Phosphoproteomic Analysis of Mouse Thymoma Cells Treated With Tributyltin Oxide: TBTO Affects Proliferation and Energy Sensing Pathways

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We report the results of phosphoproteomic analysis of mouse thymoma cells treated with tributyltin oxide (TBTO), an immunotoxic compound. After cell lysis, phosphoproteins were isolated using Phosphoprotein Purification Kit, separated by SDS-PAGE and subsequently digested with trypsin. Phosphopeptides were enriched employing titanium dioxide, and the obtained fractions were analyzed by nano-LC-MS/MS. A total of 160 phosphoproteins and 328 phosphorylation sites were identified in thymoma cells. Among the differentially phosphorylated proteins identified in TBTO-treated cells were key enzymes, which catalyze rate-limiting steps in pathways that are sensitive to cellular energy status. These enzymes included acetyl-CoA carboxylase isoform 1, which catalyzes the rate-limiting step of fatty acid synthesis. Another enzyme was glutamine: fructose-6-phosphate amidotransferase, GFAT1, the first and rate-limiting enzyme for the hexoamine synthesis pathway. Pyruvate dehydrogenase (PDH), a multicomplex enzyme that catalyzes the rate-limiting step of aerobic oxidation of fuel carbohydrates, was identified in both TBTO-treated and control cells; however, phosphorylation at residue S293, known to inhibit PDH activity, was only identified in control cells. A higher expression level of ribosomal protein S6 kinase 1, a downstream kinase of the mammalian target of rapamycin signaling pathway implicated in protein synthesis through phosphorylation of 40 ribosomal S6, was observed in the treated cells. Giant kinases like AMP-activated protein kinase (AMPK) and cAMP-dependent protein kinase (PKAR1A), which are known to mediate the phosphorylation of these enzymes, were identified in TBTO-treated cells. Down-regulation of proteins, such as MAPK, matrin-3 and ribonucleotide reductase, subunit RRM2, which are implicated in cell proliferation, was also observed in TBTO-treated cells. Together, the results show that TBTO affects proliferation and energy sensor pathways.

Key Words: TBTO; immunotoxicity; thymoma cells; proteomics; mechanism; phosphoproteins.

Tributyltin oxide (TBTO), an organotin compound, has been used as a molluscicide, agent in wood preservation, and as antifouling in marine paints, industrial water systems, and textiles. (Elsabbagh et al., 2002). Human exposure mainly occurs through the food chain by consumption of dietary marine products (Kannan et al., 1996). TBTO is primarily characterized by its immunotoxicity, which is manifested through both specific and nonspecific immune suppressive effects (Aluoch et al., 2006; Van Loveren et al., 1990; Vos et al., 1990). In rodents, TBTO causes atrophy of the thymus gland (Vos et al., 1990).

In addition to its immunotoxic effect, TBTO has adverse effects on both vertebrate and invertebrate endocrine systems (Iguchi et al., 2007) and, similar to various other organotins (Costa, 1985; Doctor et al., 1982; and references therein), has been shown to be a potent neurotoxicat; impaired learning ability and memory loss were observed in rats treated with this compound (Elsabbagh et al., 2002). Furthermore, it has been shown that TBTO inhibits protein synthesis (Osman et al., 2009; Raffray et al., 1992) and, like other-related organotins compounds, disrupts mitochondrial energy metabolism (Penninks et al., 1983; Raffray et al., 1992; Soracco and Pope, 1983; Veiga et al., 1996), and blocks mitosis and cytokinesis, disturbing spindle formation in Chinese hamster V79 cells (Jensen et al., 1991). In accord with the latter observation, triaryl- and trialkyltin compounds have been shown to inhibit in vitro polymerization of rat brain tubulin (Tan et al., 1978). Although numerous studies have been undertaken to understand the mechanisms of TBTO toxicity, including messenger RNA (mRNA) microarray investigations (Baken et al., 2006, 2007; Katika et al., 2011), the target molecules of TBTO toxicity still remain unclear. The EL-4 thymoma cell line was shown to possess an immature thymus cell phenotype (Tanaka et al., 1987) and has been used as a model for mouse thymocytes in mechanistic studies (El-Darahali et al., 2005; Lee et al., 2008). Recently, we have reported proteomic profiling of mouse thymoma cells treated with TBTO and found that this compound altered the expression levels of 12 proteins, including prothymosin alpha (Protα) (Osman et al., 2009), an essential protein in cell proliferation (Evasieva et al., 2000; Sburlati et al., 1991). Based on these results, we proposed...
that the downregulation of this immune-modulating protein could account for the previously reported antiproliferative effect of TBTO (Osman et al., 2009).

The purpose of the present study was to extend our knowledge on the effect of TBTO on protein expression by investigating whether TBTO modifies the phosphorylation state of the proteome of treated thymoma cells as compared with that of untreated cells. Phosphorylation is probably the most studied posttranslational modification since most cellular processes utilize reversible phosphorylation for regulation, e.g., metabolism, signaling pathways, cell cycle, differentiation, or as a code for protein degradation. However, the identification of phosphoproteins by mass spectrometry poses challenges mainly because of low abundance of phosphoproteins within cells compared with nonmodified proteins, low stoichiometry of phosphorylation, ionic suppression, and difficulties in locating phosphorylation sites as well as the intrinsic dynamic nature of phosphorylation in cells (Mann et al., 2000). Therefore, in the present study, we used the following approach: Upon lysis of cells and extraction of proteins, first, we enriched the phospho-protein fractions by using phosphoprotein purification columns, followed by 1D SDS-PAGE. Proteins were then digested in gel with trypsin. Second, we used titanium dioxide for enriching phosphopeptides prior to the analysis of peptide fractions by nano-LC-MS/MS analysis. The results showed that the treatment of thymoma cell line with TBTO causes differentially phosphorylated proteins and that this immunotoxic compound especially affects the phosphorylation state of key enzymes that catalyze rate-limiting steps of energy sensing pathways and proteins involved in cell proliferation.

MATERIALS AND METHODS

Chemicals. Bovine β-casein, α-casein, bovine serum albumin (BSA), ovalbumin, TBTO, α-dithiothreitol, and iodoacetamide were purchased from Sigma. Sequencing grade modified trypsin was from Roche (Mannheim, Germany). ReproSil-Pur18-AQ3 resin was obtained from Dr Maisch GmsH (Ammerbuch, Germany), and C18 200A-AQ5 was purchased from Phenomenex. Ammonium bicarbonate was obtained from Fluka. The solvents ethanol absolute (96%), acetonitrile, formic acid, and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany), and sodium chloride and lithium dodecyl sulfate sample buffer were from Promega (Madison, USA). Acetonitrile and formic acid were of LC-MS grade. Bovine serum albumin, trypsin, and lysozyme were from Sigma (St Louis, USA). Phosphatase cocktail (Magic standard) were from Invitrogen and ECL detection reagents were obtained from Millipore (Carrigtwohill, Ireland). Protein A/G Plus agarose, radioimmunoprecipitation (RIPA) lysis buffer, PMSF, sodium orthovanadate solution, and protease inhibitor cocktail were obtained from Santa Cruz Biotechnology.

Cell culture and treatment with TBTO. EL-4 cells were cultured as described before (Osman et al., 2009). Each treated sample or control contained 5 × 10⁶ cells in a volume of 20 ml. A 1 mM TBTO stock solution was prepared in ethanol, and 10 μl of this solution was added to each one of the cell suspension samples so that the final concentration of TBTO was 0.5 μM, whereas 10 μl of ethanol without TBTO was added to the control cells. Two independent replicate experiments for both treated sample and control were performed for the proteomics approach and two other replicates for immunoprecipitation of matrix-3 and Western blot (WB) analysis. Subsequently, cells were incubated at 37°C for 6 h. Cell viability was evaluated by trypan blue dye exclusion and ranged 85–90% in all experiments, which was in agreement with previous reports (Osman et al., 2009; Raffray and Cohen, 1991). TBTO concentrations higher than 1 μM and incubation times longer than 6 h led to apoptosis of cells (Osman et al., 2009; Raffray and Cohen, 1991). TBTO has a lipophilic character and, like other tributyltin compounds, is rapidly taken up by cells and extraction of proteins, first, we enriched the phospho-protein fractions by using phosphoprotein purification columns, followed by 1D SDS-PAGE. Proteins were then digested in gel with trypsin. Second, we used titanium dioxide for enriching phosphopeptides prior to the analysis of peptide fractions by nano-LC-MS/MS analysis. The results showed that the treatment of thymoma cell line with TBTO causes differentially phosphorylated proteins and that this immunotoxic compound especially affects the phosphorylation state of key enzymes that catalyze rate-limiting steps of energy sensing pathways and proteins involved in cell proliferation.

Gel electrophoresis. Electrophoresis of the samples was performed essentially as described in detail before (Osman et al., 2009). Briefly, 80 μg of protein was loaded per well of a 10% Nu-PAGE-Tris-gel and run at 160 V for 90 min. After that, the gel was rinsed three times with deionized water (5 min each time), followed by staining it with Coomassie (Simply Blue Safe Stain) for 2 h. The gel lanes of sample and control were horizontally cut into 14 fractions. Each gel slice was cut into pieces and put into a 1.5 ml microcentrifuge tube.

In-gel digestion. The fractions were destained, dehydrated, reduced, with dithiothreitol, and alkylated with iodoacetamide as described before (Osman et al., 2009). The fractions were digested with trypsin and incubated for 17 h. After that, to each fraction, 30 μl of ammonium bicarbonate buffer was added and incubated for additional 2 h. The supernatant of each fraction was put into an Eppendorf tube and subsequently 25 μl of 50% ethanol containing 5% formic acid was added to each fraction, mixed, and pooled it to the previous one. This process was repeated once. The fractions were freeze-dried by Speed-Vac, suspended in 60 μl acetonitrile, and again freeze-dried. Finally, the peptide fractions were suspended in 50 μl solution of 0.5% formic acid/2% acetonitrile and used for phosphopeptide enrichment.

Titanium dioxide enrichment of phosphopeptides. Phosphopeptides were enriched from peptide mixture obtained as described above using titanium dioxide MonoTip Tio pipette tips. Essentially, the operation protocol of the manufacturer was followed with slight modification. The MonoTip Tio was preconditioned with 100% acetonitrile, and this process was repeated twice.
Next, the tip was conditioned with 0.2M phosphate buffer, pH 7.0 and equilibrated with 50% aqueous acetonitrile solution containing 0.1% formic acid, repeating this step three times. After that, peptide fraction was drawn and ejected. This adsorption process was repeated 25 cycles per fraction, after which the tip was rinsed with 30% aqueous acetonitrile containing 0.1% formic acid and 0.1M KCl. The rinsing process was repeated six times per fraction. Finally, the tip was desalted with the equilibration buffer, drawing and ejecting three times, followed by elution with 2% aqueous ammonia. The desalting step did not affect negatively the efficiency of the enrichment method. The eluted fractions were freeze-dried, suspended with 60 µl of 100% acetonitrile per fraction, and again freeze-dried. After that, each fraction was suspended with 30 µl of 1% formic acid/2% acetonitrile aqueous solution. The fractions were kept at −20°C until analysis.

Nano-LC/MS/MS. Peptide fractions were analyzed by a nano-LC-MS/MS using an Agilent 11000 Series LC set up (vacuum degasser, autosampler, and one high pressure-mixing binary pump without static mixer) coupled to an LCQ Deca Quadrupole Ion Trap mass spectrometer (Thermo Finnigan, San Jose, CA) as previously described by Meiring et al. (2002). The conditions of elution were essentially as described in Osman et al. (2009). Briefly, we loaded 5 µl of peptide solution to a trap column (Aqua C18 [Phenomenex]; l = 15 mm–100 µm ID, packed in house) at 5 µl/min of 100% solvent A (0.1M acetic acid). Following a decrease of the flow to 150 n/min by a splitter, peptides were transferred to Reprosil C18 RP column (Dr Maisch GmbH [l = 20 cm, 50 µm ID]) with a linear gradient from 0 to 100% solvent B (0.1M acetic acid in 80% acetonitrile) in 40 min. Solvent B was maintained at 100% for 5 min, followed by decreasing solvent B to 0% in 0.1 min, washing, and reequilibrating the system at 100% A for 10 min. The LCQ operated in the data-dependent mode using a positive ion mode. Full MS spectra from m/z 400 to 2000 were acquired followed by 1400 to 2000 were acquired followed by 3. The exposure experiments for the proteomics were performed in duplicate and each treated or control sample was analyzed twice (technical duplicate). Phosphopeptides and the corresponding phosphopeptides identified in the control samples and their technical duplicates were combined in a list; the same procedure was applied to the treated samples. The comparison of these lists allowed determining the common phosphoproteins and the corresponding phosphopeptides identified in both cells as well as those detected only in either of them. The relevance of the differentially phosphorylated peptides was evaluated mainly on the number of differentially phosphorylated peptides identified and the known role of the phosphorylation sites, in addition to their potential role in explaining what is known about TBTO effects.

Preparation of total cell extracts for immunoprecipitation and immunoblot. Total cell extracts used for WB analyses and immunoprecipitation of matrin-3 were prepared by using cell pellets obtained as described above. Each sample was suspended in 1 ml of RIPA lysis buffer containing 10 µl of PMSF, 10 µl of protease inhibitor cocktail, 10 µl of sodium orthovanadate, phosphatase inhibitor cocktail 1 (10 µl), and 1mM final concentration of EDTA. For immunoprecipitation of matrin-3, sometimes (see below), EDTA was not included in the lysis buffer. Cell suspensions were incubated on ice for approximately 30 min and subsequently disrupted by repeated aspiration through a 21 gauge needle, transferred to an Eppendorf, and centrifuged at 12,000 × g for 10 min. The supernatants were used for protein concentration determination, divided into aliquots, and stored at −20°C.

Immunoprecipitation of matrin-3. Control cell lysates extracted in lysis buffer with and without EDTA, as described above, were used for the immunoprecipitation experiments. In each case, 500 µg protein was incubated with goat anti-matrın-3 antibody (2 µg) for 2 h at 4°C, after which 20 µl of A/G Plus agarose suspension was added to the lysate and incubated overnight at 4°C. The immunoprecipitates were collected by centrifugation at 2500 rpm for 5 min at 4°C. Pellets were washed four times with 1 ml of 10mTris-HCl, pH 7, containing 0.15M NaCl, each time repeating the centrifugation step. After the final wash, the pellet was suspended in 40 µl of 1× electrophoresis buffer. Samples were boiled for 3 min, centrifuged at 2500 rpm, and the supernatants used for WB analysis. Five microliters of each sample was subjected to SDS-PAGE.

WB analysis. Both the enriched phosphoproteins and the total cell extracts obtained as described above were used for immunoblotting. In the case of the enriched phosphoproteins, equal amounts of protein (60 µg per lane) from TBTO-treated and nontreated cells were separated by SDS-PAGE, followed by electrotransfer of the proteins using Hybrid-P PVDF membrane at 25 V for 90 min. For the total cell extracts, the same amount of protein as the enriched phosphoproteins was used for one of the proteins (matrin-3) and 20 µg of protein per lane for RPS6k1. The membrane was blocked by using nonfat milk powder in PBS containing 0.2% Tween and probed with goat anti-matrın-3 antibody diluted in (1:400) or with rabbit anti-p70 S6 kinase (Thr421/Ser424, corresponding to Thr444/Ser447 in mouse sequence) antibody diluted in (1:400). Detection was performed by using donkey anti-goat IgG-HRP (1:2000) and goat anti-rabbit IgG-HRP (1:2000), respectively, and ECL Western blotting detection reagents (Amersham Pharmacia). The antibody of the loading control was diluted in 1:1000.

RESULTS

Validation and Procedure of Enrichment of the Phosphoprotein Purification Affinity Column

The effectiveness of the phosphoprotein purification affinity column was evaluated by loading onto the affinity column a mixture of known phosphorylated proteins (α- and β-casein), a partially phosphorylated protein, ovalbumin, and a nonphosphoprotein, BSA. We conditioned the column according to the manufacturer’s instructions. The flow-through fraction, wash, and the eluate were collected and run on 1D SDS-PAGE. The phosphoproteins α- and β-casein and a part of ovalbumin were detected in the flow-through and wash fractions (data not shown). This result indicates the ability of the phosphoprotein affinity column to enrich phosphoproteins. Next, we applied our cell extracts, containing 2.5 mg protein at the concentration of 0.1 mg/ml, as recommended by the manufacturer, to the affinity column. Approximately 10% of the loaded protein was bound to the column (Fig. 1). The insert shows a typical elution profile of the enriched phosphoprotein fractions. Fractions 2–6 were combined, though most of the bound proteins were found in the third fraction. After separation of the isolated phosphoproteins by SDS-PAGE, followed by in-gel digestion with trypsin, we enriched phosphopeptides using titanium dioxide prior to nano-LC-MS/MS analysis of the samples.

Classification of the Phosphoproteins Identified

A total of 160 phosphoproteins and 328 phosphorylation sites were identified from thymoma cells. Examples of CID
beta (PURB). These proteins were identified in both treated and control cells.

The CID spectra from two of the identified phosphopeptides, NRNP-SNVVPYDFNR from isoform 2 of leukocyte common antigen (PTPRC); (B) phosphopeptide RGGSGGGDEpSEEGEEVDED from transcriptional activator protein Pur-beta (PURB). These proteins were identified in both treated and control cells.

Additional examples of MS/MS of identified phosphopeptides are reported in Supplementary table 1S. Of these proteins 52 were common to both TBTO-treated and non-treated cells (Table 1), whereas equal number of proteins (54) were detected in TBTO-treated (Table 2) and nontreated cells (Table 3). These data (160 phosphopeptides and the 328 sites) were compared with the public database of Phosphosite (http://www.phosphosite.org/). Of the phosphoproteins and sites identified, 3 proteins and 13 sites were not reported in this public database and hence are marked with asterisk (Tables 1–3). The three proteins are an oxidoreductase of the cytochrome P450 family (CYP4x1), G-protein–coupled receptor 45 (GPR45), a signal transducer, and a protease isofrom 2 of astacin-like metalloendopeptidase (ASTL) (Tables 2 and 3). Examples of representative MS/MS spectra of validated phosphopeptides are reported in Supplementary table 1S. The identified phosphorylation sites were distributed as follows: approximately 74% occurred on serine, 18% on threonine, and 8% on tyrosine (Supplementary fig. 2SA). Figure 3A reports the classification of the total identified phosphoproteins on the basis of their biological function. The majority of the identified proteins belonged to DNA- and RNA-binding proteins, which, combined with transcription factors, represented 31% of the total (Fig. 3A). Cytoskeletal proteins were the next abundant group (17%). Furthermore, proteins involved in signal pathways, metabolism, DNA replication and repair, transport, proteasome and ubiquitin-conjugating system, and chaperons were also identified (Fig. 3A). Upon comparison of the proteins identified in TBTO-treated cells (Fig. 3B and Table 2) with those detected in the controls (Supplementary fig. 2SB and Table 3), proteins involved in cell cycle, proliferation, and translation were relatively more representative in the controls than in the treated cells. In contrast, proteins involved in signal inhibitory effect and in apoptosis were found in TBTO-treated cells. For instance, both caspase 3 (CASP3) and programmed cell death (PDCD5), which are involved in apoptotic cell processes, were identified only in TBTO-treated cells (Table 2). CD5, a cell surface glycoprotein, which has been implicated in the negative regulation of TCR-mediated growth responses in thymocytes (Tarakhovsky et al., 1995), was detected only in TBTO-treated cells (Table 2).

Some of the differentially phosphorylated proteins identified in this study appear to be relevant for the mechanism(s) of TBTO toxicity.

Identification of Critical Phosphorylation Sites on Key Enzymes

Among the differentially phosphorylated proteins identified in the treated cells but not in control cells were key enzymes that catalyze rate-limiting steps in biosynthetic pathways. One of these enzymes was acetyl-CoA carboxylase (ACC) isofrom 1, which catalyzes the rate-limiting step of fatty acid synthesis (Ha et al., 1994). Phosphorylation sites at residues S79, S77, and S76 were identified for this protein (Table 2). Another enzyme that was identified in TBTO-treated cells only was glutamine: fructose-6-phosphate amidotransferase 1 (GFT1), which catalyzes the rate-limiting step in hexoamine pathway (Eguchi et al., 2009). Peptides with phosphorylation sites at T260 and T261 were identified for this enzyme (Table 2). A key regulatory enzyme that was identified in both TBTO-treated and control cells was the multicomplex protein pyruvate dehydrogenase (PDH) (Table 1). This enzyme links glycolysis to the tricarboxylic acid cycle by catalyzing the irreversible oxidative decarboxylation of pyruvate to form CO2, NADH, and acetyl-CoA. Although only one phosphorylation site at the residue S295, common to both cells, was identified, additional phosphorylation sites (S293, T231, Y289, and Y301) were identified in the control cells (Table 1). This enzyme’s activity is regulated via phosphorylation (Linn et al., 1969). Phosphorylation at the specific sites of S232, S293, and S300 are known to inhibit the enzyme activity (Rardin et al., 2009 and references therein). One of these phosphorylation sites, S293, was identified in control cells. Moreover, p70RPS6k1, a downstream effector of mammalian target of rapamycin (mTOR) pathway and a major kinase for 40 ribosomal S6, whose phosphorylation is often associated with increase in protein synthesis and cell growth (Fingar and Blenis, 2004; Zhou et al., 2010; and references therein), was identified in both control and TBTO-treated cells (Table 1). One phosphorylated site, at residue S447, was detected in TBTO-treated cells (Table 1), whereas in addition to this residue, two more phosphorylated sites at T444 and S452 were identified for this kinase in control cells (Table 1). Because RPS6k1 phosphorylation is used as a readout of mTOR activity (Hay and
FIG. 2. A typical elution profile of proteins obtained from a TBTO-treated sample loaded onto the phosphoprotein purification column. The insert shows the elution pattern of the enriched phosphoprotein fractions. The elution profile of the proteins obtained from vehicle-treated cells was comparable to that of the TBTO-treated cells.
Sonenberg, 2004), we evaluated the level of the phosphorylated protein in both cells using p70s6k (Thr421/Ser424) antibody, corresponding to Thr444 and Ser447 in mouse sequence. WB analysis of both enriched phosphoproteins and total cell extracts confirmed the decrease in the intensity signal of the band corresponding to Thr444 and Ser447 of the treated cells as compared with that of the control (Fig. 4A). In addition to these major enzymes, proteins implicated in cell proliferation and growth were affected by TBTO exposure.

Proteins Associated With Cell Proliferation Were Downregulated in TBTO-Treated Cells

Proteins that are implicated in cell proliferation and growth were identified in control cells. Based on the number of phosphorylated peptides identified, these proteins appeared to be downregulated in TBTO-treated cells. One of these proteins was MAPK1, known also as extracellular signal-regulated kinase, which has essential roles in cell proliferation, growth, and differentiation (Warren et al., 2009). Phosphorylation sites at T179, T183, T188, and Y185 were identified for this protein in control cells (Table 3). This protein was not detected in TBTO-treated cells. Another protein identified only in control cells and considered as a marker for cell proliferation was ribonucleotide reductase M2. The enzyme ribonucleotide reductase catalyzes the conversion of ribonucleotides to deoxyribonucleotides for DNA synthesis. The enzyme consists of two subunits: M1 and M2. The activity of M1 is stable during the whole cell cycle, whereas the activity of M2 is transient and has been shown to be present in late G1/early S phase of the cell cycle (Engström et al., 1985; Heidel et al., 2007). A phosphorylation site at S20 was identified for this protein (Table 3). Finally, another protein that seemed to be downregulated in TBTO cells was matrin-3 (Table 1). This nuclear matrix protein has been previously reported in rat liver cells (Giordano et al., 1991), in rat neuron cells (Hibino et al., 2006), and in human fetal brain (Bernert et al., 2002). In contrast, the bands with the lower molecular weights might be degradation products of matrin-3. WB analysis of immunoprecipitated matrin-3 from control lysates, prepared in RIPA lysis buffer with or without EDTA showed, in addition to a band of strong signal intensity at high molecular weight, corresponding to matrin-3, the presence of two additional bands with apparent molecular weights of 51 and 25 kDa, which were especially marked in signal intensity in the lysates extracted with the buffer without EDTA (Fig. 4C).

Finally, because it was previously shown that organotin compounds disrupt microtubule assembly causing spindle disturbance in V79 Chinese hamster cells (Jensen et al., 1991), it is worth to note that, among the cytoskeleton proteins identified in the treated cells, there were two proteins belonging to the family tubulins, both of beta chain TUBB4 and TUBB5 (Table 2). The phosphorylation sites at residues S335 and T55 were identified for these proteins, respectively (Table 2). Polymerization of α- to β-tubulins leads to the formation microtubules, which carry out a variety of functions in the cell, including cell division, where they form mitotic spindles, structures that segregate faithfully sister chromatids. A number of kinases are known to mediate phosphorylation of tubulin subunits, some of which inhibit the polymerization reaction (Macrae, 1997). Also, a microtubule-binding protein, collapsin response mediator protein-2 (DPYSL2 or CRMP-2) was identified in the treated cells but not in the control cells (Table 2). A number of phosphorylation sites, including T514, were identified for this protein (Table 2). The nonphosphorylated form of this protein has been shown to promote axon outgrowth via microtubule assembly, whereas the phosphorylated form, especially at position T514, weakens the binding of DPYSL2 to tubulins, thereby hindering axon outgrowth (Yoshimura et al., 2005). Therefore, this phosphoprotein might contribute to the reported organotin-mediated disruption of microtubule assembly.

DISCUSSION

The study of organotin toxicity has been a subject of intense investigation for decades, yet, the molecular mechanisms responsible for the toxicity of these compounds currently
| Gene symbol and protein name | Accession number | Sequences of the phosphopeptides identified | Phosphorylation sites |
|-----------------------------|------------------|---------------------------------------------|----------------------|
| Eap1 enhanced at puberty protein 1 | IP00469941.2 | R.KpSPEPDDSALSEALK.L | S526 |
| Abcf1 ATP-binding cassette subfamily F member 1 | IP00396671.1 | K.AKGNVFALIQDpSEEEEEENR.V | S138 |
| Acly putative uncharacterized protein | IP00126248.3 | R.pTSFSESRASEDVPQAK.K | T453 |
| Canx calnexin | IP00119618.1 | K.AEEDEKIDHLFGpSPRR.R | S582 |
| Cbx5 chromobox protein homolog 5 | IP00123755.1 | R.KSSFSNpSADDIKSK.K | S97 |
| Cdc12 coiled-coil domain–containing protein 12 | IP00319221.3 | R.LKGQEDSLASVADELFGpSPRR.R | S165 |
| Dbnl isoform 2 of drebrin-like protein | IP00308222.3 | R.AMpSTTVSSQPGK.V | S277 |
| Dnmt1 isoform 2 | IP00474974.1 | R.SQSSDpTLSVEpSSVPVAR.T | S138 |
| Eef1d isoform 1 of elongation factor 1-delta | IP00118875.5 | R.AMSTT | V277 |
| Eef2 elongation factor 2 | IP00466069.3 | R.FDTRK | T57 |
| Eif4b eukaryotic translation initiation factor 4B | IP00221581.1 | R.SQSSDpTLSVEpSSVPVAR.T | S138 |
| Eif4g1 isoform 1 of eukaryotic translation initiation factor 4 gamma 1 | IP00421179.1 | R.pSFESKEVEER.S | S1187 |
| Eif5b eukaryotic translation initiation factor 5B | IP00756424.3 | K.KTSFDENpSSEELEDKSDK.S | S114 |
| G3bp1 Ras GTPase-activating protein–binding protein 1 | IP00130095.1. | K.pSSTTPAPADVPAPAqEDLR.T | S229 |
| Gm11223 similar to Pr22 isoform 1 | IP00475134.1 | R.KpSHEAEVLKQLAEK.R | S63 |
| Hdgf hepatoma-derived growth factor | IP00313817.1 | R.AGDVLEDpSPIRPC.K | S165 |
| Hmgal1; Hmgal1-rs1 isoform HMG-I of high mobility group p | IP00314240.5 | K.KLKEEEGIQPEpSSEEEQ.- | S102 |
| Hnmpc putative uncharacterized protein | IP00130343.2 | K.pKVEKKEEGIQPpSSEEEQ.- | S99 |
| Hnmpd isoform 2 of heterogeneous nuclear ribonucleoprotein D0 | IP00230086.2 | K.pKVEKKEEGIQPpSSEEEQ.- | S103 |
| Hsp90ab1 MCG18238 | IP00229080.7 | K.pKVEKKEEGIQPpSSEEEQ.- | S99, S102 |
| Kif13b kinesin family member 13B | IP00761751.2 | K.pKVEKKEEGIQPpSSEEEQ.- | S246 |
| Lig1 DNA ligase (ATP) 1 | IP00473314.3 | K.pTEVQKSESGLRKpTP.R | T93 |

**TABLE 1**

List of the Common Phosphoproteins Identified in Both TBTO-Treated and Non-treated Cells and the Corresponding Phosphopeptides.
| Gene symbol and protein name | Accession number | Sequences of the phosphopeptides identified | Phosphorylation sites |
|-----------------------------|------------------|--------------------------------------------|----------------------|
| Mcm2 putative uncharacterized protein | IPI00323820.4 | R.RADALTSpSPGR.D | S41 |
|  |  | R.RKIpSDPLTSSPGR.S | S21, S27 |
|  |  | R.RRISDPLpSpSPGR.S | S26, S27 |
|  |  | R.RISDPLpTSSPGR.S | T25 |
|  |  | R.RIpSPDLTSSSpGR.S | S21 |
|  |  | R.RISDPLTSpSPGR.S | S27 |
|  |  | R.GLpYPDSSEEDERPAR.K | Y137 |
|  |  | R.RISDPLTSSPGRSpSR.R | S32 |
|  |  | R.RGILYDpSpSEEDERPAR.K | S139 |
| Mybbp1a Myb-binding protein 1A | IPI00331361.2 | K.pSPAPSNSpTpTPSpSTPAK.T | S1244, S1251, S1255 |
|  |  | Myh9 myosin-9 | R.KGpTDCSDEEVGKDAGDAK.A | T1938 |
|  |  | Ndrg1 protein NDRG1 | R.KGTGDCpTDCSDEEVGKDAGDAK.A | S1942 |
|  |  | Pdha1 pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial | K.KAPKEELApSDELMATSAK.R | S1513 |
|  |  | Nop56 nucleolar protein 56 | K.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Nsfl1c isoform 2 of NSFL1 cofactor p47 | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Nucks1 nuclear ubiquitous casein and cyclin-dependent kinases substrate | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Pdha1 pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial | K.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Phf6 isoform 1 of PHD finger protein 6 | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Pptrc isoform 2 of leukocyte common antigen | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Purr transcriptional activator protein Pur-beta | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Sept2 septin-2 | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Sept9 isoform 1 of septin-9 | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Sfrs2 splicing factor, arginine/serine-rich 2 | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Sgta isoform 1 of small glutamine-rich tetratricopeptide repeat-containing protein alpha | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Sub1 activated RNA polymerase II transcriptional coactivator p15 | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
|  |  | Tceal isoform 2 of transcription elongation factor A protein 1 | R.KVVDYQEFQESDDADEDYGR.D | Y26 |
remain unclear (Aldridge, 1958; Iguchi et al., 2007); nor is it clear how, for instance, TBTO affects the expression of so many genes and cellular processes (Baken et al., 2006, 2007).

The results of this study showed that treatment of thymoma cells with the model immunotoxic compound (TBTO) caused differential phosphorylations in the proteome of the treated cells compared with that of the control. However, not all differentially phosphorylated proteins were discussed because the currently unknown roles of their modified sites make it difficult to decipher their biological meaning. Among the differentially phosphorylated proteins were key enzymes that catalyze rate-limiting steps of major pathways that are sensitive to cellular energy status. These enzymes included PDH, ACC, and glutamine: fructose-6-phosphate amidotransferase 1 (GFPT1) as well as ribosomal S6 kinase 1 (RPS6K1), the latter is a downstream effector of mTOR pathway (see Fig. 5). PDH complex plays a central role in glucose oxidation and controls the rate-limiting step that forms acetyl-CoA, which is delivered to tricarboxylic acid cycle. PDH is regulated via phosphorylation, and three specific phosphorylation sites (S232, S293, and S300) are known to inhibit its activity (Rardin et al., 2009). Though PDH was identified in both treated and nontreated cells, one of the critical phosphorylation sites, S293 that inhibits the enzyme activity was identified only in control cells (Table 1). This suggests that PDH is more active in the treated cells. Another differentially phosphorylated enzyme that was identified in the treated cells was ACC isoform 1. This enzyme catalyzes the irreversible carboxylation of acetyl-CoA to form malonyl-CoA, a metabolite used for fatty acid synthesis. The identification of two critical phosphorylation sites S77 and S79 (Table 2), known to inhibit ACC activity (Ha et al., 1994), indicates that this enzyme is inactive in TBTO-treated cells. This would allow acetyl-CoA to be delivered to tricarboxylic acid cycle rather than for fatty acid

| Gene symbol and protein name | Accession number | Sequences of the phosphopeptides identified | Phosphorylation sites |
|------------------------------|-----------------|--------------------------------------------|-----------------------|
| Top2a DNA topoisomerase 2-alpha | IPI00122223.1 | R.KPSSSdpSpSDSDFER.A, K.RPSSSdpSpSDSDFER.A, K.RPSSSdpSpSDSDFER.A | $S1469, S1470$ |
| Tpd52l2 tumor protein D54 | IPI00319046.1 | R.NSAPKspSFEDE.R, V.R.NSAPKspSFEDE.R, V | $S189, T177$ |
| Tpm3 tropomyosin 3, gamma U2af2 splicing factor U2AF 65 kDa subunit | IPI00169707.2 | K.QLEDELapTMQK.K | $T46^*$ |
| | IPI00113746.3 | R.GAKEEHGGLIRpSPR.H | $S79$ |
| Vim vimentin | IPI0027299.6 | R.RMFGSGSpTSSR.P, R.RMFGSGTspSSR.P, R.SVSpSSpSYR.R, R.pSVpSpSSpSYR.R | $T20, S8, S10$ |
| Mat3 matrin-3 | IPI00453826.2 | R.SypSPDGDKEPSDKK.S, R.pSPDGDKEPSDKK.S, R.SpSPDGDKEPSDKK.S, R.RDpsSPDGDKEPSDKK.S | $S598, S596, Y597, S188$ |
| | | K.INKpYILMRMK.S, K.TEEGPTLspYGRDGR.S | $Y526, S157, T158^*$ |
| BC005624 uncharacterized protein C9orf78 homolog | IPI00115553.2 | VGDTEKPEPERpSPPRN | $S261$ |
| Trim28 isoform 1 of transcription intermediary factor 1-beta Rps6kb1 isoform alpha I of ribosomal protein S6 kinase beta-1 | IPI00312128.3 | R.SRspGEVEGSGLR.K | $S473$ |
| Huwe1 isoform 2 of E3 ubiquitin-protein ligase HUWE1 | IPI00453603.1 | R.TPVpSpVpKspSGDFWGR.G, R.pTPVpSpVpKspSGDFWGR.G, R.TPVpSpVpKspSGDFWGR.G | $S447, T444, S452$ |
| Setx isoform 2 of probable helicase senataxin | IPI00626271.3 | R.GSGTApSDFENRL.R, R.GSGpTASDFENRL.R, R.CgpTASDFENRL.R | $S1907, T1905, S1903$ |
| Cad-pending protein Stmn1 (stathmin1) 12 kDa protein | IPI00830256.1 | IHRApSdpGLPAEEPK, RApSdqAFELIIpSPR | $S1859, S16$ |
| | IPI00648723.1 | RApSdqAFELIIpSPR | $S25$ |

Note. The asterisk indicates that the phosphoprotein and/or the phosphorylation site was not reported in the public database of Phosphosite (http://www.phosphosite.org). Peptides indicated in the black color were identified in both TBTO-treated and nontreated cells; peptides indicated in light blue were identified only in TBTO-treated cells, whereas those in red were identified only in control cells.
TABLE 2
The Various Identified Phosphoproteins in TBTO-Treated Cells With the Corresponding Phosphopeptides

| Gene symbol and protein name | Accession number | Sequence of phosphopeptides identified | Phosphorylation sites |
|------------------------------|------------------|----------------------------------------|-----------------------|
| Acaca isoform 1 of ACC 1     | IPI00474783.4    | R.SSMpSGHLV.K, QpSGLHLV.K, RpSSMSGHLV.K | S79                   |
| Acta1 actin, alpha skeletal muscle | IPI00110827.1 | R.KDLpYANVMSSGTGMYPIA1DR.M | Y296 |
| Bysl bystin                  | IPI00118762.6    | R.LGPQLQDP.GSDDEDEEWTLEK.A | S77 |
| Casp3 caspase 3              | IPI00308498.1    | K.THGSKpVDSGILYDDLSYK.M | S26 |
| Cd5 T-cell surface glycoprotein CD5 | IPI00120466.1 | R.SSTQPDNSpSDSYDLQAQR.L, R.SSTQPDNSpSDSYDLQAQR.L | S484, S482 |
| D1Pas1 putative ATP-dependent RNA helicase PI10 | IPI00133708.1 | R.EIAPQpIPEFL.S | Y282 |
| Dpysl2 dihydropyrimidinase-related protein 2 | IPI00114375.2 | | |
| Des desmin                   | IPI00130102.4    | R.AGFGTKpSSSMTS.R | S45 |
| Dync1li1 cytoplasmic dynein 1 light intermediate chain 1 | IPI00153421.1 | R.VTSPAPVG.S412, S414 |
| Evl isoform 2 of Ena/VASP-like protein | IPI00109256.4 | R.VGTSPAPVG | S212, T21, S23 |
| Ezr; LOC100044177 ezrin     | IPI00330862.5    | K.KVpSAPVQK.V | S66 |
| Fkbp4 FK506-binding protein 4 | IPI00230139.5 | R.EKKLpYSVTP.S | S518, S517 |
| Gfpt1 isoform 1 of glutamine—fructose-6-phosphate aminotransferase [isomerizing] | IPI00406371.3 | R.VDSpTCLPVEA.K, R.VDSpTCLPVEA.K | T260, T261 |
| Gm10120 similar to Sm D2 isoform 1 | IPI00116535.1 | K.pSMPpTPELQR.A | S9, T12 |
| Gm8394 similar to zeta proteasome chain; PSMA5 isoform 2 | IPI00122562.3 | R.EYNSVpTPELQR.L | S16 |
| Hnrnpa2b1 isoform 3 of heterogeneous nuclear ribonucleoprotein A2/B1 | IPI00405058.6 | R. GS GRGNNFGFGDpAS.R | S212 |
| Hnmpd isoform 1 of heterogeneous nuclear ribonucleoprotein D0 | IPI00330958.2 | K.IDASKNNEEDGHSPR.H, K.IDASKNNEEDGHSPR.H, K.IDASKNNEEDGHSPR.H | S83 |
| Mcm4 DNA replication licensing factor MCM4 | IPI0017016.1 | R.GKVTPpTQpSL.R | T21, S23 |
| Ncl nucleolin | IPI003317794.5 | K.ATTPAKKVVSQpTPpT.K | T69 |
| Nop58 nucleolar protein 58 | IPI00463486.3 | K.IKKEEPpSSEEPCTSTAVpPPEK.K | S509, S521 |
| Nphp3 nephrocystin 3 isoform a | IPI00416030.2 | K.ILAIpYK.K, Y1181 |
| Npm1 nucleophosmin | IPI00127415.1 | K.LGLMpSGKRSAPGGNK.V | S139 |
| Pdcd5; Gm3837 programmed cell death protein 5 | IPI00116120.3 | K.G RpSGLMpSDEDDADY | S119 |
| Prkar1a putative uncharacterized protein | IPI00192973.5 | R.TDpSREDEISPPPNNpFPGD.VK.G | S206 |
| Prkrl1a putative uncharacterized protein | IPI00121460.1 | R.TDREDEISPPPNNpFPGD.VK.G | S212 |
| Pppf3 U4/U6 small nuclear ribonucleoprotein | IPI003317794.5 | K.ATTPAKKVVSQpTPpT.K | T69 |
| Psma3 proteasome subunit alpha type-3 | IPI00127415.1 | | |
| Ppp6 isoform 2 of tyrosine-protein phosphatase nonreceptor type 6 | IPI00127415.1 | | |
| Rally isoform 2 of RNA-binding protein rally | IPI00130417.2 | K.G RpSGLMpSDEDDADY | S119 |
| Slc16a1 monocarboxylate transporter 1 | IPI00137194.1 | | |
synthesis. An earlier study showed that cAMP-dependent protein kinase (PRKAR1A) mediates the phosphorylation of S77, whereas AMP-activated protein kinase (PRKAA1) phosphorylates S79 (Ha et al., 1994). Both kinases were identified in the TBTO-treated cells (Table 2).

Two other biosynthetic pathways affected by the TBTO exposure are hexoamine and mTOR pathways (Fig. 5). Glutamine:fructose-6-phosphate amidotransferase 1 is the enzyme responsible for the catalysis of the first and rate-limiting step reaction in the hexoamine pathway. A previous study showed that cells depleted of glucose resulted in the inhibition of this enzyme’s activity via phosphorylation (Eguchi et al., 2009). This phosphorylation is mediated by AMPK (Eguchi et al., 2009). Ribosomal S6 kinase 1, a downstream effector of mTOR pathway and a target of AMPK, was downregulated in TBTO-treated cells. RPSK1 has been shown to phosphorylate 40 ribosomal S6, which is believed to regulate synthesis of proteins associated with translation machinery by facilitating the translation of the transcripts corresponding to these proteins. These transcripts are characterized by the presence of 5’-terminal pyrimidine tract and are consequently referred to as Top mRNAs (Fingar and Blenis, 2004; Zhou et al., 2010). This downregulation of RPS6K1 in the treated cells could explain the reported inhibition of protein synthesis by TBTO (Osman et al., 2009; Raffray et al., 1992).

Since several studies showed that TBTO severely depleted ATP levels in various biological systems (Raffray et al., 1992; Soracco and Pope, 1983; Veiga et al., 1996), it is not surprising

| Gene symbol and protein name | Accession number | Sequence of phosphopeptides identified | Phosphorylation sites |
|------------------------------|-----------------|----------------------------------------|-----------------------|
| Smc1a structural maintenance of chromosomes protein 1A | IPI00123870.1 | R.MEEEpQSQQRDITLEENQVKK.Y | S358 |
| Tubb4 tubulin beta-4 chain | IPI00109073.5 | K.EVDEQMLSVQpSK.N | S335 |
| Ubx1 UBX domain–containing protein 1 | IPI00123589.1 | R.SpSSpTPDGPVPSpPSQEPpTK.R | S188, S200, T207 |
| Tubb5 tubulin beta-5 chain | IPI00117352.1 | R.ISVVYNeApTGK.G | T55 |
| Dkc1 H/ACA ribonucleoprotein complex subunit 4 | IPI00113635.7 | K.VKVEEMpSE | S508 |
| Nherf sodium-hydrogen exchanger regulatory factor 1 | IPI00109311.3 | R.SASpSTSEELNSQDpSK.R | S286 |
| PC4 activated RNA polymerase II transcriptional coactivator p15 | IPI00225633.3 | ELVSSSSGSDpSEVEK | S19 |
| Pdlim4 PDZ and LIM domain 4 | IPI00882072.1 | RSpSVGSiLeDNR | S119 |
| Isoform 1 of UPF0368 protein Coxr26 homolog | IPI00132352.2 | GADpSGGEKEEGANREGEK | S184 |
| CBX3 chromobox protein homolog 3 | IPI00129468.2 | pSLpSDpSEDDDpSK | S93, S95, S97, S102 |
| Tpd52l2 tumor protein D52-like 2, isoform CRA_a | IPI00753870.1 | NSATFpSFEDR | S180 |
| Zerb1 isoform 2 of zinc finger CCHC-type and RNA-binding motif–containing protein 1 | IPI00798500.1 | SAPYFSDEELpSD | Y208, S216 |
| Sgta isoform 2 of small glutamine-rich tetratricopeptide repeat–containing protein alpha | IPI00399631.1 | SRTpSASHEEQE | S307 |
| Ptnp6 isoform 3 of tyrosine-protein phosphatase nonreceptor type 6 | IPI00225422.1 | TSpSKHKEEYVENVHSK | S557 |
| Whsc2 negative elongation factor A | IPI00221446.1 | KpTLpDEVEpWpIK | T277, T280 |
| Chm choroiderma | IPI00828331.2 | FNIpDLVSKLPpYSR | Y249 |
| Klf20B isoform 2 of kinesin-like protein | IPI00828908.1 | IDELRSldpSPSHIK | S950 |
| Camsp11l isoform 1 of calmodulin-regulated spectrin-associated protein 2 | IPI00831089.3 | LNQpSpPDPNLDTDK | S589, S590 |
| OBOX1 homeobox 1* | IPI00135372.1 | ERTvYpTKEQQGLLQK | Y101, T102 |
| ATP-binding cassette, subfamily A (ABC1), member 5 | IPI00649505.1 | QpTRTLLLKpYLPILK | T14*, Y22 |
| Gpgr5a retinoic acid–induced protein 3 | IPI00321753.7 | AQAAPSpYPpYNpYEGR | S344, Y346, Y349 |
| Dmntl cytosine-specific methyltransferase | IPI00469321.2 | R.AEMADSRSpPSRS.Rp | S125 |
| Astl isoform 2 of astacin-like metalloendopeptidase* | IPI00283246.7 | R.LLSVPpTNNKpFP.G | T69* |
| Fbxw8 isoform 1 of F-box/WD repeat–containing protein | IPI00378206.1 | R.YVAIApTAGDLVpYLLK.A | Y369* |

Note. The asterisk indicates that the phosphoprotein and/or the phosphorylation site identified was not reported in the public database of Phosphoproteinsite (http://www.phosphoproteinsite).

TABLE 2—Continued
| Gene symbol and protein name | Accession number | Sequences of the phosphopeptides identified | Phosphorylation sites |
|-----------------------------|------------------|---------------------------------------------|---------------------|
| Smap small acidic protein   | IPI00127941.1    | R.SApSPDDDLGSSWNWEAADLGNEERQK.F             | S17, S15            |
| Alkbh5 alkylated DNA repair protein alkB homolog 5 | IPI00453512.3    | R.pASAPSPDDDLGSSWNWEAADLGNEERQK.F           | S362                |
| Chd4 chromodomain helicase DNA–binding protein 4 | IPI00396802.1    | R.RGpSFSSENYWR.K                           | S1569               |
| Ddx21 nuclear RNA helicase 2 | IPI00120691.1    | K.EEepSTEIGKEVK.S                         | S13                 |
| Eif2s2 eukaryotic translation initiation factor 2 subunit 2 | IPI00116302.1    | K.EEepSTEIGKEVK.S                         | S105                |
| Gbf1 golgi-specific brefeldin A-resistance factor 1 | IPI00463493.1    | R.RGpSFSSENYWR.K                           | S1298               |
| Grap2 GRB2-related adaptor protein 2 | IPI00131492.1    | R.RGpSFSSENYWR.K                           | S362                |
| Hist1h1c histone H1.2       | IPI00396802.1    | K.EEepSTEIGKEVK.S                         | S13                 |
| Hmgb2 high mobility group protein B2* | IPI00396802.1    | K.EEepSTEIGKEVK.S                         | S13                 |
| Hnrnpu heterogeneous nuclear ribonucleoprotein U | IPI00458583.3    | K.EEepSTEIGKEVK.S                         | S13                 |
| Igf2 cation-independent mannose-6-phosphate receptor | IPI00308971.1    | R.RGpSFSSENYWR.K                           | S1298               |
| Irf2b2 interferon regulatory factor 2 binding protein 2 | IPI00357145.5    | R.RGpSFSSENYWR.K                           | S348, S443          |
| Krt10 keratin complex 1, acidic, gene 10 | IPI00755181.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Larp1 isoform 1 of La-related protein 1 | IPI00929786.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Mapk1 mitogen-activated protein kinase 1 | IPI00119663.3    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Mcm3 DNA replication licensing factor | IPI00108338.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Msn moesin | IPI00308971.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Mtdh protein LYRIC | IPI00453512.3    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Ncbp1 nuclear cap-binding protein subunit 1 | IPI00308971.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Prdx1 peroxiredoxin-1 | IPI00120691.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Rbm39 isoform 1 of RNA-binding protein 39 | IPI00120691.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Rbmx RNA-binding motif protein, X-linked | IPI00120691.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Rcc2 protein RCC2 | IPI00116302.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Rm2 ribonucleoside-diphosphate reductase subunit M2 | IPI00116302.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Rfc1 Rfc1 protein | IPI00116302.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Pspj1 isoform 1 of PC4 and SFRS1-interacting protein | IPI00116302.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Sf5 splicing factor, arginine/serine-rich 5 | IPI00116302.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Stk4 serine/threonine-protein kinase 4 | IPI00116302.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Slik isoform 1 of STE20-like serine/threonine-protein kinase | IPI00116302.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
| Snpb2 isoform 2 of spectrin beta chain, brain 1 | IPI00116302.1    | R.RGpSFSSENYWR.K                           | S17, S29, T31       |
that AMPK, as a sensor of cellular energy status, monitoring the ratio AMP/ATP, especially under stress conditions (Corton et al., 1994), inhibits ATP-consuming anabolic pathways, while activating ATP-generating catabolic pathways in TBTO-treated cells. In relation to this energy homeostasis, it is of interest to note that a recent finding showed TBTO to be a potent inhibitor of fish peroxisome proliferators–activated receptor α (Colliar et al., 2011), transcription factors involved in energy homeostasis. PPARα is a potent activator of gluconeogenic gene expression (Colliar et al., 2011). AMPK was shown to phosphorylate PGC-1α (peroxisome proliferators–activated receptor coactivator), affecting its transcriptional capacity (Jäger et al., 2007). AMPK was shown to phosphorylate PGC-1α both in vitro and in primary muscle cells, affecting positively the expression of mitochondrial genes (Campbell et al., 2002). In contrast, in liver AMPK repressed the induction of PGC-1α by preventing the nuclear import of TORC2, the transcriptional coactivator of CREB (Shaw et al., 2005). It should be noted that PGC-1α is a potent activator of gluconeogenic gene expression in the liver, whereas AMPK suppresses this anabolic pathway (Campbell et al., 2002). These observations underline the possible regulatory role of AMPK on the gene expressions

TABLE 3—Continued

| Gene symbol and protein name | Accession number | Sequences of the phosphopeptides identified | Phosphorylation sites |
|-----------------------------|------------------|--------------------------------------------|----------------------|
| Tmpo thymopoietin isof orm delta | IPI00828461.1 | R.EFpTPVLPgSGASVGR.G | T74, S79 |
| Top2b DNA topoisomerase 2-beta | IPI00135443.2 | R.pSRSGGEVSGDLL.R.K | S1537 |
| Hist1h2hp putative uncharacterized protein | IPI00459318.1 | K.AgGIMNSFVNDIFER.I | T60 |
| Ubal ubiquitin-like modifier-activating enzyme 1 | IPI00123313.1 | K.PGpSNCSPAQASLpSEVSpSVPTNGMAK.N | S21, S31*, S35* |
| Snp2 isoform 2 of spectrin alpha chain, brain | IPI00753793.2 | K.KLDPAQpSASRENLEEQGIAL.R.Q | S1029 |
| Tceal isoform 1 of transcription elongation factor α protein | IPI00224168.1 | KKEPAISSQPnSPEAR | S100 |
| Grap2 similar to adaptor protein | IPI00756731.1 | R.HpTDPVQLQAGGR | T254 |
| Ena-vasodilator stimulated phosphoprotein isoform 4 | IPI00753522.1 | SNpSVKPVSSLLSR | S329 |
| Isoform 2 of DDB1- and CUL4-associated factor 8 | IPI00407739.2 | GGHHpSDEDEEQPR | S100 |
| Hnf4a isoform short of hepatocyte nuclear factor 4-alpha | IPI00230333.2 | pSQVQVSLEDYINDR | S313 |
| Sept9 septin-9 | IPI00648786.1 | LVDSLQRpSPKPSLR | S85 |
| Hsp 90ab1 MCG18238 | IPI0029080.7 | IEDVpGSpDDEDSGKDk | S255 |
| P4kb isoform 2 of phosphatidylinositol 4-kinase beta | IPI00341034.4 | IEDVpGSDEDDpSpGKDk | S261 |
| Snx1 sorting nexin-1 | IPI00622364.4 | R.FpSDFLGLYEk | S188 |
| Aakl isoform 2 of Ap2-associated protein kinase 1 | IPI00356608.4 | RILSDYpTHSAYFGVPASK | T638 |
| Gapdvl 74 kDa protein | IPI00757126.1 | KApsSPEPDSAESALK | S526 |
| Acly ATP-citrate synthase | IPI00762047.1 | SRpSSDIVSVR | S902 |
| Ptrpc putative uncharacterized protein | IPI00876155.1 | NNNpSNVVPYDFNR | S962 |
| Eif4g1 protein | IPI00856453.1 | KAapSLTEDR | S1211 |
| Muc 13 mucin-13 | IPI00115423.1 | K.CQDILQCPtCKPGDL.RL | T409* |
| Cyp4x1 cytochrome P450* | IPI00347382.1 | K.FLQEDNMpTLDEIVK.K | T68* |
| GPR45 probable G-protein–coupled receptor 45* | IPI0011422.1 | R.QQQASDLpSFK.T | S261* |
| Ndufv2 isoform 1 of NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial | IPI00169942.1 | K.A | T11* |

Note. The asterisk indicates that the phosphoproteins and/or the phosphorylation site identified was not reported in the public database of Phosphosite (http://www.phosphosite.org).
mediated by PGC-1α and might also account for the observed effect of TBTO on the PPAR α/β transcription factors (Colliar et al., 2011). Additionally, since AMPKinase has been implicated in the regulation of gene expression by phosphorylating directly a number of transcription factors and coactivators as well as components of the transcriptional machinery (Leff, 2003), this kinase may play a role in the observed effect on gene expression pattern induced by TBTO (Baken et al., 2006, 2007).

Furthermore, we identified differentially phosphorylated proteins that are implicated in cell proliferation and growth. One of these proteins is matrin-3, a multifunctional protein of the nuclear matrix. WB analysis confirmed the downregulation of matrin-3 in TBTO-treated cells (Fig. 4B). WB analyses of immunoprecipitates of matrin-3 prepared from control cell lysates in the presence or absence of EDTA allowed us to identify two degradation products of the protein, which were markedly increased when EDTA was omitted from the lysis buffer (Fig. 4C). This result suggests that the observed down-regulation of matrin-3 might be due to accentuated degradation of the protein triggered by TBTO, probably, by altering calcium homeostasis. That organotin compounds affect Ca²⁺ homeostasis has been documented (Chow et al., 1992; Corsini et al. 1998; Gennari et al., 2000). Furthermore, it has been previously

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**FIG. 3.** (A) Classification of the phosphoproteins identified from thymoma cells according to the biological process the proteins are implicated. (B) Classification of phosphoproteins detected in TBTO-treated cells.

**FIG. 4.** TBTO downregulates p70RS6K1 and matrin-3. Enriched phosphoproteins and total cell extracts obtained from TBTO-treated (lane +TBTO) and control cells (lane −TBTO) were subjected to SDS-PAGE.
shown that matrin-3 mediates neuronal death following NMDA activation of rat neurons and that this protein acts as a substrate of cAMP-dependent protein kinase, whose phosphorylations leads to degradation of matrin-3 (Giordano et al., 2005). A recent study has shown that matrin-3 is a downstream substrate of caspase 3 and caspase 8 and that matrin-3 forms a complex with calmodulin-binding protein in the presence of Ca$^{2+}$ (Valencia et al., 2007). Interestingly, both cAMP-dependent kinase and caspase 3 were identified in TBTO-treated cells (Table 2). Most probably, Ca$^{2+}$ perturbation destabilizes the interaction of matrin-3 with other proteins, like CaM, and this may render matrin-3 susceptible to degradation thus explaining why the band corresponding to matrin-3 was not detectable in the enriched phosphoproteins and was barely visible in the total extracts of TBTO-treated cells (Fig. 4B). Another phosphoprotein used as a marker of cell proliferation and identified in the control cells is ribonucleotide reductase, subunit RRM2. This protein has been shown to be present in late G1/early S phase of the cell cycle, contrary to the other subunit, RRM1, whose activity is stable throughout the cell cycle (Engström et al., 1985; Heidel et al., 2007). For this protein, the phosphorylation site S20 was identified (Table 3). Earlier study showed that RRM2 is phosphorylated at the S20 site by p34Cdc2 kinase (Chan et al., 1999). Using mutagenesis, the Wnt gene assay and RNA interference Tang et al. (2007) provided evidence that RRM2 may act as inhibitor of β-catenin, a downstream effector of Wnt signaling pathway and that phosphorylation at S20 may relieve this inhibition. In other words, in addition to DNA synthesis, RRM2 upon phosphorylation at S20 decreases the threshold of β-catenin effect, hence increasing the latter’s transcriptional effect on target genes. These results suggest that TBTO affects not only cell proliferation by inducing apoptosis as described before (Osman et al., 2009; Raffray et al., 1992) but also could, at lower concentrations, affect the rate of cell cycle. The observed down-regulation of phosphoproteins involved in cell cycle and proliferation, like RPS6K1, might contribute to our understanding of how TBTO suppresses immune cell activation since T-cell activation and proliferation play a crucial role in immune responses.

In conclusion, our results provide evidence that TBTO, a model immunotoxic compound, affects important pathways that are sensitive to energy charge status by modifying the phosphorylation state of key enzymes that catalyze rate-limiting steps of these pathways. Important kinases like cAMP-dependent kinase and AMPK, which are responsible for phosphorylating some of the sites identified in these enzymes, were also identified in the treated cells. Furthermore, proteins implicated in cell proliferation were found down-regulated in TBTO-treated cells, lending support to the notion that TBTO, at higher concentrations, induces cells to apoptosis and at lower concentrations slows down the rate of cell cycle.

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followed by immunoblotting (A) p70RPS6Kinase1 probed with p70S6K1 (Thr421/Ser424)-R antibody; (B) matrin-3 probed with anti-matrin-3 goat polyclonal antibody. The loading control in the total extracts was probed with rabbit polyclonal antibody against actin; (C) WB analysis of matrin-3 immunoprecipitated from control cell lysates extracted in lysis buffer with EDTA (lane +EDTA) or without EDTA (lane −EDTA). The products may also result from aspecific interactions of proteins or fragments of proteins with the antibodies. Details of sample preparations and Western blotting procedures are described in “Materials and Methods.”
The observed phosphorylation of tubulin proteins (this study) and the identification of collapsin response mediator protein-2 in the treated cells, a protein whose phosphorylated form affects negatively the normal dynamics and the proper functioning of microtubules (Yoshimura et al., 2005), are of interest and worth further investigation as this modification may be responsible for triggering the previously reported disrupting effects of organotin compounds on mitotic spindle in Chinese hamster V79 cells as well as in vitro tubulin polymerization inhibition, especially because the oxidation of the –SH group of the tubulin proteins was ruled out before (Jensen et al., 1991).

SUPPLEMENTARY DATA

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REFERENCES

Aldridge, W. N. (1958). The biochemistry of organotin compounds. Biochem. J. 69, 367–376.

Alexandra, N. S., Meijne, M. L., van der Pol, A. J., and de Jong, L. (1990). The nuclear matrix from cells of different origin. Evidence for a common set of matrix proteins. J. Biol. Chem. 265, 5460–5465.

Alouch, A. O., Odman-Ghazi, S. O., and Whalen, M. M. (2006). Alteration of microtubule (Yoshimura et al., 2005) negatively the normal dynamics and the proper functioning of the treated cells, a protein whose phosphorylated form affects negatively the normal dynamics and the proper functioning of microtubules (Yoshimura et al., 2005), are of interest and worth further investigation as this modification may be responsible for triggering the previously reported disrupting effects of organotin compounds on mitotic spindle in Chinese hamster V79 cells as well as in vitro tubulin polymerization inhibition, especially because the oxidation of the –SH group of the tubulin proteins was ruled out before (Jensen et al., 1991).

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Aldridge, W. N. (1958). The biochemistry of organotin compounds. Biochem. J. 69, 367–376.

Alexandra, N. S., Meijne, M. L., van der Pol, A. J., and de Jong, L. (1990). The nuclear matrix from cells of different origin. Evidence for a common set of matrix proteins. J. Biol. Chem. 265, 5460–5465.

Alouch, A. O., Odman-Ghazi, S. O., and Whalen, M. M. (2006). Alteration of an essential NK cell signaling pathway by low doses of tributyltin in human natural killer cells. Toxicology 224, 229–237.

Baken, K. A., Arkusz, J., Pennings, J. L. A., Vanderbriel, R. J., and van Loveren, H. (2006). Gene expression profiling of bis(tri-n-butyl)tinoxide (TBTO) studied by toxicogenomics. Toxicology 237, 35–48.

Baken, K. A., Pennings, J. L. A., de Vries, T. M., van Steeg, H., and van Loveren, H. (2006). Gene expression profiling of bis(tri-n-butyl)tinoxide (TBTO)-induced immunotoxicity in mice and rats. J. Immunotoxicol. 3, 227–244.

Bernet, G., Fountoulakis, M., and Lubec, G. (2002). Manifold decreased protein levels of matrix 3, reduced motor protein HMP and hlfark in fetal Down’s syndrome brain. Proteomics 2, 1752–1757.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein dye binding. Anal. Biochem. 72, 245.

Campbell, F. M., Kozak, R., Wagner, A., Altarajjos, J. Y., Dyck, J. R. B., Belde, D. D., Severson, D. L., Kelly, D. P., and Lopaschuk, G. D. (2002). A role for peroxisome proliferator-activated receptor α (PPARα) in the control of cardiac malonyl-CoA levels. J. Biol. Chem. 277, 4098–4103.

Chan, A. K., Persad, S., Litchfield, D. W., and Wright, J. A. (1999). Ribonucleotide reductase R2 protein is phosphorylated at S-20 by P34cdc2 kinase. Biochim. Biophys. Acta 1448, 363–371.

Chow, S. C., Kass, G. E. N., McCabe, M. J., and Orenius, S. (1992). Tributyltin increases cytosolic free Ca^2+ concentration in thymocytes by mobilizing intracellular Ca^2+ stores, activating a Ca^2+ entry pathway, and inhibiting Ca^2+ efflux. Arch. Biochem. Biophys. 298, 143–149.

Colliar, L., Sturr, A., and Lever, M. J. (2011). Tributyltin is a potent inhibitor of piscine peroxisome proliferators-activated receptor α and β. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 168–173.

Corsi, E., Viviani, B., Marinovich, M., and Galli, C. L. (1998). Primary role of mitochondria and calcium ions in the induction of reactive oxygen species by external stimuli such as triorganotins. Toxicol. In Vitro 12, 551–556.

Corton, J. M., Gillespie, J. G., and Hardie, D. G. (1994). Role of the AMP-activated protein kinase in the cellular stress response. Curr. Biol. 4, 315–324.

Costa, L. G. (1985). Inhibition of γ-[3H]aminobutyric acid uptake by organotin compounds in vitro. Toxicol. Appl. Pharmacol. 79, 471–479.

Doctor, S. V., Costa, L. G., Kendall, D. A., and Murphy, S. D. (1982). Trimethyltin inhibits uptake of neurotransmitters into mouse forebrain synaptosomes. Toxicology 25, 213–221.

Eguchi, S., Oshiro, N., Miyamoto, T., Yoshino, K.-I., Okamoto, S., Ono, T., Kikkawa, U., and Yonezawa, K. (2009). AMP-activated protein kinase phosphorylates glutamine: Fructose-6-phosphate amidotransferase 1 at Ser243 to modulate its enzymatic activity. Genes Cells 14, 179–189.

El-Darahali, A., Fawcett, H., Mader, J. S., Conrad, D. M., and Hoskin, D. W. (2005). Adenosine-induced apoptosis in EL-4 thymoma cells is caspase-independent and mediated through non-classical adenosine receptor. Exp. Mol. Pathol. 79, 249–258.

Elsabbagh, H. S., Moussa, S. Z., and El-Tawil, O. S. (2002). Neurologic sequelae of tributyltin intoxication in rats. Pharmacol. Res. 45, 201–206.

Engström, Y., Eriksson, S., Jilvedik, I., Skog, S., Thelander, L., and Tribukait, B. (1985). Cell cycle-dependent expression of mammalian ribonucleotide reductase. J. Biol. Chem. 260, 9114–9116.

Erazo, A., Yee, M. B., Banfield, B. W., and Kinchington, P. R. (2011). The alphaherpesvirus US3/ORB6 protein kinases directly phosphorylate the nuclear matrix protein matrix 3. J. Virol. 85, 568–581.

Evans, A. G., Belov, G. A., Kalkum, M., Chichova, N. V., Bogdanov, A. A., Agol, Y. I., and Vartapetian, A. B. (2000). Prothymosin-α fragmentation in apoptosis. FEMS Lett. 467, 150–154.

Fingar, D. C., and Blenis, J. (2004). Target of rapamycin (TOR): An integrator of nutrient and growth factor signals and coordinator of cell growth and cell progression. Oncogene 23, 3151–3171.

Gennari, A., Viviani, B., Galli, C. L., Marinovich, M., Pieters, R. H. H., and Corsi, E. (2000). Organotins induce apoptosis by disturbance of [Ca^{2+}], and mitochondrial activity, causing oxidative stress and activation of caspases in rat thymocytes. Toxicol. Appl. Pharmacol. 169, 185–190.

Giordano, G., Pérez, A. M. S., Montolui, C., Berezney, R., Malavantham, K., Costa, L. G., Calvete, C. J., and Felipo, V. (2005). Activation of NMDA receptors induces protein kinase A-mediated phosphorylation and degradation of matrin 3. Blocking these effects prevents NMDA-induced neuronal death. J. Neurochem. 94, 808–818.

Ha, J., Daniel, S., Bryoles, S. S., and Kim, K.-H. (1994). Critical phosphorylation sites for acetyl-CoA carboxylase activity. J. Biol. Chem. 269, 22162–22168.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev. 18, 1926–1945.

Heidell, J. D., Liu, J. Y.-C., Yen, Y., Zhou, B., and Heale, B. S. E. (2007). Potent siRNA inhibitors of ribonucleotide reductase subunit RRM2 reduce cell proliferation in vitro and in vivo. Clin. Cancer Res. 13, 2207–2215.

Hibino, Y. T., Usui, Y., Morita, N., Hirose, M., Okazaki, M. S., and Higara, K. (2006). Molecular properties and intracellular localization of rat liver nuclear scaffold protein P130. Biochim. Biophys. Acta 1759, 195–207.
Iguchi, T., Katsu, Y., Horiguchi, T., Watanabe, H., Blumberg, B., and Ohta, Y. (2007). Endocrine disrupting organotin compounds are potent inducers of imposex in gastropods and adiopogenesis in vertebrates. Mol. Cell. Toxicol. 3, 1–10.

Jäger, S., Handschin, C., Pierre, J. S., and Speigelman, B. M. (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. Proc. Natl. Acad. Sci. U.S.A. 104, 12017–12022.

Jensen, K. G., Oufelt, A., Wallin, M., and Andresen, O. (1991). Effects of organotin compounds on mitosis, spindle structure, toxicity and in vitro microtubule assembly. Mutagenesis 6, 409–416.

Kannan, K., Corsolini, S., Focardi, S., Tanabe, S., and Tatsukawa, R. (1996). Nuclear matrix proteins: Identification of the major fraction in rat liver. Neurochem. Res. 21, 135–139.

Katika, M. R., Hendriksen, P. J. M., van Loveren, H., and Peijnenburg, A. J. M. (2009). Proteomic analysis of mouse kidney and lung tissues from tri-n-butyltin oxide treated mice. Toxicon 53, 157–164.

Klein, D., Soracco, R. J., and Pope, D. H. (1983). Bacteriostatic and bactericidal modes of action of bis(tributyltin)oxide on Legionella pneumophila. Appl. Environ. Microbiol. 45, 48–57.

Tan, L. P., Ng, M. L., and Kumar Das, V. G. (1978). The effect of trialkyltin compounds on tubulin polymerization. Neurochemistry 31, 1035–1041.

Tanaka, K., Koga, Y., Taniguchi, K., and Nomoto, K. (1987). T-cell recruitment from the thymus to the spleen in tumor bearing mice: Phenotypical alteration and recruitment of thymocytes in a tumor bearing state. Cancer Res. 47, 2136–2141.

Tang, L. Y., Deng, N., Wang, L. S., Dai, J., Wang, Z. L., Jiang, X. S., Li, S. J., Li, L., Sheng, Q. H., Wu, D. Q., et al. (2007). Quantitative phosphoproteome profiling of Wnt3a-mediated signaling network indicating the involvement of ribonucleoside diphosphate reductase M2 subunit phosphorylation at residue serine 20 in canonical Wnt signal transduction. Mol. Cell Proteomics 6, 1952–1967.

Tarakhovsky, A., Kanner, S. B., Hombach, J., Ledbetter, J. A., Muller, W., Killen, N., and Rajewsky, K. A. (1995). A role for CD5 in TCR-mediated signal transduction and thymocyte selection. Science 269, 535–537.

Valencia, C., Ju, W., and Liu, R. (2007). Matrin 3 is a Ca2+/calmodulin-binding protein cleaved by caspases. Biochem. Biophys. Res. Commun. 361, 281–286.

Van Loveren, H., Krajinovic, E. I., Rombout, P. J., Blommaert, E. A., and Vos, J. G. (1990). Effects of ozone, hexachlorobenzene, and tributyltin oxide on natural killer activity in the rat lung. Toxicol. Appl. Pharmacol. 102, 21–33.

Veiga, A., Pinto, A. F., and Loureiro-Dias, M. C. (1996). Tributyltin oxide affects energy production in the yeast Rhodotorula fusiformis, a utilizer of phenolic compounds. Can. J. Microbiol. 43, 683–687.

Vos, I. G., De Klerk, A., Krajinovic, E. I., van Loveren, H., and Rozing, J. (1990). Immunotoxicity of bistri(tributyltin)oxide in the rat: Effects on thymus dependent immunity and on non-specific resistance following long term exposure in young versus aged rats. Toxicol. Appl. Pharmacol. 105, 144–155.

Warren, D. T., Tasic, T., Mellad, J. A., Searles, R., Zhang, Q., and Shanahan, C. M. (2009). Novel nuclear nesprin-2 variants tether active extracellular signal-regulated MAPK1 and MAPK2 at promyelocytic leukemia protein nuclear bodies and act to regulate smooth muscle cell proliferation. J. Biol. Chem. 285, 1311–1320.

WHO. (1990). Tributyltin compounds. In Environmental Health Criteria 116, p. 70. WHO, Geneva, Switzerland.

Yates, J. R., Eng, J. K., McCormack, A. L., and Schultz, D. (1995). Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. Anal. Chem. 67, 1426–1436.

Yoshimura, T., Kawano, Y., Arimura, N., Kawabata, S., Kikuchi, A., and Kaibuchi, K. (2005). GSK-3β regulates phosphorylation of CRMP-2 and neuronal polarity. Cell 120, 137–149.

Zhou, X., Lin, D. S., Zheng, F., Sutton, M. A., and Wang, H. (2010). Intracellular calcium and calmodulin link brain-derived neurotrophic factor to p70S6 kinase phosphorylation and dendritic protein synthesis. J. Neurosci. Res. 88, 1420–1432.