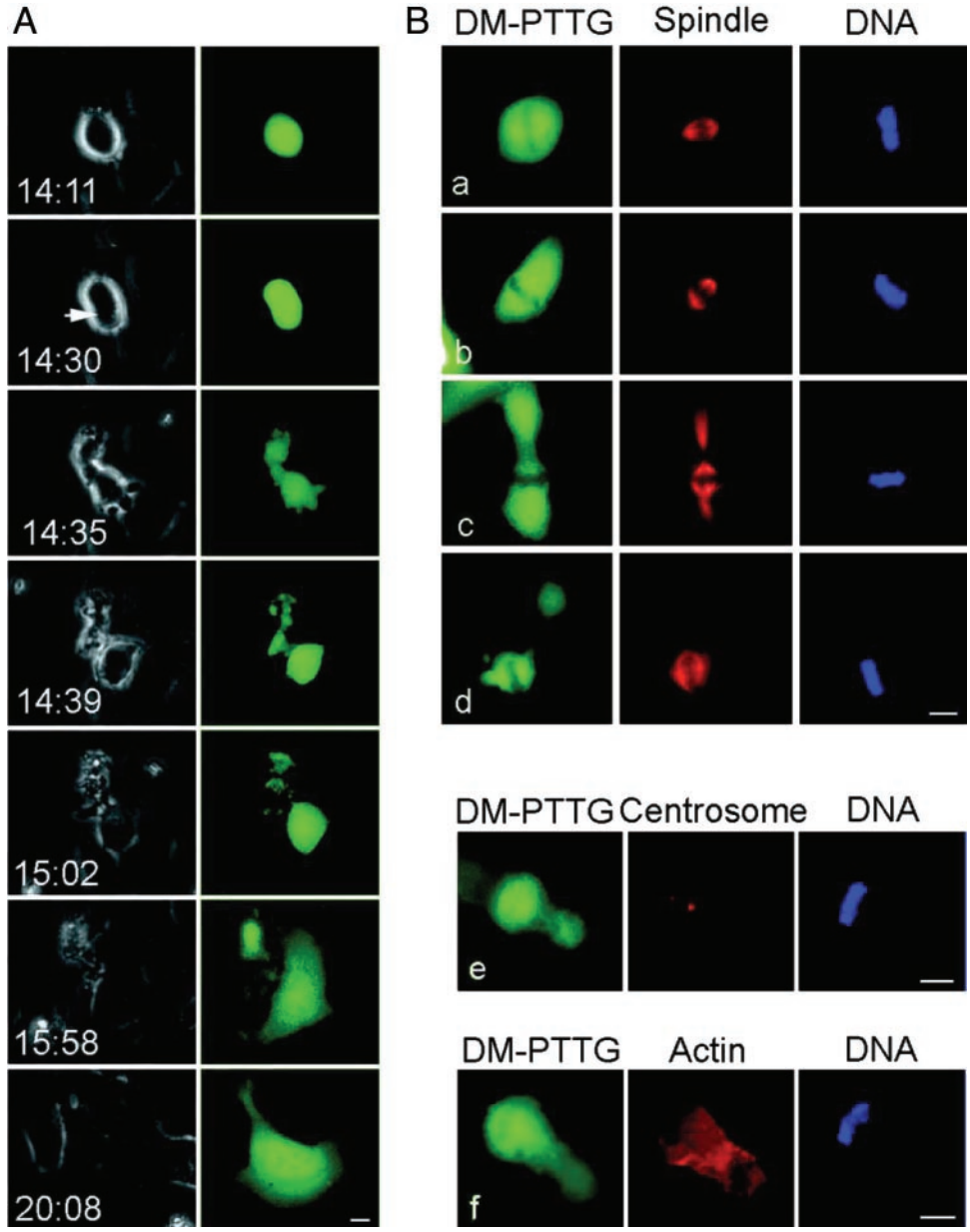

FIG. 6. Chromosome nonsegregation and aneuploidy in H1299 cells expressing nondegradable mutant PTTG-EGFP (DM-PTTG-EGFP). A, Chromosome nonsegregation and cytokinesis in a live cell expressing DM-PTTG-EGFP. Single, live cells expressing DM-PTTG-EGFP alone were continuously observed and representative images shown. Both phase contrast (*bright field*) and DM-PTTG-EGFP images (*green*) are shown. *Arrow*, Nonsegregated chromosomes. B, Cells expressing DM-PTTG-EGFP (*green*) were fixed and mitotic spindles (a–d, *red*) or centrosomes (e, *red*) immunofluorescently stained (see *Materials and Methods*) with an antibody to α- or γ-tubulin, respectively; α-tubulin is a marker of mitotic spindles and γ-tubulin a marker of centrosomes. Cells were also stained for actin (f, *red*) and DNA stained by Hoechst 33342 (*blue*). Cell a was at metaphase; cell b was at early cytokinesis; cells c, e, and f were at late cytokinesis; and cell d, post cytokinesis. Cells a–d corresponded roughly to the first four frames in A. *Bar*, 10 μm.

ing for asymmetrical cytokinesis, although the observed actin localization suggests an intact actomyosin mechanism. If spindle pulling remains equal and chromosome movement does not occur, metaphase chromosomes then decondense and cells enter G1 directly, which is the second mitotic disorder observed. Asymmetrical cytokinesis and chromosome decondensation both result in macronuclei. Persistent anaphase bridges and the ensuing double nuclei and micronuclei are also observed here. It is prudent to point out that most aneuploidy observed in our study is secondary to aberrant chromosome segregation. As a consequence of disordered mitoses consequent to PTTG abundance, two chromosome copies and two centrosomes are retained in the same cell. A chaotic mitosis may occur in the subsequent cell cycle, resulting in genetic instability. The delayed prophase and delayed metaphase are another mitotic disruption caused by PTTG overexpression. Our results suggest that the mitotic checkpoint protein MAD2 and phosphoepitope 3F3/2 are not mediators of these delays. Thus, these results imply a novel checkpoint for PTTG overexpression; or more simply, the cell requires more time to degrade the overexpressed PTTG before anaphase.

Aneuploidy is one of the hallmarks of tumors. Although multiple mechanisms may cause aneuploidy, it has not previously been demonstrated how a specific aneuploidy is produced in the tumorigenesis process. This study demonstrates that PTTG directly causes chromosome copy doubling. Because the cells only expressed PTTG for hours, the resultant aneuploidy is likely a direct consequence of PTTG expression. In previous aneuploidy studies using stable oncogene transfectants or tumor suppressor gene-deficient mice, aneuploidy is usually generated weeks to months after generations of cell division (2–7). It is unclear whether aneuploidy observed in those studies occurs directly, or indi-

TABLE 3. Mitosis of JEG-3 and AtT20 cells expressing various EGFP constructs

	n (%)	PTTG degradation	Chromosome segregation	Chromosome decondensation	Cytokinesis	Daughter cell nucleus	Cell death n (%)
JEG-3 cells							
Control (25 cells)	25 (100)	N/A	Yes	No	Yes	Normal	0 (0)
EGFP (27 cells)	27 (100)	N/A	Yes	No	Yes	Normal	0 (0)
PTTG-EGFP (22 cells)	8 (36)	Yes	Yes	No	Yes	Normal	12 (55)
	2 (9)	No	No	No	Yes	Macro	
DM-PTTG-EGFP	18 (72)	No	No	No	Yes	Macro	3 (12)
(25 cells)	4 (16)	No	No	Yes	No	Macro	
AtT20 cells							
DM-PTTG-EGFP (10 cells)	3 (30)	No	No	No	Yes	Macro	7 (70)

Mitosis of single, live JEG-3 cells untransfected (Control), expressing EGFP only (EGFP), PTTG-EGFP, or nondegradable mutant PTTG-EGFP (DM-PTTG-EGFP), and mitosis of AtT20 cells transfected with nondegradable mutant PTTG-EGFP (DM-PTTG-EGFP) were observed for presence (Yes) or absence (No) of PTTG degradation, chromosome segregation and condensation, and cytokinesis. All PTTG-EGFP AtT20 transfectants died and could not be analyzed. Macro, Macronucleus.

rectly as a consequence of genetic manipulations. Continuous observation of the same cells, both before and after mitosis (as used here), confirmed that the amount of DNA is indeed doubled in one daughter cell compared with that in the parental cell.

In summary, the results demonstrate that PTTG disrupts mitosis and causes aneuploidy in single, live human cancer cells because of failure of PTTG degradation, as a result of overexpression, whereas PTTG causes apoptosis in nontransformed cells. The results imply that PTTG-induced aneuploidy contributes to genetic instability in tumors expressing PTTG.

Acknowledgments

The authors thank Drs. Gary J. Gorbsky and Robert Morris for providing materials.

Received March 10, 2003. Accepted July 15, 2003.

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This work was supported by NIH Grant CA75979, the Doris Factor Molecular Endocrinology Laboratory, the Annenberg Foundation, and the Lilly Pituitary Scholars Award (to R.Y.).

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