PTTG's C-terminal PXXP motifs modulate critical cellular processes *in vitro*

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Abstract

Human pituitary tumor-transforming gene (PTTG), known also as securin, is a multifunctional protein implicated in the control of mitosis and the pathogenesis of thyroid, colon, oesophageal and other tumour types. Critical to PTTG function is a C-terminal double PXXP motif, forming a putative SH3-interacting domain and housing the gene's sole reported phosphorylation site. The exact role of phosphorylation and PXXP structure in the modulation of PTTG action *in vitro* remains poorly understood. We therefore examined the mitotic, transformation, proliferation and transactivation function of the C-terminal PXXP motifs of human PTTG. Live-cell imaging studies using an EGFP-PTTG construct indicated that PTTG's regulation of mitosis is retained regardless of phosphorylation status. Colony-formation assays demonstrated that phosphorylation of PTTG may act as a potent inhibitor of cell transformation. In proliferation assays, NIH-3T3 cells stable transfected and overexpressing mutations preventing PTTG phosphorylation (*Phos*−) showed significantly increased [³H]thymidine incorporation compared with WT, whereas mutants mimicking constitutive phosphorylation of PTTG (*Phos*+) exhibited reduced cell proliferation. We demonstrated that PTTG transactivation of FGF-2 in primary thyroid and PTTG-null cell lines was not affected by PTTG phosphorylation but was prevented by a mutant disrupting the PXXP motifs (*SH3*−). Taken together, our data suggest that PXXP structure and phosphorylation are likely to exert independent and critical influences upon PTTG's diverse actions *in vitro*.

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Introduction

Pituitary tumor-transforming gene, originally isolated from rat pituitary tumour cells (Pei & Melmed 1997), has subsequently been identified as a human securin (Zhang *et al.* 1999b). PTTG has numerous cellular roles, including the control of mitosis (Zou *et al.* 1999, Yu *et al.* 2000b, Zur & Brandeis 2001), cell transformation (Pei & Melmed 1997, Zhang *et al.* 1999b), DNA repair (Romero *et al.* 2001) and gene transactivation (Zhang *et al.* 1999b, Pei 2001). PTTG overexpression has been reported in tumours of the pituitary (Zhang *et al.* 1999a), thyroid (Heaney *et al.* 2001, Boelaert *et al.*

2003a), colon (Heaney *et al.* 2000), ovary (Puri *et al.* 2001) and breast (Puri *et al.* 2001), as well as in haematopoietic neoplasms (Dominguez *et al.* 1998). In thyroid, pituitary, oesophageal and colorectal tumours, high PTTG expression correlates with tumour invasiveness (Zhang *et al.* 1999a, Heaney *et al.* 2000, Shibata *et al.* 2002, Boelaert *et al.* 2003a). Furthermore, PTTG has recently been identified as a key metastatic 'signature gene', with high expression in multiple tumour types predicting metastasis (Ramaswamy *et al.* 2003).

Numerous studies in human and yeast cells have demonstrated interaction of PTTG/securin with separase during cell division, with PTTG

proteolysis during late mitosis facilitating sister chromatid separation. Failure of this process results in inappropriate sister chromatid exchange, resulting in genetic instability as an early tumourigenic event (Zou *et al.* 1999, Yu *et al.* 2000b, Zur & Brandeis 2001). Recent studies have identified unusually frequent rates of aneuploidy in high PTTG-expressing MG-63 (Yu *et al.* 2000b) and NIH-3T3 (Zur & Brandeis 2001) cells. Interestingly, both under- and overexpression of PTTG cause inappropriate cell division (Yu *et al.* 2000a, Wang *et al.* 2001). In addition, PTTG's role in cell turnover remains complex. NIH 3T3 cells overexpressing rat PTTG show slower rates of proliferation (Pei & Melmed 1997), and PTTG overexpression results in cell cycle arrest in JEG-3 cells (Yu *et al.* 2000b). In contrast, PTTG induction in HeLa cells increases *c-myc* and MEK expression, as well as cell proliferation (Pei 2001), and PTTG overexpression leads to raised cell turnover in rat FRTL5 cells (Heaney *et al.* 2001). From these disparate findings, it has been proposed that the effects of PTTG on cell proliferation may be a function of the level of expression (Yu & Melmed 2001). In support of this, we have recently demonstrated that PTTG is able both to repress and stimulate cell turnover in human fetal NT-2 cells, depending on the level of PTTG expression (Boelaert *et al.* 2003b).

Aside from mitotic regulation, one of PTTG's other key functions is the regulation of FGF-2 expression (Zhang *et al.* 1999b). FGF-2 has previously been implicated in the growth and development of numerous tumour types, including those of the pituitary, thyroid and colon. Perpetuation of tumour growth beyond a few millimetres depends on adequate vascularisation (Folkman *et al.* 1971), and a functional link between PTTG, FGF-2 and angiogenesis has recently been described (Ishikawa *et al.* 2001). In addition, we have reported upregulation of VEGF by PTTG (McCabe *et al.* 2002), generally providing compelling evidence that PTTG-mediated transactivation of angiogenic factors may promote tumour vascularisation. Taken together, these findings suggest that PTTG has a dual role in tumourigenesis: firstly as an early cause of genetic instability through aberrant cell division, and secondly as a promoting factor, encouraging tumour growth through FGF-2 and VEGF induction.

A key domain of PTTG involved in FGF-2 and VEGF transactivation, as well as cell transformation and *in vivo* tumourigenesis, is the C-terminal double PXXP motif. Ablation of this region abrogates gene transactivation (Zhang *et al.* 1999b, McCabe *et al.* 2002) and prevents transformation and tumourigenesis (Zhang *et al.* 1999b). Given that the PXXP motifs form a predicted SH3-interacting domain, it has been proposed that such processes may depend on PTTG binding a protein at this site (Zhang *et al.* 1999b). PTTG has been reported to interact with p53 (Bernal *et al.* 2002), separase (Zou *et al.* 1999), Ku heterodimer (Romero *et al.* 2001), the anaphase-promoting complex (Zur & Brandeis 2001), PTTG-binding factor (PBF) (Chien & Pei 2000) and the testicular proteins S10 and HSJ2 (Pei 1999). However, none of these have been shown to interact specifically at the double PXXP motif.

The sole reported site of phosphorylation of human PTTG (serine 165) lies within the first of the two PXXP motifs (Pei 2000, Ramos-Morales *et al.* 2000). Transcriptional regulation of FGF-2 expression, as well as subcellular localisation, is influenced by PTTG phosphorylation in the rat (Pei 2000). Furthermore, human PTTG is phosphorylated during mitosis at this site (Ramos-Morales *et al.* 2000). However, the precise role of phosphorylation within the SH3-interacting domain is unknown, and the effects of altered phosphorylation status on PTTG's mitotic, transforming and proliferative function have not been studied.

We have therefore undertaken a wide-ranging assessment of the influence of PTTG's C-terminal double PXXP motif upon four of the gene's fundamental functions: mitotic regulation, cell transformation, cell proliferation and gene transactivation. We have utilised a number of mutations which enhance or abrogate PTTG phosphorylation and function, and hence defined the mechanisms of action of the SH3-interacting domain in modulating the actions of PTTG. We show, for the first time, that securin function is not influenced by phosphorylation, whereas cell transformation and proliferation are critically regulated by PTTG phosphorylation. Retention of the key proline residues of the PXXP motifs is essential to gene transactivation, a process unaffected by PTTG's phosphorylation status. Overall, our data reveal that PXXP structure and phosphorylation status may exert profound

and independent influences upon PTTG's actions *in vitro*.

Materials and methods

Site-directed mutagenesis of wild-type (WT) PTTG

We utilised pCI-neo-PTTG, which houses the full length in-frame human PTTG cDNA, as we have previously described (Zhang *et al.* 1999b). The previously reported 'S165A' mutation (Ramos-Morales *et al.* 2000), which prevents PTTG from being phosphorylated (Pei 2000, Ramos-Morales *et al.* 2000), was created in the pCI-neo-PTTG vector by the GeneEditor System (Promega), according to the manufacturer's instructions, and is subsequently referred to as '*Phos*⁻'. The mutagenic primer, which resulted in a single amino-acid substitution of serine to alanine at position 165, was of the sequence 5'-G CTG GGC CCC CCT **GCA** CCT GTG AAG ATG CCC.

The PTTG '*Phos*⁺' mutation, which mimics a constitutively phosphorylated threonine residue by substituting the serine for glutamic acid (Morrison *et al.* 1993), was created with a mutagenic primer of the sequence 5'-TTT CAG CTG GGC CCC CCT **GAA** CCT GTG AAG ATG CCC. Negatively charged amino acids have been shown to act as specific structural mimics for phosphorylated threonine or serine residues (Schneider & Fanning 1988, Wittekind *et al.* 1989). This approach has subsequently been used in numerous other studies (Morrison *et al.* 1993, Tourriere *et al.* 2001, Reimer *et al.* 2003, Siam & Marczynski 2003). Since such mutations are designed to mimic phosphorylation, rather than actually being phosphorylated (Morrison *et al.* 1993, Tourriere *et al.* 2001, Reimer *et al.* 2003, Siam & Marczynski 2003), we were unable to confirm the phosphorylation status of the *Phos*⁺ mutant.

The PTTG '*SH3*⁻' mutation was created by substituting two key proline residues of the double PXXP motif and retaining the key phosphorylation site. The mutagenic primer was of the sequence 5'-CTG GGC CCC CCT TCA **GCT** GTG AAG ATG **GCC** TCT CCA CCA TGG G. This resulted in amino-acid changes P166A and P170A.

PTTG constructs were also tagged with EGFP and were used essentially as we have described previously (Yu *et al.* 2000b), with EGFP at the

C-terminus of PTTG. The different PTTG constructs were sequenced and verified to ensure that they contained the correct mutations.

Cell lines and transfections

PTTG-null HCT116 cells were kindly supplied by Drs Vogelstein and Lengauer (Johns Hopkins School of Medicine, Baltimore, MD, USA) (Jallepalli *et al.* 2001), and were maintained in McCoy's 5A medium, with 10% fetal bovine serum, penicillin (10⁵ U/l) and streptomycin (100 mg/l) (Life Technologies, Grand Island, NY, USA). Cells were passaged twice weekly. Prior to transfection experiments, cells were washed in PBS or Hanks' balanced salt solution (for primary cultures). Primary thyroid cells were transfected in 12- or 24-well plates with Eugene 6 reagent (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions. Cells were harvested in 0.5 ml Tri Reagent (Sigma-Aldrich, UK) 48 h later. Control transfections utilised equal amounts of vector-only plasmids. Transfection efficiency was assessed by cotransfection with a RSV β-galactosidase expression vector. Measurement of β-galactosidase expression, either through Western blot analysis or cell staining, was used to equilibrate transfection data. Transfections were performed on at least two separate occasions, each with at least three replicates.

Primary thyroid cell culture

Human thyroid follicular cells were prepared from surgical specimens as previously described (Eggo *et al.* 1996, Ramsden *et al.* 2001). In brief, thyroid tissue was digested by 0.2% collagenase. Follicles were plated in medium described by Ambesi-Impimbato *et al.* (1980), supplemented with thyrotrophin (300 mU/l), insulin (100 µg/l), penicillin (10⁵ U/l), streptomycin (100 mg/l) and 1% newborn bovine calf serum. After 72 h, serum was omitted, and experiments were performed after 5–7 days of culture. Cells were transfected as above. Cultures were terminated by lysis of the cells with the Sigma Trisol kit or with protein lysis buffer. RNA extraction, reverse transcription and quantitative RT-PCR, as well as Western blotting, were performed as above.

Single, live-cell imaging

Human lung cancer H1299 cells were grown in Dolbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (10^5 U/l) and streptomycin (100 mg/l). Prior to transfection experiments, cells were washed in PBS and were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Live-cell imaging was carried out as we have described before (Yu *et al.* 2003). Briefly, H1299 cells were perfused with CO₂-independent L15 medium (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin and saturated with ambient air in an FCS-2 closed perfusion system (Bioprotech, Butler, PA, USA) at 37 °C on a Nikon fluorescence microscope. Phase-contrast and EGFP fluorescent images were taken simultaneously with a $\times 40$ objective and digitalised by a CCD digital camera. Relative fluorescence intensity was objectively determined with the application of two neutral density filters (NDFs) (Yu *et al.* 2003). Each NDF reduces incident light by 50%. High and low expression was determined as previously described (Yu *et al.* 2003). High fluorescence was defined as a cell clearly visualised after application of two NDFs. Low fluorescence was defined as a cell visualised only when neither NDF was applied. Cells were studied (microscopy or Western blot) 18–24 h after transfection.

Stable transfection and cell invasion assays

Mouse fibroblast NIH3T3 (ATCC CCL-92) cells were maintained in low-glucose DMEM (Life Technologies) with 10% fetal bovine serum, penicillin (10^5 U/l) and streptomycin (100 mg/l). Cells were transfected with expression vectors for WT PTTG, *Phos*[−], *Phos*⁺ and *SH3*[−] and G418 selection started after 48 h. PTTG expression was determined in individual colonies by TaqMan RT-PCR and Western blot analysis. Colonies which expressed similarly high levels of wild-type (WT) and mutant PTTG were selected for soft agar assays, as previously described (Campbell *et al.* 1997). Briefly, trypsinised and washed single-cell suspensions of cells from 80% confluent cultures were counted and plated into 24-well, flat-bottom plates with a two-layer soft-agar system, with a total of 1×10^4 cells per well in a total volume of 400 μ l (Campbell *et al.* 1997). Both layers were prepared

with sterile agar (1%) that had been equilibrated at 42 °C. Cells were mixed into the top layer and plated onto the preset feeder layer. After 14-day incubation in a humidified atmosphere of 5% CO₂ at 37 °C, the colonies (>50 cells) were counted under an inverted microscope. All experiments were performed three times and in quadruplicate.

Analysis of cell proliferation

The rate of proliferation of unsynchronised, stable-transfected NIH3T3 cells overexpressing WT-PTTG, *Phos*[−], *Phos*⁺ and *SH3*[−] constructs, as well as vector-only controls, was assessed by measurement of nuclear [³H]thymidine incorporation, as we have described previously (Boelaert *et al.* 2003b). Cells were incubated with 0.2 μ Ci [³H]thymidine (specific activity 80 Ci/mmol; Amersham) for the last 6 h of culture incubation. Cells were then washed twice in PBS, followed by 1 ml cold 5% trichloroacetic acid (TCA) to precipitate proteins, and left on ice for 20 min. The liquid layer was then removed and drained. An aliquot (200 μ l) of 0.1 M sodium hydroxide was added to the cells and left at room temperature overnight on a shaker, before adding a further 100 μ l NaOH. The resulting solubilised nuclear material was then transferred to 4 ml scintillant, and radioactive counts were determined by scintillation counting. Proliferation was assessed at 24, 48 and 72 h.

RNA extraction and reverse transcription

Total RNA was extracted from primary thyroid cell cultures, HCT116-PTTG^{−/−} or NIH3T3 cells with the Tri Reagent kit (Sigma-Aldrich) – a single-step acid guanidinium phenol-chloroform extraction procedure – following the manufacturer's guidelines. RNA was reverse transcribed with avian myeloblastosis virus (AMV) reverse transcriptase (Promega) in a total reaction volume of 20 μ l, with 1 μ g total RNA, 30 pmol random hexamer primers, 4 μ l 5 AMV reverse transcriptase buffer, 2 μ l deoxynucleotide triphosphate (dNTP) mix (200 μ M each), 20 units ribonuclease inhibitor (Rnasin; Promega) and 15 units AMV reverse transcriptase (Promega).

Quantitative PCR

Expression of specific messenger RNAs was determined by the ABI PRISM 7700 Sequence

Table 1 Oligonucleotide sequences of PCR primers and TaqMan probes used. All TaqMan primers run at 59 °C and yield amplicons of 70–150 bp

	Forward Primer	Reverse Primer
PTTG	GAGAGAGCTTGAAAAGCTGTTTCAG Probe: TGGGAATCCAATCTGTTGCAGTCTCCTTC	TCCAGGGTCGACAGAATGCT
FGF-2	CGACCCTCACATCAAGCTACAA Probe: TTCAAGCAGAAGAGAGAGGAGTTGTGTCTATCAAA	CCAGGTAACGGTTAGCACACACT

Detection System. RT-PCR was carried out in 25 µl volumes on 96-well plates, in a reaction buffer containing 1 × TaqMan Universal PCR Master Mix, 100–200 nmol TaqMan probe and 900 nmol primers, as we have described previously (McCabe *et al.* 2002). All reactions were multiplexed with a preoptimised control probe for 18S ribosomal RNA (PE Biosystems, Warrington, UK), enabling data to be expressed in relation to an internal reference, to allow for differences in RT efficiency. Primer and probe sequences are given in Table 1. According to the manufacturer's guidelines, data were expressed as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine Δ Ct values (Δ Ct = Ct of the target gene (PTTG) minus Ct of the housekeeping gene). To exclude potential bias due to averaging data which had been transformed through the equation $2^{-\Delta\Delta Ct}$ to give fold changes in gene expression, all statistics were performed with Δ Ct values.

Western blot analysis

Proteins were prepared in lysis buffer (100 mmol/l sodium chloride, 0.1% Triton X-100, and 50 mmol/l Tris, pH 8.3) containing enzyme inhibitors (1 mmol/l phenylmethylsulphonylfluoride, 0.3 µmol/l aprotinin, and 0.4 mmol/l leupeptin) and denatured (2 min, 100 °C) in loading buffer. Protein concentration was measured by the Bradford assay with bovine serum albumin as standard. Western blot analyses were performed as we have described previously (Gittoes *et al.* 1997, Heaney *et al.* 2000, Boelaert *et al.* 2003a,b). Briefly, soluble proteins (30 µg) were separated by electrophoresis in 12.5% sodium dodecyl sulphate polyacrylamide gels, transferred to polyvinylidene fluoride membranes, and incubated in 5% non-fat milk in PBS with 0.1% Tween, followed by incubation with an antibody to FGF-2 (Santa Cruz

Biotechnology, Santa Cruz, CA, USA) at 1:1000 for 16 h at 4 °C. The polyclonal PTTG antibody was created and used as previously described (Boelaert *et al.* 2003a,b). After washing in PBS plus 0.1% Tween, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. After further washes, antigen–antibody complexes were visualised by the ECL chemiluminescence detection system. Actin expression was determined in all Western blot analyses (monoclonal anti-β-Actin Clone AC-15 (Sigma-Aldrich), used at 1:10 000) to assess potential differences in protein loading.

Statistical analyses

Data were analysed by SigmaStat. Student's *t*-test and the Mann–Whitney U test were used for comparison between two groups of parametric and non-parametric data respectively. The analysis of variance and Kruskal–Wallis tests were used for between-group comparisons of multiple groups of parametric and non-parametric data respectively. Correlations between levels of mRNA expression

Table 2 Summary of mitosis of single, live H1299 cells expressing *Phos*–EGFP PTTG or *Phos*+EGFP PTTG. When *Phos*–EGFP PTTG or *Phos*+EGFP PTTG levels were low, *Phos*–EGFP or *Phos*+EGFP was invariably degraded during mitosis and the cell divides normally. When *Phos*–EGFP or *Phos*+EGFP levels were high, chromosome segregation was inhibited and a unique cytokinesis occurred

	<i>Phos</i> -EGFP	<i>Phos</i> -EGFP
Expression		
Low	Degraded (11/11 cells)	Degraded (6/6 cells)
High	Mitosis Inhibited (8/8 cells)	Mitosis Inhibited (4/4 cells)

A

B

C

D

were performed with the Pearson rank sum test. Significance was taken as $P < 0.05$.

Results

PXXP Structure and securin function

Human PTTG is a key regulator of cell division. We therefore tested whether phosphorylation of PTTG affects its mitotic function. We recently established a validated live-cell observation system which allows us to study the behaviour of WT PTTG and various PTTG mutants during mitosis in H1299 cells (Yu *et al.* 2003). These human cells express low endogenous PTTG and undergo normal cell division, and are hence a suitable model for monitoring mitosis. We have previously shown (Yu *et al.* 2003) that an SH3 mutant loses PTTG function in H1299 cells, in that the EGFP-conjugated protein failed to induce aneuploidy or other mitotic abnormalities when overexpressed, in contrast to WT PTTG. We therefore examined *Phos*[−] and *Phos*⁺ mutations in this context. We observed the mitosis of a number of live H1299 cells expressing mutant PTTG-EGFPs (Table 2). A mutant was considered to retain PTTG function if two criteria were met. First, the mutant had to be degraded during mitosis; second, overexpression of the mutant had to inhibit chromosome separation. EGFP levels remain unchanged throughout mitosis, and EGFP alone does not affect mitosis at all (Yu *et al.* 2003). We found that both *Phos*[−] EGFP and *Phos*⁺ EGFP PTTGs were degraded during mitosis, which was more evident in cells where their levels were low (Fig. 1A and C). In those cells, no abnormal mitosis was observed. When their levels were high, however, chromosome separation was inhibited, and the cell underwent an unsymmetrical cytokinesis which resulted in one daughter cell having two

chromosome copies and the other having none (Fig. 1B and D). The *Phos*[−] and *Phos*⁺ PTTG mutants therefore behave exactly like WT in meeting the two criteria for normal PTTG function.

Phosphorylation modulates PTTG-mediated cell transformation

Having examined the influence of phosphorylation on the securin function of PTTG, we next determined whether the induction of cell transformation is affected by PTTG phosphorylation. Stably transfected NIH3T3 cell lines overexpressing WT and mutated PTTG were constructed. Similarly high PTTG-expressing clones were selected for colony-formation assays (Fig. 2A). FGF-2 protein was also assessed in stable lines (Fig. 2A), and demonstrated findings in keeping with those predicted from our transient transfection studies; that is, WT PTTG induced FGF-2 at similar levels to *Phos*[−] and *Phos*⁺, whereas the SH3[−] mutant failed to demonstrate FGF-2 induction.

As expected, in comparison to vector-only (VO) controls, WT PTTG demonstrated abundant colony formation in soft agar assays (VO: 8 ± 1.3 colonies/well; WT: 59 ± 1.6 colonies/well, $P < 0.001$ compared with VO, $n = 12$ wells, mean $\pm$ S.E.M.) (Fig. 2B). Interestingly, *Phos*[−] stable 3T3 lines consistently formed significantly more colonies than both VO and WT (201 ± 16.2 colonies/well, $P < 0.001$ compared with WT; $P < 0.001$ compared with VO, $n = 13$ wells). In contrast, *Phos*⁺ mutants showed significantly reduced colony formation compared with VO, WT and *Phos*[−] (30.4 ± 4.3 colonies/well, $P < 0.001$ compared with WT; $P < 0.001$ compared with *Phos*[−]; $P < 0.01$ compared with VO, $n = 11$ wells). When we investigated the disrupted SH3 structure mutant,

Figure 1 Lack of PTTG phosphorylation or a phosphorylation-mimicking mutation does not affect securin function. Mitosis of live H1299 cells (arrows) expressing *Phos*[−] EGFP PTTG (A and B) or *Phos*⁺ EGFP PTTG (C and D) was continuously observed, and representative images are shown, phase-contrast and EGFP-fluorescent images are taken at $\times 40$ magnification. (A and C) A cell expressing low levels of *Phos*[−] EGFP (A) or *Phos*⁺ EGFP (C) was initially at metaphase, as manifested by the alignment of chromosomes in the middle of the cell (top panel). The cell then progressed to anaphase with the chromosomes segregated (middle panel), and *Phos*[−] EGFP or *Phos*⁺ EGFP was degraded; the cell eventually divided (bottom panel). When *Phos*[−] EGFP or *Phos*⁺ EGFP levels were high (B and D), the metaphase cell (top panel) expressing *Phos*[−] EGFP or *Phos*⁺ EGFP could not segregate the chromosomes, and the cell underwent unsymmetrical cytokinesis, which resulted in one daughter cell having two copies of the chromosome and the other having none (middle panel). The daughter cell with the non-segregated chromosomes survived, but the daughter cell without chromosomes died (bottom panel).

Figure 2 Cell-transformation assays in stably transfected NIH3T3 cells. (A) PTTG protein expression in stable lines, confirming similarly raised levels in all four PTTG lines compared with vector-only (VO) control. Also shown are actin and FGF-2 expression, the latter being similar in wild-type (WT) PTTG, *Phos*[−] and *Phos*⁺ PTTG, but reduced in *SH3*[−] expressing lines. (B) Top panel: photomicrographs of cell colonies at ×10 magnification in VO (control), WT, *Phos*[−], *Phos*⁺ and *SH3*[−] mutant PTTG cell lines. Lower panel: quantification of mean colony number per dish, ±S.E.M. ****P* < 0.001 for VO versus WT and *Phos*[−]; ****P* < 0.001 for WT compared with *Phos*[−], *Phos*⁺ and *SH3*[−], and for *Phos*[−] compared with *Phos*⁺. ***P* < 0.01 for VO versus *Phos*⁺.

we observed that *SH3-* resulted in greatly abrogated transforming ability (22.5 ± 4.7 colonies/well, $P < 0.001$ compared with WT, $n = 11$ wells).

Taken together, these data imply that PTTG's transforming ability is modulated by its phosphorylation status, and that transformation is contingent upon an intact C-terminal PXXP motif.

PXXP structure and cell proliferation

Having examined the securin and cell-transformation properties of PTTG's SH3-interacting domain, we next determined its influence upon cell proliferation through [3 H]thymidine incorporation assays. Human PTTG inhibits mitosis until an appropriate time point. Its influence on cell proliferation is not well understood, although previous evidence points to specific effects dependent upon cell type and expression level (Pei & Melmed 1997, Ramos-Morales *et al.* 2000, Yu *et al.* 2000b). We used stable NIH-3T3 cell lines overexpressing similarly high levels of WT-PTTG, *Phos-*, *Phos+* and *SH3-* constructs, as well as vector-only (VO) controls. [3 H]Thymidine incorporation was assessed at 24 and 48 h from the start of the experiment (Fig. 3A). At all time points, cells expressing *Phos-* demonstrated increased, and *Phos+* decreased, proliferation compared with WT. At 48 h, when cells were growing maximally, cell lines overexpressing *Phos-* PTTG showed a significant 38% increase in proliferation compared with WT ($n = 8$ wells, $P < 0.001$ compared with both VO (control) and WT), whereas *Phos+* cells demonstrated a 41% reduction in proliferation ($n = 8$ wells, $P < 0.001$ compared with both VO and WT). *SH3-*-expressing 3T3 cells demonstrated no significant difference in proliferation compared with either VO or WT. In comparison to VO control stable lines, cells overexpressing WT-PTTG showed similar proliferation at 48 h (Fig. 3A and B), indicating that under these conditions, WT PTTG is not pro-proliferative.

Overall, these experiments demonstrate that absence of PTTG phosphorylation induces a significant proliferative effect, and that a specific mimic of phosphorylation inhibits cell turnover. The structural integrity of the PXXP motif per se, however, does not influence this process.

PXXP structure, but not phosphorylation, influences FGF-2 stimulation

Transactivation of FGF-2 by PTTG is well recognised (Pei & Melmed 1997, Heaney *et al.* 1999, Ishikawa *et al.* 2001, Zhang *et al.* 1999b), but the effects of PTTG phosphorylation on this process are largely confined to rodent models (Pei 2000, Wang & Melmed 2000). As we have recently reported PTTG and FGF-2 overexpression in human thyroid tumours (Boelaert *et al.* 2003a), we examined PTTG transactivation of FGF-2 in primary cultures of human thyroid cells. FGF-2 mRNA levels were significantly increased in primary thyroid cells 48 h after transient overexpression of WT-PTTG compared with VO controls (3.2-fold induction, $P = 0.01$, $n = 12$ wells) (Fig. 4A). However, the phosphorylation status of PTTG failed to influence FGF-2 mRNA upregulation (*Phos-*: 3.0-fold induction, $P = 0.06$, $n = 9$ wells; *Phos+*: 3.0-fold, $P = 0.01$, $n = 9$ wells, compared with VO). In contrast, abrogation of the PXXP motifs resulted in the inability of PTTG to transactivate FGF-2 (0.9-fold induction, *SH3-* compared with VO, $P = \text{NS}$, $n = 9$ wells). Western blot analyses of protein expression (Fig. 4B) confirmed the upregulation of FGF-2 regardless of PTTG phosphorylation. In accord with mRNA data, the *SH3-* mutant showed reduced FGF-2 protein compared with WT.

To examine these findings in a PTTG-negative background, we repeated our experiments in HCT116-PTTG $^{-/-}$ cells (Jallepalli *et al.* 2001) (Fig. 4C). Transient transfections yielded similar results to those obtained in primary thyroid cultures (WT: 3.8-fold induction in FGF-2 mRNA expression, $n = 6$ wells, $P = 0.01$; *Phos-*: 3.5-fold induction, $P = 0.02$, $n = 6$ wells; *Phos+*: 3.4-fold, $P = 0.02$, $n = 6$ wells; *SH3-*: 1.1-fold induction compared with VO, $n = 6$ wells, $P = \text{NS}$).

Thus, PXXP structure, but not phosphorylation, influences FGF-2 transactivation in human cells *in vitro*. Taken together, our data suggest that phosphorylation status does not influence PTTG's promotion of abnormal mitosis *in vitro*, but does play an essential role in cell transformation and proliferation.

Discussion

Human PTTG, known also as securin, has a wide range of functions, including regulating mitosis

A

B

(Zou *et al.* 1999) and angiogenesis (Ishikawa *et al.* 2001), modulating p53 action, stimulating DNA repair (Romero *et al.* 2001), transactivating FGF-2 (Zhang *et al.* 1999b), VEGF (McCabe *et al.* 2002) and *c-myc* (Pei 2001), and inducing transformation and tumorigenesis (Zhang *et al.* 1999b). Critical to transactivation, transformation and tumorigenesis is a putative C-terminal SH3-interacting domain, comprised of two PXXP motifs (Zhang *et al.* 1999b). Although PTTG has been reported to interact specifically with p53, separase, Ku heterodimer, the anaphase-promoting complex (APC), PTTG-binding factor (PBF) and the testicular proteins S10 and HSJ2, none of these has been conclusively shown to bind at the double PXXP motif. Furthermore, despite the sole reported site of phosphorylation of human PTTG (serine 165) lying within this complex region, the precise role of phosphorylation is unknown.

We have therefore undertaken a detailed investigation into the influence of the double PXXP motif upon four of PTTG's major functions: mitotic function, cell transformation, cell proliferation and FGF-2 transactivation (Table 3). We report, for the first time, that phosphorylation is likely to be a critical regulator of cell proliferation and transformation, but does not alter securin function. Furthermore, retention of the key proline residues of the PXXP motifs, but not the phosphorylation site, is essential to gene transactivation. Our findings indicate therefore that the phosphorylation status and structure of this region of PTTG are likely to be critical to the diversity of the gene's actions *in vitro*.

In the rat, PTTG is phosphorylated at the analogous site to serine 165 (Ser162) by mitogen-activated protein kinase (MAPK) (Pei 2000). MAPK is directly phosphorylated by MEK, and rat PTTG's SH3-interacting domain is able to bind a complementary domain on MEK. As integrity of the SH3-binding domain is necessary for PTTG to elicit cell transformation (Zhang *et al.* 1999b), PTTG phosphorylation via MEK/MAPK may be

a critical pathway in human tumours. However, for the human gene, serine 165 appears to be a Cdc2 phosphorylation target (Ramos-Morales *et al.* 2000), and PTTG may therefore interact with a different protein at this region. The identity of this putative protein remains unclear, and we have recently instigated a large-scale IPP/mass spectrometry approach to define proteins which bind to the site, in both its phosphorylated and unphosphorylated forms.

Overexpression of PTTG has been noted in a growing number of malignancies (Dominguez *et al.* 1998, Zhang *et al.* 1999a, Heaney *et al.* 2000, 2001, Puri *et al.* 2001). We recently reported upregulation of PTTG and FGF-2 in follicular and papillary thyroid cancers (Boelaert *et al.* 2003a), with gene expression predicting markers of tumour recurrence, invasion and metastasis. Aneuploidy is a common feature of thyroid follicular adenomas and carcinomas, as well as of many clonal human thyroid carcinoma cell lines (Joensuu *et al.* 1986, Joensuu & Klemi 1988). As a key regulator of mitosis, PTTG represents a potential initiator of chromosomal instability in thyroid and other cancers, given that overexpression leads to inappropriate cell division (Yu *et al.* 2000a, Zur & Brandeis 2001). However, our data show that phosphorylation status does not disrupt this process. *Phos*[−] and *Phos*⁺ mutants both retained securin function, in that they were degraded during mitosis and, when overexpressed, elicited abnormal mitosis. Our previous data (Yu *et al.* 2003) demonstrated that an SH3-binding domain mutant lost securin function, suggesting that PTTG may require binding of a protein at this site to effect mitotic regulation, a process which is not modulated by phosphorylation.

In addition to interfering with securin function (Yu *et al.* 2003), disruption of the SH3-interacting domain led to reduced colony formation. Interestingly, however, the SH3- mutation did not affect cell turnover rates significantly. Our cell proliferation and cell transformation assays measure very

Figure 3 Cell-proliferation assays in stably transfected NIH3T3 cells. (A) [³H]Thymidine incorporation (d.p.m. ± S.E.M.) in VO (control), WT, *Phos*[−], *Phos*⁺ and *SH3*[−] after 24 and 48 h. WT cells demonstrated similar rates of proliferation when compared with VO cells. *Phos*[−] cells showed significantly increased proliferation compared with WT at all time points. In contrast, *Phos*⁺ demonstrated reduced proliferation. The *SH3*[−] mutant failed to influence [³H]thymidine incorporation compared with WT PTTG. ****P* < 0.001 *Phos*[−] and *Phos*⁺ PTTG compared with WT PTTG. (B) Cell proliferation at 48 h, averaged over two experiments with four replicates each. **P* < 0.05, ****P* < 0.001 compared with VO (control).

Table 3 Summary data of wild type and mutated PTTG functions *in vitro*

	Mitotic regulation	Transformation	Proliferation	Transactivation
V/O	–	–	–	–
Wild type	+++	++	–	+++
<i>Phos</i> –	+++	+++	+++	+++
<i>Phos</i> +	+++	+	---	+++
<i>SH3</i> –	–*	–	–	–

*Previously published data (Yu *et al.* 2003).

different components of gene action, which are clearly affected in different ways by disruption of the SH3-interacting domain. Indeed, PTTG's role in cell turnover is complex, given that it stimulates pro-proliferative genes (FGF-2 (Zhang *et al.* 1999b), VEGF (McCabe *et al.* 2002) and *c-myc* (Pei 2001)), while simultaneously inhibiting sister chromatid exchange during mitosis (Zou *et al.* 1999, Zur & Brandeis 2001). One previous study (Ramos-Morales *et al.* 2000) indicated an upregulation of PTTG in response to cell growth, and a further report described increased proliferation in HeLa cells expressing an inducible PTTG (Pei 2001). However, rat PTTG has also been shown to reduce the growth of NIH3T3 cells (Pei & Melmed 1997). Our own recent investigations indicate that PTTG's influence upon proliferation is dependent on cell type and expression level (Boelaert *et al.* 2003b). In the current study, it is clear that PTTG is not a pro-proliferative gene per se in NIH3T3 cells, since stable overexpression of WT PTTG failed to increase cell turnover significantly. However, our current data demonstrate that phosphorylation at Ser165, as well as expression level, is critical to this process: *Phos*– PTTG significantly increased cell turnover, while *Phos*+, a Ser165 mutant employing the well-established approach of phospho-serine mimicry with a glutamic acid residue (Morrison *et al.* 1993, Tourriere *et al.* 2001, Reimer *et al.* 2003,

Siam & Marczyński 2003), showed significantly reduced proliferation compared with WT. Since it is impossible to demonstrate the presence of phosphorylation by this mimicking approach, we were unable to confirm that *Phos*+ represents an actual phosphorylated PTTG mutant. However, the observation of opposing effects of *Phos*+ and *Phos*– mutants on cell transformation and cell proliferation appears to provide some justification for this approach.

Our data shed new light on the transactivation properties of human PTTG. One previous study in the rat demonstrated that lack of phosphorylation results in reduced FGF-2 transactivation (Pei 2000). Moreover, in the mouse, substitution of the residue Ser159, which is analogous to human Ser165, results in a reduction of general transactivating function (Wang & Melmed 2000). In contrast, our findings demonstrated that the phosphorylation status of human PTTG does not influence FGF-2 stimulation in primary thyroid or HCT116-PTTG^{–/–} cells, at either the mRNA or protein level. Stable 3T3 lines overexpressing WT, *Phos*– and *Phos*+ PTTG also showed identical FGF-2 protein expression. The discrepancy between our findings and those of Pei (2000) may be due to differences between human and rat PTTG, or the cell lines used. However, our findings mirrored those of others (Zhang *et al.* 1999b, Ishikawa *et al.* 2001), in

Figure 4 (A) FGF-2 upregulation by PTTG. Gene expression was determined after 48 h through quantitative TaqMan RT-PCR. Transfection efficiency was determined through β -Gal staining, and values were adjusted for 18S (housekeeping) gene expression before normalising to 1.0 for control (vector-only) treatments. In this and following histograms, gene expression is given relative to a value of 1.0 for VO controls. Number of wells and Δ Ct values ($\pm$ S.E.M.) are given beneath each histogram bar. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. PTTG transfection caused a significant (3.1-fold) induction in FGF-2 mRNA ($P < 0.01$, $n = 12$). *Phos*– and *Phos*+ PTTG induced similar FGF-2 upregulation, whereas the *SH3*– PTTG mutant was unable to stimulate FGF-2. (B) Representative Western blot analysis of FGF-2 and PTTG protein expression in transiently transfected thyroid cells. Protein loading was confirmed by β -actin determination. In keeping with the mRNA data, WT PTTG induced FGF-2 protein expression, which was unaltered by phosphorylation status, whereas the *SH3*– mutant showed impaired FGF-2 stimulation. (C) Repeated transient transfections in HCT116-PTTG^{–/–} cells, showing similar findings to primary cultures of thyroid cells.

that ablation of the PXXP motifs of the SH3-binding domain resulted in an absence of FGF-2 stimulation.

Taken together, our data yield new insight into the mechanisms of PTTG function mediated by the complex C-terminal PXXP motifs. As PTTG is a gene implicated in tumour initiation, progression and metastasis, we investigated the role of one of its key functional domains in mediating its main *in vitro* roles. We show that phosphorylation is likely to modulate PTTG's ability to influence cell proliferation and transformation, but not its function as a mitotic regulator or gene transactivator. As integrity of the PXXP motifs is necessary for the majority of PTTG's functions *in vitro*, we propose a mechanism by which human PTTG interacts with a putative protein to effect its actions, a process which is ultimately modulated by the phosphorylation status of the gene.

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