RESEARCH ARTICLE

Phosphorylation of human eukaryotic elongation factor 1Bγ is regulated by paclitaxel

Miguel A. Prado, Pedro Casado, Pedro Zuazua-Villar, Eva del Valle, Noelia Artime, Lucía Cabal-Hierro, Carlos M. Martínez-Campa, Pedro S. Lazo and Sofía Ramos*

Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología del Principado de Asturias, Universidad de Oviedo, Oviedo, Spain

Paclitaxel (Ptx) is an antitumoural drug that inhibits microtubule dynamics, causes G2/M arrest and induces cell death. 2-D PAGE and MALDI-TOF-MS analysis of HeLa cells extracts revealed that Ptx up-regulates a form of the eukaryotic elongation factor 1Bγ (eEF1Bγ) and down-regulates another one. This event, linked to the lack of Ptx effect over eEF1Bγ mRNA or protein levels suggested a PTM of this elongation factor. Further 2-D PAGE analysis followed by a phospho-specific staining with PRO-Q Diamond showed the staining of the Ptx up-regulated form only. Moreover, this Ptx up-regulated form of eEF1Bγ disappears upon treatment with protein phosphatase. Thus, we demonstrate that human eEF1Bγ phosphorylation is regulated by Ptx.

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1 Introduction

Eukaryotic elongation factor 1 (eEF1) is a macromolecular structure that catalyses the transfer of aminoacyl-tRNA to ribosomes. Following Merrick's proposed nomenclature [1], eEF1 is formed by two components named eEF1A and eEF1B; eEF1A is a G protein that transfers the aminoacyl-tRNA to the ribosomes with the hydrolysis of GTP to GDP, while eEF1B is a nucleotide exchange factor that exchanges the GDP for GTP in order to regenerate active eEF1A. The G protein is composed only by the subunit named eEF1A. The eEF1B component is formed by a low molecular weight and a high molecular weight nucleotide exchange proteins named eEF1Bα and eEF1Bδ respectively, and a third protein named eEF1Bγ [2]. Since no nucleotide exchange activity has been found for eEF1Bγ, this has been considered as a structural protein that serves as a scaffold for the other subunits and anchors eEF1B to the cytoskeleton [3] or to the ER membrane [4]. In addition, a slight GST activity of this protein has been reported [5].

It has been shown that eEF1Bγ mRNA levels are 20-fold higher in transformed cells than in normal tissues [6]. Furthermore, the up-regulation of this protein is related to aggressiveness in oesophageal tumours [7] and it is also overexpressed in gastrointestinal [8], pancreatic [9] and colorectal carcinomas [10, 11]. A functional link between eEF1Bγ and cancer has not been clearly established. However, there are some data relating eEF1B and cell cycle regulation. Thus, in *Xenopus*, this component is phosphorylated by cdc2 [12], a key regulator of the mitotic checkpoint [13].

Paclitaxel (Ptx) (Taxol®), a natural taxane produced by *Taxus brevifolia* and *Taxus baccata* is a widely used anticancer drug [14]. This microtubule interfering agent binds to the taxane-binding domain of β tubulin. It is known that this

Correspondence: Dr. Sofía Ramos, Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo, 33071 Oviedo, Spain
E-mail: sramos@uniovi.es
Fax: +34-985104213

Abbreviations: eEF1, eukaryotic elongation factor 1; **λ-PPase,** λ-phosphatase; **Ptx,** paclitaxel

* Current address: Departamento de Fisiología y Farmacología, Facultad de Medicina Universidad de Cantabria, 39011 Santander, Spain

union inhibits the mitotic spindle dynamics [14], leading to c-Jun-NH2-terminal kinase (JNK) activation [15], Bcl-2 phosphorylation [16], G2/M arrest and cell death [17]. However, the events that occur between the drug binding to the microtubules and the triggering of cell death have not been fully clarified.

In this work, we use 2-D PAGE to find that Ptx treatment induces, in HeLa cells, the up-regulation of a protein identified as eEF1Bγ by MALDI-TOF-MS. This induction is not accompanied by an increment of eEF1Bγ mRNA or changes in the protein level, but rather it correlates with the down-regulation of another more basic protein, also identified as eEF1Bγ. We demonstrate that the form of eEF1Bγ up-regulated by Ptx is a phosphorylated form of this elongation factor.

2 Materials and methods

2.1 Cell culture and treatments

HeLa cells were propagated in phenol red DMEM (Cambrex) containing 100 μg/mL gentamicine and 10% of heat inactivated FBS (Cambrex). For experiments, cells were transferred to phenol-red free DMEM containing 0.5% of charcoal/dextran-treated FBS, 100 μg/mL gentamicine and 4 mM L-glutamine. After 3 days, the cells were treated with Ptx (Sigma) dissolved in ethanol. The final concentration of ethanol was 0.1%.

2.2 Flow cytometry

Cells were collected by trypsinization and incubated sequentially, according to Vindelov's technique in 300 μL of buffer A (0.5 mM Tris-HCl (pH 7.6), 0.1% NP-40 v/v, 3.4 mM trisodium citrate, 1.5 mM spermin, 30 μg/mL trypsin (Sigma)) for 10 min, in 250 μL of buffer B (0.5 mM Tris-HCl (pH 7.6), 0.1% NP-40 v/v, 3.4 mM trisodium citrate, 1.5 mM spermin, 500 μg/mL trypsin inhibitor (Sigma), 100 mg/mL RNase A (Sigma)) for 10 min and in 250 μL of buffer C (0.5 mM Tris-HCl (pH 7.6), 0.1% NP-40 v/v, 3.4 mM trisodium citrate, 4.83 mM spermin, 416 μg/mL propidium iodide) for 10 min. Cell cycle was analysed in a FACscan flow cytometer (Becton Dickinson).

2.3 RNA extraction, cDNA synthesis and real-time PCR analysis

Total RNA was extracted using Tri® Reagent (Sigma) and the yield was measured spectrophotometrically. Quality and integrity of the total RNA was assessed on 2% agarose gel. Single stranded cDNA was synthesized from 2 μg of total RNA using MMLV reverse transcriptase (Invitrogen) and oligo dT (Invitrogen). All the procedures were performed following the manufacturer's instructions. PCR reactions were performed in an ABI PRISM® 7000 (Applied Biosystems) using SYBR® GREEN PCR Master Mix (Applied Biosystems) and 300 nM primer concentration. PCR conditions were an initial denaturation and DNApol activation step of 95°C for 10 min., followed by 40 cycles of denaturation for 15 s at 95°C and annealing-extension for 1 min at 60°C. All samples were amplified in triplicate and PCRs were repeated two times. β-2-Microglobulin (β2M) gene was used as an internal control. The following primers were designed with the Primer Express® software v2.0: eEF1Bγ forward 5′-TGACATTGGCTGACATCACAGTT-3′ and reverse 5′-AGGCCTGGCGGAAAGAAG-3′, and β2M forward 5′-TGAGTGCTGTCTCCATGTTTGA-3′ and reverse 5′-TCTGCTCCCCACCTCTAAGTTG-3′.

2.4 Western blot analysis

Cells extracts were obtained in Laemmli buffer, heat denatured and 30–60 μg of protein was electrophoresed on a 10% SDS-PAGE. After electrophoresis proteins were transferred to PVDF membranes. Membranes were blocked with TBS/Tween-20 supplemented with 5% w/v nonfat milk for 1 h at room temperature, then incubated with primary antibodies overnight at 4°C, with secondary antibodies for 1 h at room temperature and developed with ECL reagents (GE-Healthcare). Anti-eEF1Bγ (Abnova, Cat. H00001937-M01), anti-β-actin (Sigma, Cat. A-5441) anti-cyclin B1 (Santa Cruz, Cat. sc-245) and antimouse peroxidase (Sigma, Cat. A-9044) were used at 1:3000, 1:20 000, 1:4000 and 1:10 000 dilution, respectively. Band intensity was quantified with Phoretix software.

2.5 2-D PAGE

Cells were solubilized in UTATH buffer (7 M urea, 2 M thiourea, 1% amidosulfobetaine-14, 50 mM 2-hydroxyethyl disulphide (HED), 0.5% IPG buffer pH 3–10 (BioRad)), desalted with a desalting spin column (Pierce) and 60–100 μg of protein loaded onto a strip holder. First dimension was run in 7 cm Immobiline™ DryStrips at pH 3–11 (GE-Healthcare) for 12 h at 30 V, 250 V·h at 500 V, 500 V·h at 1000 V and 8000 V·h at 5000 V. For the second dimension, strips were equilibrated in equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris (pH 6.8), 2% SDS, 0.002% bromophenol blue w/v) and run in 10% polyacrylamide gels supplemented with 50 mM HED and 6 M urea. For Coomassie staining, gels were fixed with fixing-solution (20% methanol v/v, 10% acetic acid v/v) for 24 h, stained with Coomassie solution (0.25% brilliant blue R-250 w/v, 45% methanol v/v, 10% acetic acid v/v) for 2 h and distained with fixing-solution for 24 h. For Western analysis, proteins were transferred to PVDF membranes and processed as described previously. For PRO-Q Diamond staining (Invitrogen), gels were processed following the manufactures protocol and proteins were detected with a UV transilluminator.

2.6 Trypsin digestion, MS analysis and informatic treatment of data

Gel spots were excised, distained with 25 mM ammonium bicarbonate: ACN (70:30 v/v) and incubated with 12 ng/mL trypsin (Promega) in 25 mM ammonium bicarbonate at 60°C for 1 h. 4 μL of 1% TFA was added to each sample. Then, the samples were sonicated for 10 min and desalted with a ZipTip (Millipore). Peptides were eluted with 2 μL of CHCA (10 mg/mL) in ACN/ 0.1% TFA (50:50 v/v). MALDI-TOF-MS was performed on a Voyager-DE STR (Applied Biosystems). Data from 50–200 laser shots were collected to produce a mass spectrum of 1000–4000 Da range. The mass spectrum data were analysed with Aldente software (http://www.expasy.org/tools/aldente/) [18].

2.7 *In vitro* dephosphorylation assay

Ptx treated cells were lysed in UTATH. Once UTATH was removed using a Y-10 microcone (Millipore), proteins were recovered in water and quantified by Bradford assay. 560 μg of protein was dephosphorylated with λ-Phosphatase (λ-Ppase) (New England BioLabs) as described in the manufacturers protocol. After elimination of reaction buffer with a Y-10 microcone, proteins were recovered in UTATH and run in a 2-D PAGE electrophoresis as described previously.

3 Results

3.1 Cellular response to Ptx

It is well established that Ptx induces G2/M arrest and cell death. Thus, we use propidium iodide staining followed by flow cytometry analysis to confirm whether Ptx could induce this events in our culture conditions. HeLa cells were treated with vehicle (control) or 1 μM Ptx for 24 h. The results show that Ptx increases G2/M cells from 12.88–30.45% and dead cells from 18.60–41.03% (Fig. 1a). Since cyclin B1 is a protein that increases at late S phase and remains elevated until its degradation at the end of mitosis, we have used this cyclin as a molecular marker to further confirm that Ptx induces G2/M arrest. Thus, Western blot analysis using β-actin as internal control, showed a 2.5-fold increase in cyclin B1 levels after a 24 h Ptx treatment (Fig. 1b).

3.2 Proteomic analysis of Ptx effects

To study the proteins altered upon Ptx treatment, HeLa cells were treated with either vehicle (control) or 1 μM Ptx for 24 h. Protein expression after drug treatment was analysed by 2-D PAGE. Careful observation of the 2-D electropherogram showed an up-regulated spot, named as A, and a down-regulated spot named as B which were found in Ptx treated cells as compared with control cells (Fig. 2b). In Fig. 2b it is also indicated spot C representing a protein

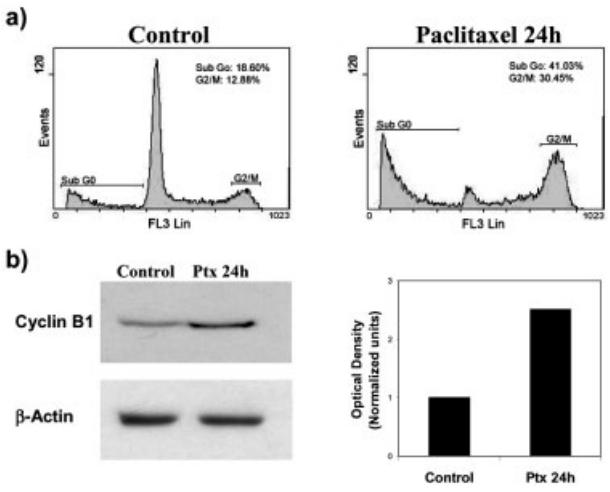

Figure 1. Flow cytometry analysis of Ptx effects. HeLa cells were treated with vehicle (control) or 1 μM Ptx for 24 h. (a) Cells were processed for propidium iodide staining and flow cytometry analysis as described in Section 2. (b) Western blot analysis of cell extracts from control cells and Ptx treated cells (left) and quantification of cyclin B1 using β-actin as internal control (right).

which does not undergo any changes upon Ptx treatment. The spots were quantified by densitometry analysis with Scion Image software using spot C for normalization (Fig. 2c). A 1-D SDS-PAGE was run to demonstrate that identical amounts of total protein were resolved in each 2-D PAGE (Fig. 2a).

3.3 Identification of Ptx-regulated proteins

To identify the proteins of interest MALDI-TOF-MS in positive reflector mode was used. The two Ptx-regulated spots (A and B, Fig. 2b) were excised from the gel and digested with trypsin. The MS data obtained for spots A and B showed a high number of coincident peptides, strongly indicating that the two spots were different forms of the same protein. Aldente software confirmed that both spots were eEFB1γ with a score of 23.28 and 7.79, respectively (see Supporting Information). Of especial interest is the peptide VLSAPPHFHFGQ*T*NR with a molecular mass of 1707.86, which contains S32 and T42, which are susceptible to phosphorylation (see Section 4). Western blot analysis using antibodies against eEF1Bγ confirmed that spots A and B contained eEFB1γ (Fig. 3).

3.4 Analysis of eEF1γ expression

To study whether Ptx has any effect on eEF1Bγ expression either at RNA or protein levels, HeLa cells were treated with vehicle (control) or 1 μM Ptx for various times. Then, the cell extracts were processed for real-time PCR or Western blot analyses as described in Section 2. The results show no significant effect of Ptx over eEF1Bγ mRNA at any of the times

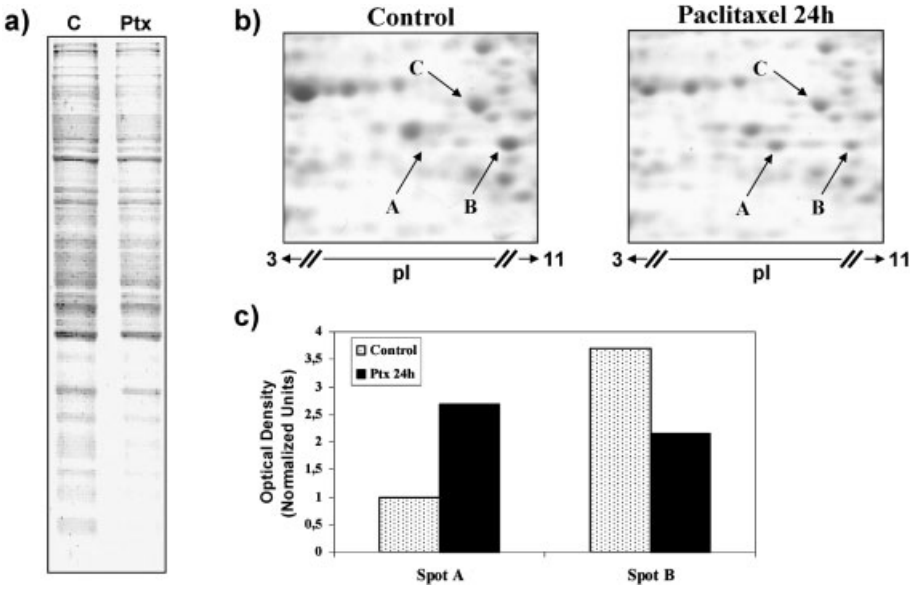

Figure 2. 2-D SDS-PAGE analysis of proteins in paclitaxel treated cells. HeLa cells were treated with vehicle (Control) or 1 μM Ptx for 24 h. (a) Coomassie staining of a 1-D SDS-PAGE used as input control, (b) magnification of a 2-D SDS-PAGE zone stained with CBB, (c) quantification of A and B spot density, using C spot for normalization.

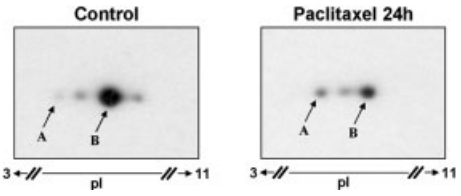

Figure 3. Western blot identification of eEF1Bγ as the Ptx regulated spots. HeLa cells were treated with vehicle (control) or 1 μM Ptx for 24 h and processed for 2-D PAGE and Western blot analysis as described in Section 2.

assayed (Fig. 4a). Furthermore, Western blot analysis, using β-actin as internal control, shows no change in the eEF1Bγ levels after Ptx treatment (Fig. 4b).

3.5 Identification of a phosphorylated form of eEF1γ

The absence of Ptx effects over eEF1Bγ mRNA or protein levels suggests that the drug is regulating a PTM of the protein. To determine if this modification is a phosphorylation, a phospho-specific analysis was performed. For this purpose,

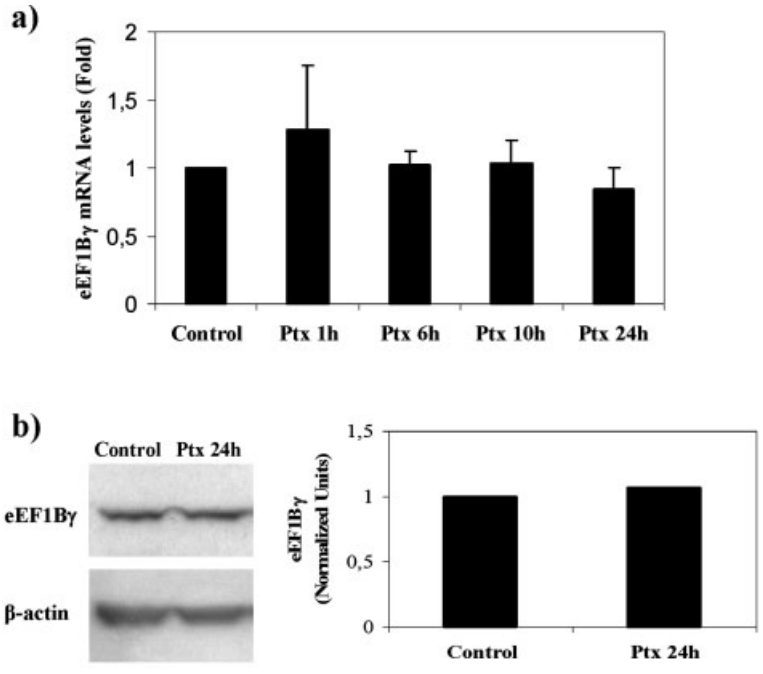

Figure 4. Effect of Ptx on steady-state level of eEF1Bγ mRNA and protein. HeLa cells were treated with vehicle (control) or 1 μM Ptx for the indicated times. (a) Total RNA was isolated, the cDNA was synthesized and eEF1Bγ mRNA expression was quantified by real-time PCR using β2M as internal control. (b) Western blot analysis of the cell extracts from control cells and Ptx treated cells (left) and quantification of eEF1Bγ using β-actin as internal control (right). For simplicity purposes, only the 24 h sample is shown.

HeLa cells were treated with vehicle (control) or 1 μM Ptx for 24 h. After processing protein extracts for 2-D PAGE, gels were stained with PRO-Q Diamond. The results show that only the Ptx up-regulated form of eEF1Bγ (named as A) is stained, while no signal for the Ptx down-regulated form of eEF1Bγ (named as B) is detected (Fig. 5a, upper panels). This suggests that form A is phosphorylated while form B is not phosphorylated. Coomassie staining of these gels determined that the levels of the PRO-Q negative form B are higher than the levels of the PRO-Q positive form A, which clearly indicates the specificity of the PRO-Q staining (Fig. 5a, lower panels). To further confirm that the A form is phosphorylated, an *in vitro* dephosphorylation assay was performed. Thus, protein extracts from cells treated with 1 μM Ptx for 24 h were incubated in the presence or absence of λ-PPase as described in Section 2. After the phosphatase treatment extracts were subjected to 2-D PAGE analysis. The indicated spots were subjected to MALDI-TOF-MS analysis and identified as eEF1Bγ (Fig. 5b, upper panels). These data were further confirmed by Western blot analysis (Fig. 5b, lower panels). These results, therefore, indicate that Ptx triggers the specific phosphorylation of eEF1Bγ.

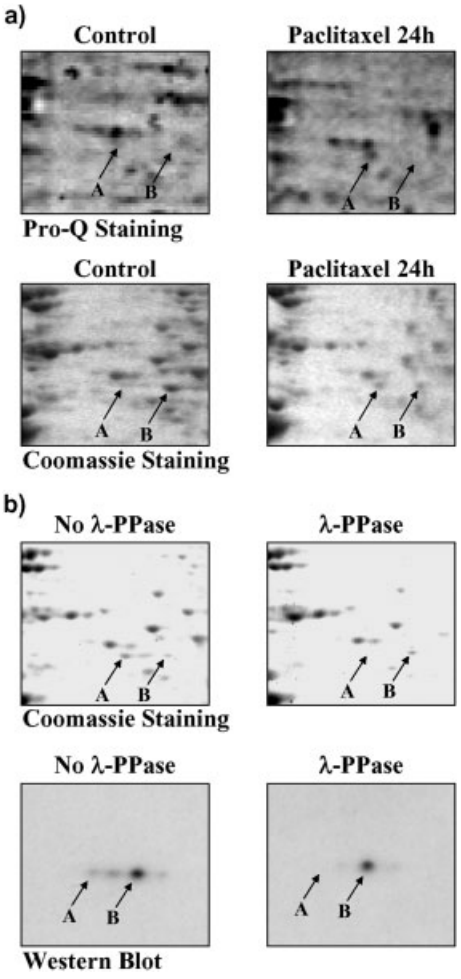

Figure 5. Identification of phosphorylated eEF1Bγ as the protein up-regulated by Ptx. (a) HeLa cells were treated with vehicle (Control) or 1 μM Ptx for 24 h and processed for 2-D PAGE. Gels were stained with PRO-Q Diamond (upper panels) or with CBB (lower panels). (b) Extracts from cells treated with 1 μM Ptx were incubated in the presence or in the absence of λ-PPase. After phosphatase treatment, 2-D PAGE was carried out and gels stained with CBB (upper panels) or subjected to Western blot analysis using anti-eEF1Bγ antibodies (lower panels).

4 Discussion

Taxanes such as Ptx are drugs that block the mitotic spindle dynamics that is required for chromosome migration during mitosis. It is also known that these compounds activate JNK [15], induce Bcl-2 phosphorylation [16], G2/M arrest and cell death [17]. However, the biochemical events occurring between taxane binding to tubulin and cell death are not well understood yet.

In the last few years, the development of proteomic techniques has made 2-D PAGE/MALDI-TOF-MS a powerful tool for the analysis of complex protein mixtures. This methodology has been recently used to investigate proteins that are regulated by chemotherapy agents [19, 20].

In this report, we used HeLa cells to study proteomic changes caused by Ptx treatment. After confirming that in our experimental conditions Ptx retains its capacity to induce G2/M arrest and cell death, we performed the 2-D PAGE analysis followed by MALDI-TOF-MS. In these experiments, a Ptx up-regulated form and a Ptx down-regulated form of eEF1Bγ were identified. The absence of Ptx regulation on eEF1Bγ mRNA or protein levels suggested a PTM of the protein. We observed the appearance of a more acidic form of the protein, which is compatible with a phosphorylation. We have used a phospho-specific staining with PRO-Q Diamond and an *in vitro* dephosphorylation assay to determine that the Ptx up-regulated form of eEF1Bγ is phosphorylated and the Ptx down-regulated form of eEF1Bγ is the substrate for this phosphorylation.

It has been recently described that eEF1Bγ can be phosphorylated at threonine 42 after EGF treatment [21]. We find in spot A, a peptide with an m/z of 1701.86 Da that corresponds to residues 30–44 which is not phosphorylated. Since spot A contains phosphorylated forms of the protein, it follows that this elongation factor can be phosphorylated in residues other than threonine 42.

In *Xenopus*, eEF1Bγ is phosphorylated by the mitotic promoting factor [12], which is activated during the mitotic process [13]. Despite the fact that the role of this phosphorylation has not been established, there are indications that this change does not modify eEF1B GTPase activity. Rather it can regulate the interactions of eEF1Bγ with other proteins such as valyl-tRNA synthetase [22]. In addition, this phosphorylation could induce the localization of eEF1B around the spindle pole [23]. These events could decrease global protein translation [24] and also modulate the translation of

specific proteins [22]. We have determined that this phosphorylation occurs in response to the treatment with the antitumoural agent Ptx. It is conceivable that the translation of some of the proteins implicated in Ptx resistance is specifically regulated by the phosphorylation of eEF1Bγ and therefore the elongation factor could be implicated in Ptx-induced cell death. In addition, the change of eEF1B localization could regulate chromosome migration. Since Ptx induces cell death by blocking chromosome migration, it is possible that this event also plays a role in drug-induced cell death.

In summary then, we report that the human eEF1Bγ is *in vivo* phosphorylated in response to the antitumoural agent Ptx; an event whose function is unknown but that could play a role in the drug-induced cell death.

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