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The T-cell protein tyrosine phosphatase is phosphorylated on Ser-304 by cyclin-dependent protein kinases in mitosis

Patricia BUKCZYNSKA*[1], Manuela KLINGLER-HOFFMANN*[1], Kenneth I. MITCHELHILL†[2], Mark H. C. LAM*[3], Melissa CICCOMANCINI*[4], Nicholas K. TONKS‡, Boris SARCEVIC§, Bruce E. KEMP† and Tony TIGANIS*[5]

*Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Victoria 3800, Australia, †St. Vincent's Institute of Medical Research, Melbourne, Victoria 3065, Australia, ‡Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, U.S.A., and §Cancer Research Program, Garvan Institute of Medical Research, Darlinghurst, New South Wales 2010, Australia

Two alternatively spliced forms of the human protein tyrosine phosphatase TCPTP (T-cell protein tyrosine phosphatase) exist: a 48 kDa form that is targeted to the endoplasmic reticulum (TC48) and a shorter 45 kDa form that is targeted to the nucleus (TC45). In this study we have identified Ser-304 (Phe^{301}-Asp-His-Ser^{304}-Pro-Asn-Lys^{307}) as a major TCPTP phosphorylation site and demonstrate that TC45, but not TC48, is phosphorylated on this site *in vivo*. Phosphorylation of TC45 on Ser-304 was cell cycle-dependent, and increased as cells progressed from G_2 into mitosis, but subsided upon mitotic exit. Ser-304 phosphorylation was increased when cells were arrested in mitosis by microtubule poisons such as nocodazole, but remained unaltered when cells were arrested at the G_2/M checkpoint by adriamycin. Phosphorylation of Ser-304 did not alter significantly the phosphatase activity or the protein stability of TC45, and had no apparent effect on TC45 localization. Ser-304 phosphorylation was ablated when cells were treated with the CDK (cyclin-dependent protein kinase) inhibitors roscovitine or SU9516, but remained unaltered when ERK1/2 activation was inhibited with the MEK (mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase) inhibitor PD98059. In addition, recombinant CDKs, but not the Polo-like kinase Plk1, phosphorylated Ser-304 *in vitro*. Our studies identify Ser-304 as a major phosphorylation site in human TCPTP, and the TC45 variant as a novel mitotic CDK substrate.

Key words: alternative splicing and phosphorylation, cell cycle, cyclin-dependent protein kinase (CDK), mitosis, protein tyrosine phosphatase, TC45.

INTRODUCTION

PTPs (protein tyrosine phosphatases) catalyse the dephosphorylation of tyrosine-phosphorylated proteins and regulate signalling events that control fundamental cellular processes, such as growth, proliferation, differentiation, migration and survival [1]. Although the human genome encodes approximately 100 PTP genes [1,2], many of these genes can be alternatively spliced to generate structurally distinct enzymes with different subcellular localizations [1]. It is becoming increasingly apparent that, for many signalling proteins, alternatively spliced gene products can have unique biological properties [3]. Hence the number of functionally distinct PTPs may be significantly greater than the number of PTP genes identified in the human genome.

Human TCPTP (T-cell PTP) is an intracellular non-receptor tyrosine-specific phosphatase that is expressed ubiquitously at all stages of mammalian development [4]. TCPTP was the first PTP to be cloned, and one of the first PTP transcripts shown to be alternatively spliced [4–7]. TCPTP mRNA can be alternatively spliced to generate two TCPTP variants: a 48 kDa protein (TC48) that is targeted to the endoplasmic reticulum by a hydrophobic C-terminus, and a shorter 45 kDa form (TC45) that lacks the hydrophobic C-terminus and is targeted to the nucleus by a bipartite nuclear localization sequence [4,7–11]. Despite TC45 having an apparent exclusively nuclear localization in resting cells, TC45 can shuttle between the nuclear and cytoplasmic environments [12] and gain access to cytoplasmic substrates, such as the EGF (epidermal growth factor) receptor [13,14], the insulin receptor [15], the adaptor protein p52^{Shc} [14] and JAKs (Janus protein tyrosine kinases) [16], thereby regulating mitogen/growth factor- and cytokine-induced signalling [13,14,16,17]. To date, STATs (signal transducer and activators of transcription) such as STAT1 are the only known nuclear substrates for TC45 [18,19]. TCPTP may also regulate the progression of cells through the G_1 phase of the cell cycle [20,21], but the molecular mechanism by which this occurs is not known.

Despite TC48 and TC45 having identical catalytic domains and recognizing common substrates, such as the EGF receptor [14] and the insulin receptor [15], several lines of evidence indicate that the two phosphatases might also have distinct substrates and divergent cellular roles. Utilizing substrate-trapping mutants, we have shown previously that TC45, but not TC48, recognizes the tyrosine-phosphorylated adaptor protein p52^{Shc} as well as two other unidentified substrates in response to EGF stimulation [14]. Moreover, even though both phosphatases can dephosphorylate the EGF receptor in a cellular context, only TC45 suppresses EGF

Abbreviations used: CDK, cyclin-dependent protein kinase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; HEK, human embryonic kidney; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PTP, protein tyrosine phosphatase; RCML, reduced carboxyamidomethylated and maleylated lysozyme; STAT, signal transducer and activator of transcription; TCPTP, T-cell protein tyrosine phosphatase; TC45 and TC48, TCPTP variants of 45 and 48 kDa respectively, formed by alternative splicing; TFA, trifluoroacetic acid.

[1] These authors contributed equally to this work.
[2] Present address: Peter MacCallum Cancer Institute, East Melbourne, Victoria 3002, Australia.
[3] Present address: The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia.
[4] Present address: St. Vincent's Institute of Medical Research, Melbourne, Victoria 3065, Australia.
[5] To whom correspondence should be addressed (e-mail Tony.Tiganis@med.monash.edu.au).

receptor-mediated protein kinase B/Akt signalling [13], whereas others have reported that an overexpressed substrate-trapping mutant of TC48, but not TC45, suppresses tumour cell adhesion and anchorage-independent growth [22]. Taken together, such observations suggest that TC45 and TC48 can serve as functionally distinct PTPs *in vivo*.

In the present paper, we demonstrate for the first time that endogenous TC45, but not the endoplasmic reticulum-localized TC48, is phosphorylated on Ser-304. We report that Ser-304 phosphorylation occurs primarily during mitosis, and may be mediated by CDKs (cyclin-dependent protein kinases). Our studies suggest that the alternatively spliced forms of TCPTP may be phosphorylated differentially by CDKs in mitosis, and may thus have distinct roles in cellular division.

MATERIALS AND METHODS

Materials

PD98059, calyculin A, nocodazole, thymidine, roscovitine and Pansorbin were purchased from Calbiochem Oncogene Research Products (Cambridge, MA, U.S.A.); anisomycin and propidium iodide were from Sigma (St, Louis, MO, U.S.A.); EGF and monoclonal anti-(cyclin B1) antibody were from BD Biosciences (Bedford, MA, U.S.A.); monoclonal pan-actin antibody was from NeoMarkers (Fremont, CA, U.S.A.); a polyclonal antibody against phospho-ERK (extracellular-signal-regulated kinase) 1/2 was from Promega (Madison, WI, U.S.A.); [^{32}P]P_i was from Amersham Biosciences (Little Chalfont, Bucks., U.K.); doxorubicin hydrochloride (adriamycin) and Trans^{35}S-Label™ were from ICN Biomedicals (Irvive, CA, U.S.A.). We are grateful to M. Weber (University of Virginia, Charlottesville, VA, U.S.A.) for the monoclonal anti-ERK2 antibody 1B3B9, and to E. A. Nigg (Max Planck Institute of Biochemistry, Martinsried, Germany) for the purified recombinant human Polo-like kinase Plk1. The monoclonal anti-TCPTP antibody CF4 has been described previously [14].

Plasmid constructs

TC45-pMT2, TC45-pCG, pET-GST and pET-TC45 have been described previously [12,23]. Two rounds of PCR were undertaken to generate the Ser-304 to Ala or Glu phosphorylation site mutants using recombinant *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.). In the first round, TC45-pBluescript II KS [10] was used as a template to generate two overlapping PCR products encompassing the 5′ and 3′ ends of the TC45 cDNA. The PCR product encompassing the 3′ end of the TC45 cDNA incorporated the mutation for converting Ser-304 into either Ala or Glu; 5′-end PCR products were generated using 5′-GGCTCCCCTGCAGATGCCCACCACCATCGAGCGGGAG-3′ and 5′-ATGATCAAAGGCAGGAGATAA-3′, and 3′-end PCR products were generated with 5′-CGCCGCCTCGAGTTAGGTGTCTGTCAATCTTGGCCT-3′ and 5′-CCTGCCTTTGATCATGCTCCAAACAAAATAATGAC-3′ (for mutating Ser-304 to Ala) or 5′-CCTGCCTTTGATCATGAGCCAAACAAAATAATGAC-3′ (for mutating Ser-304 to Glu). For the second round of PCR, a mixture (approx. 1:1 molar ratio) of the 5′- and 3′-end first-round PCR products was utilized as template to generate the full-length TC45 mutant cDNAs. The oligonucleotides incorporated a *Pst*I site immediately 5′ to the initiating codon (5′-GGCTCCCCTGCAGATGCCCACCACCATCGAGCGGGAG-3′) and a *Xho*I site immediately 3′ to the terminating codon (5′-CGCCGCCTCGAGTTAGGTGTCTGTCAATCTTGGCCT-3′). *Pst*I/*Xho*1-digested second-round PCR products were cloned into the same site of the mammalian expression vector pMT2.

The TC45-S304A-pCG and TC45-S304E-pCG constructs were generated by PCR using Platinum *Pfx* DNA polymerase (Invitrogen) and the TC45-S304A-pMT2 and TC45-S304E-pMT2 constructs as templates respectively. The 5′ oligonucleotide used was 5′-GGCTCCCACTAGTATGCCCACCACCATCGAGCGGGAG-3′ and the 3′ oligonucleotide was 5′-CCCAGTCATGGATCCTTAGGTGTCTGTCAATCTTGGCCT-3′. The oligonucleotides incorporated an *Spe*I site immediately 5′ to the initiating codons and a *Bam*HI site immediately 3′ to the termination codons. The *Spe*I/*Bam*HI-digested PCR product was cloned into the *Xba*I/*Bam*HI site of the mammalian expression vector pCG.

pET-TC45(1–349) and pET-TC45(1–349)S304E bacterial expression constructs were generated by PCR using Platinum *Pfx* DNA polymerase and the TC45-pMT2 and TC45-S304E-pMT2 constructs as templates respectively. The 5′ oligonucleotide used was 5′-GGCTCCCGGATCCATGCCCACCACCATCGAGCGGGAG-3′, and incorporated a *Bam*HI site immediately 5′ of the initiating methionine; the 3′ oligonucleotide was 5′-AGCTTACTTAAGAATATCTCGTGAGAGTGACAAGAGGAG-3′, and incorporated a TAA stop codon followed by an *Eco*RI site immediately after the codon for residue 349. The PCR products were digested with *Bam*HI/*Eco*RI and cloned into the same sites of pET-GST to generate pET-GST-TC45(1–349) and pET-GST-TC45(1–349)S304E. The *Nco*I/*Eco*RI fragment from pET-TC45 was then replaced with the same fragment from either pET-GST-TC45(1–349) or pET-GST-TC45(1–349)S304E to generate pET-TC45(1–349) and pET-TC45(1–349)S304E respectively.

The structures of all constructs generated were confirmed by restriction endonuclease analysis, and the fidelity of the cloned cDNAs was confirmed by sequencing.

Cell culture, transfections, cell-cycle synchronization and flow cytometry

HEK (human embryonic kidney) 293, HeLa cervical adenocarcinoma, U2OS osteosarcoma, and monkey CV1 and COS1 cells were cultured at 37 °C and 5 % CO_2 in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) FBS (fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin. Where indicated, cells were serum-starved for 20–24 h in DMEM containing 0.1 % (v/v) FBS plus antibiotics.

COS1 cells were transfected by the calcium phosphate precipitation method as described previously [14], whereas HeLa cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Where indicated, HEK293, CV1 and U2OS cells were electroporated at 975 mM and 200 V as described previously [13]. For cell cycle synchronization, cells were arrested in S-phase in the presence of 2 mM thymidine for 20 h and then washed with PBS and released in DMEM plus 10 % (v/v) FBS. To arrest cells in mitosis, cells were treated for 20 h with 100 ng/ml nocodazole. FACS was performed using propidium iodide-stained cells and a FACSCalibur flow cytometer (Becton Dickinson).

Metabolic labelling and TCPTP immunoprecipitation

COS1 cells were electroporated as described previously [13] with 20 μg of TC45-pMT2. At 36 h post-transfection, the cells were washed twice with phosphate-free DMEM and then incubated in phosphate-free DMEM for 60 min. Cells were labelled with 1.5 mCi of [^{32}P]P_i per 10 cm dish for 5 h at 37 °C. Labelled cells were lysed in IP lysis buffer [50 mM Tris, pH 7.5, 1 % (w/v)

Nonidet P-40, 150 mM NaCl, 50 mM NaF, leupeptin (5 μg/ml), pepstatin (1 μg/ml), 1 mM benzamidine and 2 mM PMSF] plus 1 μM okadaic acid, and precleared with 0.1 ml of Pansorbin for 30 min at 4 °C. Precleared lysates were centrifuged (12 000 ***g*** for 10 min at 4 °C), and TCPTP was immunoprecipitated from the supernatant with the monoclonal anti-TCPTP antibody CF4 as described previously [14].

Reduction, alkylation, tryptic digestion, and phosphopeptide extraction and purification

The ^{32}P-labelled immunoprecipitated TCPTP was resuspended in 3 × Laemmli sample buffer plus 10 mM DTT (dithiothreitol) and boiled for 5 min before being cooled to room temperature; acrylamide was then added to a final concentration of 1 % (w/v). Alkylation of the denatured and reduced TCPTP was allowed to proceed for 30 min at room temperature and the immunoprecipitates were resolved by SDS/PAGE. After staining with Coomassie Blue, the gel slice containing the immunoprecipitated TCPTP was destained, dried and then rehydrated in the presence of trypsin. Digestions were allowed to proceed overnight at 37 °C, and tryptic peptides were eluted with three sequential 30 min washes with 2 % (v/v) TFA (trifluoroacetic acid), 0.1 % TFA with 30 % (v/v) acetonitrile, and 0.1 % TFA with 60 % (v/v) acetonitrile, as described previously [24,25]. The combined extracts were dried and desalted, and the phosphopeptides were either analysed by phosphopeptide mapping or separated by reverse-phase C18 chromatography (300 Å, 5 μm, 1 mm × 250 mm) using the Amersham Biosciences SMART system (60 min; linear 0–80 % acetonitrile gradient in 0.1 % TFA at 50 μl/min) for MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS analysis as described previously [24–26]. Fractions containing ^{32}P-labelled phosphopeptides were identified by Cerenkov counting.

[^{32}P]Phosphopeptide mapping

Phosphopeptides were separated in two dimensions on thin-layer cellulose plates by high-voltage electrophoresis at pH 1.9 [pH 1.9 buffer: 50 ml of formic acid (88 %, w/v), 156 ml of glacial acetic acid, and deionized water to 2 litres] in the first dimension and ascending phosphochromatography in the second dimension as described previously [24,25]. [^{32}P]Phosphopeptides were visualized on a PhosphorImager using Image-Quant software (Molecular Dynamics).

Phosphate-release sequencing

Reverse-phase chromatography fractions containing [^{32}P]phosphopeptides were subjected to [^{32}P]phosphopeptide mapping on thin-layer cellulose plates [26]. [^{32}P]Phosphopeptides were then recovered from cellulose in pH 1.9 buffer and attached covalently to a Sequelon™-AA membrane according to the manufacturer's instructions (Millipore, Milford, MA, U.S.A.). Covalently attached [^{32}P]phosphopeptides were sequenced on a Hewlett Packard G100A protein sequencer as described previously [24], with extracted cycles diverted to a fraction collector and fractions spotted on to cellulose plates and quantified on a PhosphorImager using Image-Quant software.

Phosphoamino acid analysis

[^{32}P]Phosphopeptides separated by phosphopeptide mapping were recovered from cellulose in pH 1.9 buffer and hydrolysed in 5.7 M HCl at 110 °C for 60 min. ^{32}P-labelled phosphoamino acids were dried and resuspended in pH 1.9 buffer, and then separated on thin-layer cellulose plates by high-voltage electrophoresis at pH 3.5 with internal phosphoamino acid standards as described previously [26].

MALDI-TOF MS

Tryptic peptides were spotted on to the sample stage with α-cyano-4-hydroxycinnamic acid, and masses were analysed using a linear Voyager DE (PerSeptive Biosystems) MALDI-TOF instrument running in delayed extraction mode.

SDS/PAGE and immunoblotting

Unless otherwise indicated, proteins were resolved on 7.3 cm long SDS/PAGE (10 % polyacrylamide) gels using the Laemmli discontinuous buffer system [27] and the Bio-Rad (Hercules, CA, U.S.A.) Mini-PROTEAN® II electrophoresis unit according to the manufacturer's instructions. For resolving the dephosphorylated and Ser-304 phosphorylated forms of TC45, proteins were resolved on 16 cm long SDS/PAGE (10 %) gels using the Bio-Rad Protean II electrophoresis unit; proteins were electrophoresed until the 50 kDa protein standard (Bio-Rad Precision Protein Standards, prestained) was approx. 2 cm from the bottom of the gel. Resolved proteins were transferred on to an Immobilon™-P transfer membrane (Millipore) using a Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer apparatus according to the manufacturer's instructions, and immunoblotted with the indicated antibodies as described previously [14]. All blots were developed using enhanced chemiluminescence (Amersham Life Sciences, Cleveland, OH, U.S.A.).

Microscopy

For immunofluorescence studies, cells on glass coverslips were fixed with 3.2 % (v/v) paraformaldehyde in PBS and processed as described previously [14] using the monoclonal anti-TCPTP antibody CF4, either on its own or in combination with a polyclonal antibody specific for the protein phosphorylated on Ser-304 (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/380/bj3800939add.htm). Alternatively, cells were stained with polyclonal anti-TCPTP 159 antibodies in combination with the monoclonal anti-(cyclin B1) antibody. Alexa Fluor 488 or 568 goat anti-mouse or anti-rabbit IgGs were used as secondary antibodies (Molecular Probes, Eugene, OR, U.S.A.). For staining of DNA, cells were incubated with 5 μg/ml RNAase (Roche Diagnostics, Indianapolis, IN, U.S.A.) in PBS for 30 min, incubated with either propidium iodide or TOTO-3 (Molecular Probes) in PBS containing RNAase for 15 min, and then washed extensively with PBS. Where indicated, nuclei were visualized with Hoechst stain. Coverslips were mounted on to glass slides in DAKO® fluorescent mounting medium (DAKO Corp., Carpinteria, CA, U.S.A.), and immunofluorescence was visualized on an Olympus BX60 microscope or a Bio-Rad MRC 1024 confocal microscope.

Protein kinase and phosphatase assays

CDK assays

For assessing CDK-mediated phosphorylation of Ser-304 *in vitro*, recombinant TCPTP-(1–349) and the corresponding Ser-304 phosphorylation site mutant [TCPTP-(1–349)S304E] were utilized rather than full-length TCPTP because of ease of expression and purification. These proteins were expressed in bacteria and purified by ion-exchange chromatography as described previously [23]. The CDK/cyclin proteins were prepared

and purified as described previously [28]. Phosphorylations were undertaken in CDK assay buffer (20 mM Hepes, pH 7.0, 1 mM DTT, 10 mM $MgCl_2$, 10 mM NaF, 1 mM sodium vanadate and 10 mM β-glycerophosphate) in the presence of 100 μM ATP (plus 5 μCi of [γ-^{32}P]ATP) with or without 10 μg of TCPTP-(1–349) or TCPTP-(1–349)S304E, in the presence or absence of 15 units of the indicated CDK/cyclin proteins, in a total volume of 30 μl for 30 min at 37 °C {1 unit is defined as 1 pmol of phosphate transferred/min per μg of glutathione S-transferase–pRb-(773–928) substrate at 37 °C [28]}. Reactions were terminated by the addition of 15 μl of 3 × Laemmli sample buffer, boiled for 5 min and then resolved by SDS/PAGE and analysed by Coomassie Blue staining and autoradiography.

Polo-like kinase Plk1 assays

Aliquots of 5 μg of dephosphorylated α-casein (Sigma) or 7 μg of TC45-(1–349) or TC45-(1–349)S304E were incubated with 0.75 μg of purified recombinant Plk1 in kinase buffer (50 mM Tris, pH 7.7, 10 mM $MgCl_2$, 2 mM EGTA, 5 mM DTT, 0.5 mM sodium vanadate, 5 mM NaF and 0.1 μM okadaic acid) in the presence of 25 μM ATP (plus 10 μM [γ-^{32}P]ATP) with or without 50 μM roscovitine in a total volume of 40 μl for 30 min at 30 °C. Reactions were terminated by the addition of 20 μl of hot 3 × Laemmli sample buffer, boiled for 5 min and then resolved by SDS/PAGE and analysed by Coomassie Blue staining and autoradiography.

PTP assays

HEK293 cells were electroporated as described previously [15] with 5 μg of TC45-R222M-pCG, TC45-pCG or TC45-S304A-pCG, and at 24 h post-transfection cells were treated with 100 ng/ml nocodazole for an additional 20 h. Cells were then homogenized (Dounce homogenizer; 20 strokes) in ice-cold hypotonic lysis buffer (20 mM Tris, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM benzamidine, 2 mM PMSF, 1 μM okadaic acid and 50 nM calyculin A) and clarified by centrifugation (100000 ***g***, 20 min, 4 °C). DTT was added to the supernatants to a final concentration of 1 mM before PTP activity was assayed. RCML (reduced carboxyamidomethylated and maleylated lysozyme) or MBP (myelin basic protein) was phosphorylated with the baculovirally expressed protein tyrosine kinase domain of the human insulin receptor [29]. PTP activity was measured as described previously [23] using 10–20 μM tyrosine-phosphorylated ^{32}P-labelled RCML or MBP at 30 °C for 10–20 min in reaction buffer containing 25 mM imidazole, pH 7.2, 5 mM EDTA, 1 mM DTT and 1 mg/ml BSA. No more than 10–20 % of the substrate was dephosphorylated in each reaction.

RESULTS

Identification of TCPTP phosphorylation sites

Previous studies have indicated that TCPTP can be phosphorylated in a cellular context [30]. To identify phosphorylation sites on TCPTP, asynchronous COS1 cells overexpressing TC45 were labelled with [^{32}P]P_i for 5 h. The overexpressed and ^{32}P-labelled TC45 was immunoprecipitated, resolved by SDS/PAGE and digested *in situ* with trypsin. The ^{32}P-labelled tryptic phosphopeptides were then extracted from the polyacrylamide gel, achieving approx. 98 % recovery of ^{32}P. First, the extracted [^{32}P]phosphopeptides were separated in two dimensions by high-voltage electrophoresis and TLC, revealing two apparently distinct phosphopeptides (A and B) (Figure 1A). When the two phosphopeptides were extracted from the cellulose plates and analysed for their phosphoamino acid content, both contained ^{32}P-labelled phosphoserine (Figure 1B); ^{32}P-labelled phosphothreonine and phosphotyrosine were not detected even in total TC45 tryptic digests (results not shown). These results indicate that TC45 may be phosphorylated exclusively on serine in asynchronous cells.

Next, the extracted [^{32}P]phosphopeptides were purified by reverse-phase chromatography, and [^{32}P]phosphopeptide-containing fractions were identified by Cerenkov counting (Figure 1C). Fractions 33 and 37 contained [^{32}P]phosphopeptides and were analysed further. First, these fractions were subjected to phosphopeptide mapping followed by phosphoamino acid analysis. Based on phosphopeptide mapping, fractions 33 and 37 were homogeneous and their separation was consistent with the separation of [^{32}P]phosphopeptides A and B (Figure 1A) respectively (results not shown). In addition, fractions 33 and 37 contained only phosphoserine (results not shown). Thus the ^{32}P-labelled phosphopeptides in fractions 33 and 37 may correspond to the TC45 tryptic [^{32}P]phosphopeptides A and B in Figure 1(A). Next, fractions 33 and 37 were analysed by MALDI-TOF MS (Figure 1D). Peptides phosphorylated on a single site differ from their dephosphorylated counterparts by 80 mass units (corresponding to phosphate). We found that in fractions 33 and 34 a pair of peptides at masses of 1537.81 and 1455.46 were detected that corresponded to the phosphorylated and dephosphorylated forms of the theoretical tryptic peptide E^{295}DLSPAFDHSPNK^{307} (predicted mass of 1457.54). In fraction 37, a pair of peaks at 1915.37 and 1994.69 differed by approx. 80 mass units and corresponded to the dephosphorylated and phosphorylated forms of the peptide E^{291}LSKEDLSPAFDHSPNK^{307} (predicted mass of 1914.06). These results indicate that the phosphopeptides in fractions 33 and 37 may represent the same phosphorylation site(s) with E^{291}LSK^{295}EDLSPAFDHSPNK^{307} arising from incomplete tryptic digestion. Two possible phosphorylation sites, corresponding to Ser-298 and Ser-304, are present in the E^{295}DLSPAFDHSPNK^{307} tryptic peptide. Phosphate release sequencing of the E^{295}DLSPAFDHSPNK^{307} [^{32}P]phosphopeptide revealed phosphorylation in the tenth cycle (Figure 1E), consistent with phosphorylation of Ser-304.

Our results indicated that, in asynchronous COS1 cells, ectopically expressed TC45 was phosphorylated on Ser-304. To determine the extent of Ser-304 phosphorylation relative to other potential TC45 phosphorylation sites, Ser-304 was mutated to alanine (TC45-S304A) and the phosphorylation state of TC45-S304A was compared with that of wild-type TC45 (Figure 2). COS1 cells expressing TC45 or TC45-S304A transiently were labelled with [^{32}P]P_i, and incorporation into immunoprecipitated TC45 and TC45-S304A was monitored by autoradiography. Mutation of Ser-304 inhibited the phosphorylation of TC45 in asynchronous cells by as much as 90 % (Figure 2). In contrast, mutation of Ser-298 to Ala had no significant effect on ^{32}P incorporation into TC45 (results not shown). These results indicate that ectopic TC45 is phosphorylated predominantly on Ser-304 in asynchronous cells.

Endogenous TC45, but not TC48, is phosphorylated on Ser-304

Since the Ser-304 phosphorylation site was identified using ectopic TC45, we could not exclude the possibility that phosphorylation may have occurred due to TC45 overexpression. To assess the phosphorylation of endogenous TCPTP on Ser-304, we immunoprecipitated endogenous TCPTP from COS1 cells transfected with the pMT2 vector control using the anti-TCPTP antibody CF4 (Figure 3A); we also immunoprecipitated TCPTP from

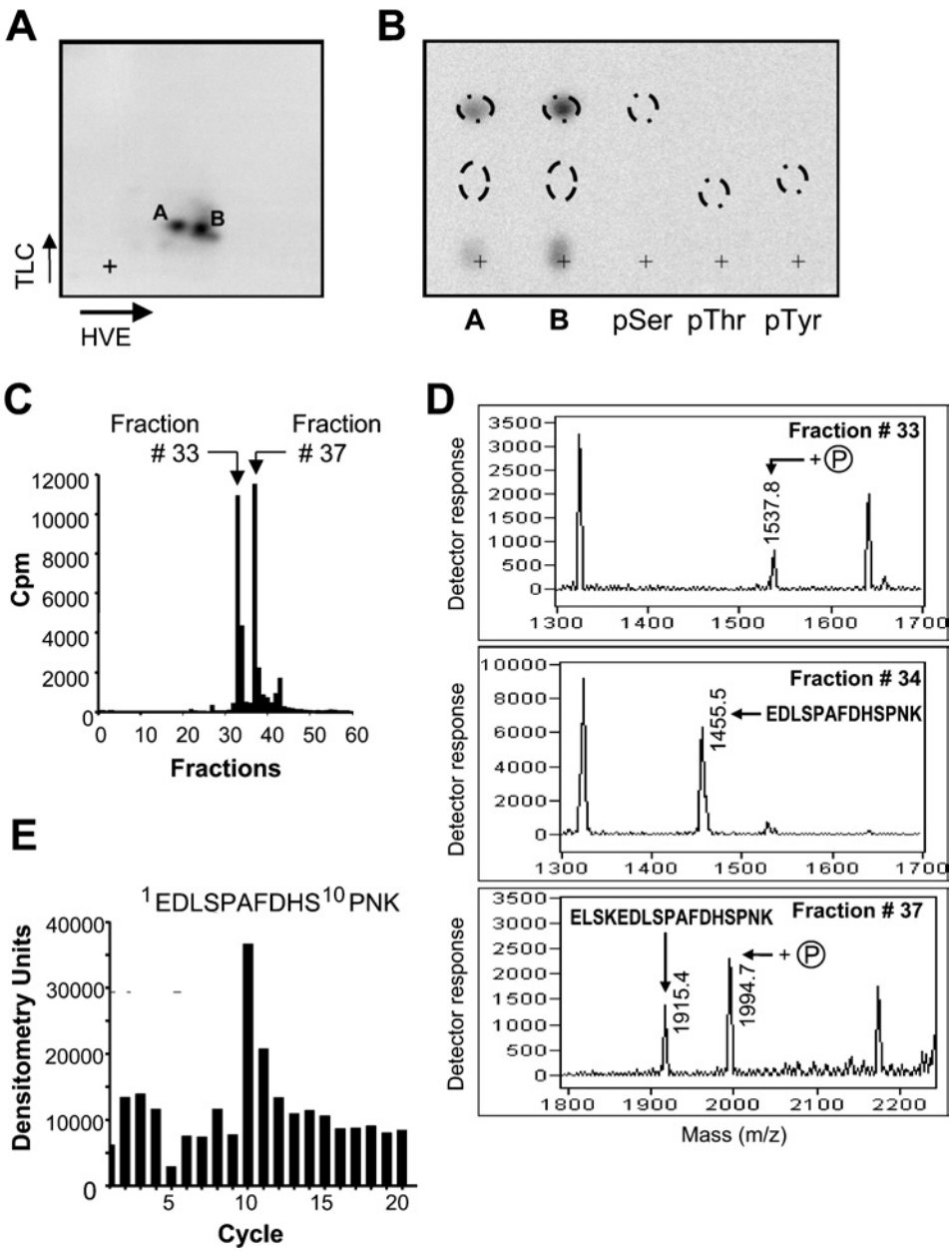

Figure 1 Identification of TC45 phosphorylation sites

TC45 was overexpressed transiently in asynchronous COS1 cells and labelled for 5 h with 1 mCi of [^{32}P]P_i per 10 cm dish. Radiolabelled TC45 was immunoprecipitated, resolved by SDS/PAGE and digested *in situ* with trypsin, and ^{32}P-labelled peptides were extracted as described in the Materials and methods section. More than 98 % of radioactivity was recovered from the gel. (**A**) [^{32}P]Phosphopeptide map of TC45 from in-gel tryptic digests, with + marking the origin. HVE, high-voltage electrophoresis. (**B**) [^{32}P]Phosphopeptides A and B from the phosphopeptide map were extracted and subjected to phosphoamino acid analysis and visualized by autoradiography (+ marks the origin). Unlabelled phosphoserine (pSer), phosphothreonine (pThr) and phosphotyrosine (pTyr) standards were run both individually and in combination as internal standards for the [^{32}P]phosphopeptide samples A and B. Shown is the autoradiograph overlaid with the migration of phosphoamino acid standards (dotted circles) that were visualized by ninhydrin staining. (**C**) [^{32}P]Phosphopeptides isolated from in-gel tryptic digests were separated by reverse-phase HPLC. The fractions were collected and radioactivity monitored by Cerenkov counting. (**D**) The masses of the [^{32}P]phosphopeptides separated by reverse-phase HPLC were determined by MALDI-TOF MS. The top two panels identify the phosphopeptide in reverse-phase HPLC fraction 33. Phosphopeptide and dephosphopeptide pairs differing by approx. 80 mass units are indicated, as are the corresponding amino acid sequences. (**E**) The phosphopeptide present in fraction 33 (E^{295}DLSPAFDHSPNK^{307}) was covalently attached on to a Sequelon-AA^{TM} (PerSeptive Biosystems) membrane and subjected to phosphate-release sequencing on a Hewlett Packard protein sequencer as described in the Materials and methods section. Radiolabelled PTC-amino acids in fractions were monitored by spotting on to a silica plate followed by PhosphorImager analysis and quantified using Image-Quant software (Molecular Dynamics).

TC45- and TC45-S304A-overexpressing cells using the same antibody to serve as immunoblot controls for the Ser-304 phosphorylation-specific antibody (Figure 3A). The TCPTP immunoprepitates were divided in two, resolved by SDS/PAGE and immunoblotted either with affinity-purified polyclonal antibodies specific for TCPTP phosphorylated on Ser-304 (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/380/bj3800939add.htm) or with affinity-purified polyclonal anti-TCPTP 159 antibodies (see Supplementary Figure 1) (Figure 3A). Despite endogenous TC48 and TC45 being present

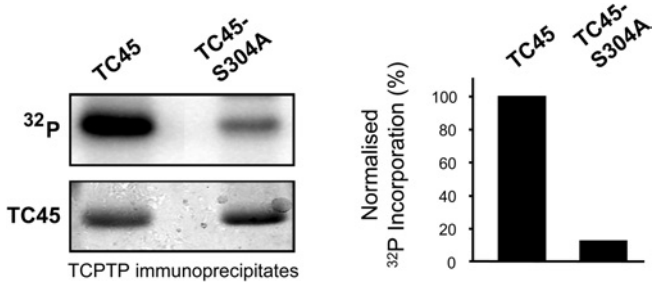

Figure 2 Ser-304 is the major site of phosphorylation on TC45

COS1 cells were transfected with constructs for the expression of TC45 or TC45-S304A. Transfected COS1 cells were labelled with [^{32}P]P_i (2 h with 1 mCi/10 cm dish), and radiolabelled TCPTP was immunoprecipitated, resolved by SDS/PAGE and analysed by Coomassie Blue staining. ^{32}P incorporation into the immunoprecipitated TC45 or the TC45-S304A mutant was monitored on a Phosphorimager and quantified using Image-Quant software (Molecular Dynamics). ^{32}P incorporation was normalized for TCPTP protein and expressed as percentage incorporation compared with wild-type TC45.

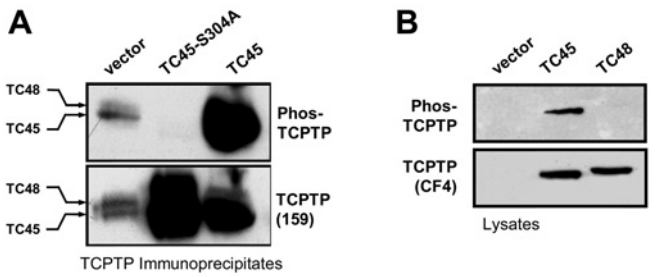

Figure 3 TC45, but not TC48, is phosphorylated on Ser-304

(**A**) COS1 cells were transfected with the pMT2 vector control or constructs for the expression of TC45 or the TC45-S304A mutant. TCPTP immunoprecipitates were resolved by SDS/PAGE and immunoblotted with affinity-purified polyclonal antibodies specific for the TCPTP phosphorylated on Ser-304 (Phos-TCPTP) or affinity-purified polyclonal TCPTP 159 antibodies (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/380/bj3800939add.htm). Indicated are the endogenous TC48 and TC45 proteins in the vector control immunoprecipitates. (**B**) COS1 cells were transfected using the calcium phosphate precipitation method as described previously [14] with 200 ng of pMT2 vector control, TC45-pMT2 or TC48-pMT2. At 24 h post-transfection, cells were collected in 3 × Laemmli sample buffer and lysates were resolved by SDS/PAGE and immunoblotted with the monoclonal anti-TCPTP antibody CF4, and then reprobed with polyclonal Phos-TCPTP antibodies.

in equal amounts in immunoprecipitates from vector control-transfected cells, only endogenous TC45 was phosphorylated to any appreciable extent on Ser-304 (Figure 3A). Consistent with this, TC48 was phosphorylated poorly in comparison with TC45 when low amounts of the TCPTP variants were overexpressed in COS1 cells (Figure 3B). These results demonstrate (1) that phosphorylation of Ser-304 is not an artefact of overexpression, but instead that Ser-304 phosphorylation may be a physiologically relevant event, and (2) that phosphorylation of Ser-304 may be specific to TC45 *in vivo*.

Phosphorylation of TC45 occurs in a cell cycle-dependent manner and is optimal in mitosis

We next examined the circumstances under which Ser-304 phosphorylation may occur. We found that diverse stress-inducing agents, such as hyperosmotic shock (600 mM, 30 min), cold shock (18 °C, 2 h), heat (42 °C, 30 min), oxidative stress (5 mM H_2O_2, 30 min) and anisomycin (100 μg/ml, 30 min), as well mitogens such as EGF (100 ng/ml, 5–30 min) and FBS (20 %, v/v; 30 min), had no significant effect on Ser-304 phosphorylation (results not shown). Similarly, treatment of cells for up to 60 min with the microtubule-disrupting agent nocodazole (500 ng/ml), a potent activator of stress-response pathways, had no significant effect on Ser-304 phosphorylation (results not shown). In contrast, treatment of TC45-overexpressing HEK293 cells for prolonged periods of time (16–20 h) with nocodazole (200 ng/ml), so that cells were arrested in mitosis (Figure 4A, left panel), caused a significant increase in Ser-304 phosphorylation and retarded the electrophoretic mobility of TC45 (Figure 4A, right panel; see also Supplementary Figure 1 at http://www.BiochemJ.org/bj/380/bj3800939add.htm). A similar increase in the phosphorylation of Ser-304 was observed when cells were arrested in mitosis with the microtubule-stabilizing agent colcemid (results not shown). These results indicate that Ser-304 phosphorylation may be cell cycle-dependent.

To examine further the phosphorylation status of Ser-304 during cell cycle progression, CV1 cells expressing ectopic TC45 were accumulated at the G_1/S transition (thymidine block) and released into G_2, mitosis (M) and subsequently G_1 of the next cell cycle (Figure 4B). Cell cycle progression was monitored by assessing DNA content as measured by FACS and by assessing cyclin B1 protein levels; cyclin B1 levels are low in G_1, then increase in G_2, peaking at metaphase of mitosis; the protein is degraded at anaphase of mitosis, when sister chromatids segregate and cell division ensues [31]. Between 5 and 7 h after release from the G_1/S block, the majority of cells had a 4*n* DNA content and increased expression of cyclin B1, consistent with cells being in late G_2 or M phase (Figure 4B). After 8 h of release, the proportion of cells with a 4*n* DNA content had declined, coinciding with a dramatic loss of cyclin B1 protein, consistent with cells exiting mitosis (Figure 4B). Ser-304 phosphorylation was low for the first 6 h, but increased dramatically at 7 h (Figure 4B). This delay in phosphorylation relative to the increase in cells with 4*n* DNA and cyclin B1 protein indicates that Ser-304 phosphorylation may occur late in G_2 and in mitosis. Ser-304 phosphorylation declined at 8 h, correlating with cyclin B1 loss and mitotic exit (Figure 4B). Consistent with these results, fluorescence microscopy of asynchronous HEK293 cells expressing ectopic TC45 indicated that Ser-304 phosphorylation was optimal in mitotic cells, but absent in cells undergoing cytokinesis (Figure 4C).

Finally, to assess whether Ser-304 phosphorylation may occur in late G_2 prior to the onset of mitosis, HEK293 cells expressing TC45 or the TC45-S304A mutant as a control were treated with the DNA double-strand-break-inducing agent adriamycin, which arrests cells at the G_2/M checkpoint by activating the ATR (ataxia telangiectasia-related)/Chk1 protein kinase signalling cascade [32]. The phosphorylation state of TC45 in response to adriamycin was monitored by assessing the electrophoretic mobility of TC45 and comparing it with that of TC45 in asynchronous cells or cells arrested in mitosis by overnight treatment with nocodazole (Figure 5). Treatment of HEK293 cells with adriamycin resulted in the accumulation of a G_2/M cell population (4*n* DNA content), consistent with G_2/M checkpoint activation (Figure 5A, upper panels), but did not alter the electrophoretic mobility of TC45 significantly when compared with asynchronous cells (Figure 5A, lower panels). In contrast, nocodazole caused an increase in electrophoretically retarded (Figure 5A, lower panels) and phosphorylated (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/380/bj3800939add.htm) TC45. To assess further whether Ser-304 phosphorylation may occur at the G_2/M checkpoint, we compared Ser-304 phosphorylation in cells accumulated at G_1/S (thymidine block) with that in cells

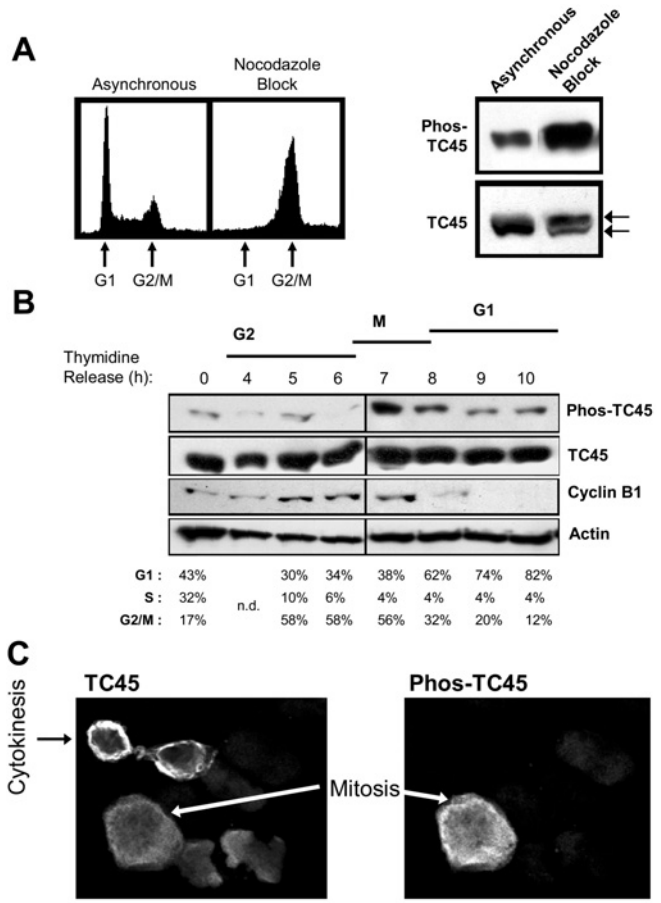

Figure 4 Phosphorylation of Ser-304 is cell cycle-dependent and optimal in mitosis

(**A**) HEK293 cells were transfected with TC45-pCG for the expression of TC45. At 24 h post-transfection, cells were left untreated or treated for 20 h with 200 ng/ml nocodazole. Left panel: cells were fixed and DNA content (horizontal access) was measured by propidium iodide staining and FACS. Cells with $2n$ (G_1) and $4n$ (G_2/M) DNA contents are indicated. Right panel: cells were collected in hot 3 × Laemmli sample buffer containing 6 % (v/v) 2-mercaptoethanol, resolved extensively by SDS/PAGE (16 cm long gels; see the Materials and methods section) and immunoblotted with affinity-purified polyclonal antibodies specific for TC45 phosphorylated on Ser-304 (Phos-TC45) or the monoclonal anti-TCPTP antibody CF4. The retarded electrophoretic mobility of TC45 in response to the nocodazole block is indicated by arrows. (**B**) CV1 cells were electroporated with constructs for the expression of TC45. At 24 h post-transfection, cells were arrested at G_1/S with 2 mM thymidine and then released in DMEM plus 10 % (v/v) FBS for the indicated times. Cells were collected in 3 × Laemmli sample buffer containing 6 % (v/v) 2-mercaptoethanol, resolved by SDS/PAGE and immunoblotted with Phos-TC45, TC45 (CF4), anti-(cyclin B1) or anti-actin antibodies. Alternatively, cells were fixed and DNA content was measured by propidium iodide staining and FACS, and cells in G_1, S and G_2/M were quantified using CellQuest (Becton Dickinson) software. (**C**) Asynchronous HEK293 cells overexpressing TC45 were processed for immunofluorescence microscopy with Phos-TC45 and TC45 (CF4) antibodies. Cells consistent with a mitotic (identified by their rounded morphology with diffuse TC45 staining due to disruption of nuclear envelope) or cytokinetic phenotype are indicated.

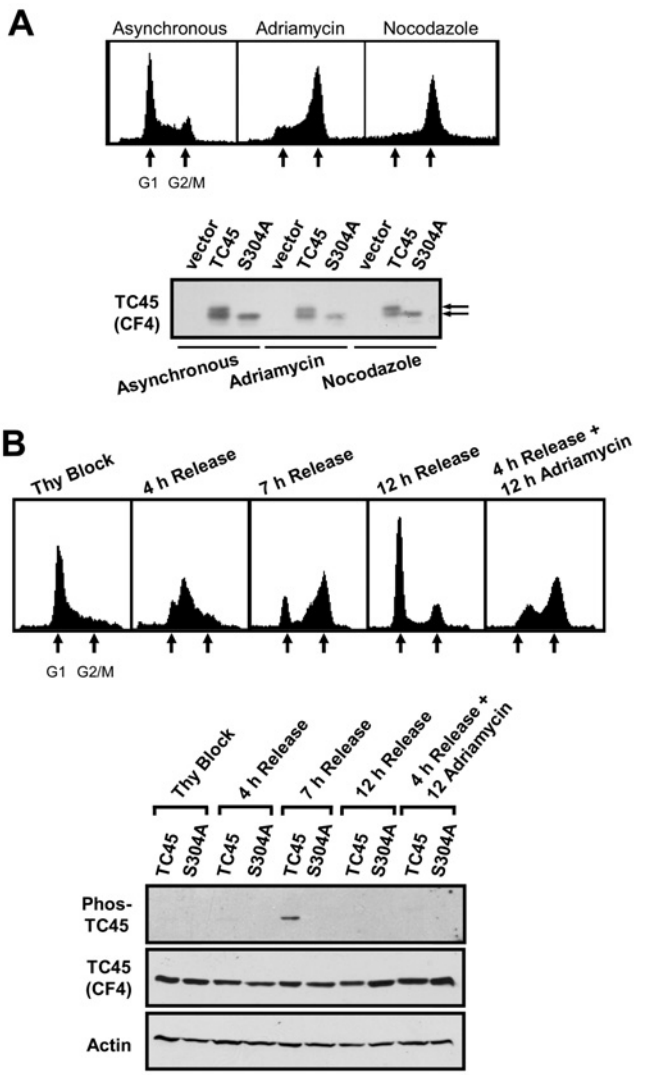

Figure 5 Phosphorylation of Ser-304 is not induced upon G_2/M checkpoint arrest with adriamycin

(**A**) HEK293 cells were transfected with 5 μg of pCG vector control or constructs for the expression of TC45 or TC45-S304A (S304A). At 24 h post-transfection, cells were left untreated or treated for 20 h with 100 ng/ml nocodazole or 1 μM adriamycin. Upper panels: TC45-pCG-transfected cells were fixed and DNA content measured by propidium iodide staining and FACS. Cells with $2n$ (G_1) and $4n$ (G_2/M) DNA contents are indicated. Lower panels: cells were collected in hot 3 × Laemmli sample buffer containing 6 % (v/v) 2-mercaptoethanol, resolved extensively on SDS/PAGE (16 cm long gels; see the Materials and methods section) and immunoblotted with anti-TCPTP antibody CF4 to detect TC45. The electrophoretic mobilities of the dephosphorylated and phosphorylated forms of TC45 are indicated. (**B**) HEK293 cells were transfected with 100 ng of pCG vector control or constructs for the expression of TC45 or TC45-S304A (S304A). At 20 h post-transfection, cells were accumulated at G_1/S with 2 mM thymidine (Thy) and then released in DMEM plus 10 % (v/v) FBS for the indicated times, or otherwise released for 4 h and then treated with 1 μM adriamycin for an additional 12 h. Upper panels: TC45-pCG transfected cells were analysed by FACS as indicated above. Lower panels: cells were collected, resolved by SDS/PAGE and immunoblotted with antibodies specific for TC45 phosphorylated on Ser-304 (Phos-TC45), total TC45 (CF4) or actin.

that had been released for 4, 7 or 12 h, or otherwise released for 4 h and then treated for another 12 h with adriamycin to arrest cells at the G_2/M checkpoint (Figure 5B). Consistent with the results in Figures 4 and 5(A), Ser-304 phosphorylation increased significantly in mitotic cells 7 h after release from G_1/S, but not in cells arrested at the G_2/M checkpoint with adriamycin (Figure 5B). Taken together, these results indicate that TC45 Ser-304 phosphorylation may be restricted to the mitotic phase of the cell cycle.

Phosphorylation of Ser-304 does not modulate TC45 activity

Previous studies have shown that the non-catalytic C-terminus of TC45 can suppress TC45 activity through a yet to be defined autoinhibitory mechanism [23]. Phosphorylation of Ser-304 may alter intrasteric interactions to modulate TC45 activity [23]. To ascertain whether this might be the case, we examined first whether the phosphatase activity of TC45 was increased in

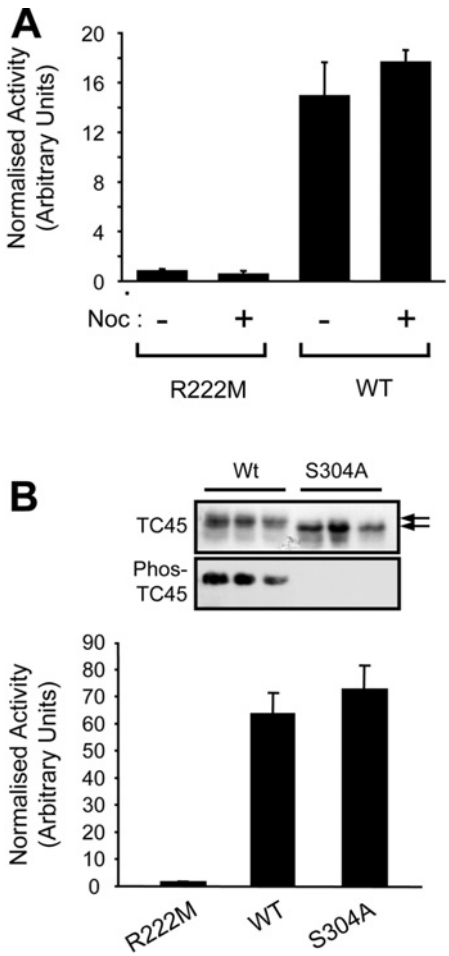

Figure 6 Phosphorylation of Ser-304 does not alter the phosphatase activity of TC45

(**A**) HEK293 cells were transfected with constructs for the expression of a phosphatase-dead TC45-R222M control or wild-type TC45 (WT). At 24 h post-transfection, cells were left untreated or treated for 20 h with 100 ng/ml nocodazole (Noc). (**B**) HEK293 cells transfected with constructs for the expression of TC45-R222M, wild-type TC45 (Wt) or TC45-S304A were treated with 100 ng/ml Noc for 20 h. Cells in (**A**) and (**B**) were homogenized in hypotonic lysis buffer and clarified by centrifugation as described in the Materials and methods section. PTP activity in supernatants was measured using tyrosine-phosphorylated ^{32}P-labelled MBP as substrate. The experiments shown are representative of at least three independent experiments with triplicate transfections assayed in duplicate. PTP activity was normalized for the overexpressed TCPTP protein in the corresponding supernatants. In (**B**), a representative immunoblot of supernatants from wild-type TC45- or TC45-S304A-expressing cells that were resolved extensively by SDS/PAGE (16 cm long gels; see the Materials and methods section) is shown; the electrophoretic mobilities of the phosphorylated and dephosphorylated forms of TC45 are indicated.

mitotic cells (Figure 6) where Ser-304 phosphorylation was optimal (Figure 4). We measured PTP activity in lysates from HEK293 cells transfected with constructs for the expression of wild-type TC45 or the catalytically inactive TC45-R222M mutant (Figure 6A). Asynchronous cells or nocodazole-treated metaphase-arrested cells were homogenized in hypotonic lysis buffer and PTP activity measured using tyrosine-phosphorylated RCML (results not shown) or MBP (Figure 6A) as substrate. We found no significant difference in TC45 activity in lysates from metaphase-arrested cells where TC45 was largely phosphorylated, as compared with asynchronous cells where TC45 was predominantly dephosphorylated (Figure 6A). Next, we compared the activity of TC45 with that of the S304A mutant from nocodazole-treated metaphase-arrested cells (Figure 6B). Mutation of Ser-304 and ablation of phosphorylation had no significant effect on phosphatase activity (Figure 6B) under conditions where phosphorylation of wild-type TC45, as determined by its retarded electrophoretic mobility relative to TC45-S304A (see Supplementary Figure 1), was stoichiometric (Figure 6B, inset). Taken together, these results indicate that phosphorylation of Ser-304 may not alter the phosphatase activity of TC45.

Phosphorylation of Ser-304 is mediated by CDKs

The Ser-304 phosphorylation site of TC45 is followed immediately by a proline residue (AFDHS^{304}P) and conforms to the consensus recognition sequence for two classes of protein kinases: the MAPKs (mitogen-activated protein kinases) and the CDKs. In addition, the Ser-304 site (AFDHS^{304}P) may also conform to the consensus site for phosphorylation by the Polo-like kinase Plk1 ([E/D]X[S/T]) [33]. All three protein kinases are essential for mitotic progression [34–37]. Since a variety of cellular stresses failed to modulate the phosphorylation status of Ser-304 (results not shown), we surmised that stress-activated MAPKs such as c-Jun N-terminal kinase and p38 were unlikely to phosphorylate Ser-304. To examine the possible contribution of the MAPKs ERK1/2 to Ser-304 phosphorylation, TC45-expressing COS1 cells were treated with PD98059, a specific pharmacological inhibitor of ERK1/2 activation. Although PD98059 inhibited completely the activation of ERK1/2, it had no effect on TC45 Ser-304 phosphorylation, even after overnight treatment (Figure 7A). These results demonstrate that Ser-304 phosphorylation is independent of ERK1/2 activity.

To examine whether the phosphorylation of Ser-304 may be mediated by CDKs, TC45-expressing HEK293 cells were treated with roscovitine, a specific pharmacological inhibitor of CDKs 1, 2 and 5 (inhibits mammalian cellular proliferation with an average IC_{50} of 16 μM) [38]. Treatment with roscovitine inhibited almost completely the phosphorylation of Ser-304 (Figure 7B). Furthermore, treatment of TC45-overexpressing cells with another specific CDK inhibitor, SU9516 (inhibits G_2/M progression at concentrations of approx. 12.5–25 μM) [39], also suppressed Ser-304 phosphorylation (Figure 7C). Taken together, these results indicate that CDKs may phosphorylate TC45 *in vivo*. Consistent with this, we found that recombinant CDKs could phosphorylate the Ser-304 site directly *in vitro* (Figure 8A). Although, the recombinant CDKs could also phosphorylate other site(s) on TCPTP-(1–349), the stoichiometry of these *in vitro* phosphorylation events was significantly lower (Figure 8A). In contrast, Plk1 activity was not inhibited by roscovitine *in vitro*, indicating that Plk1 is unlikely to phosphorylate Ser-304 *in vivo*. Moreover, Plk1 could not phosphorylate TCPTP either on Ser-304 or on other sites under conditions where phosphorylation of the heterologous substrate α-casein was robust (Figure 8B). Taken together, these results indicate that CDKs may mediate the phosphorylation of TC45 on Ser-304 *in vivo*.

DISCUSSION

Mitosis is morphologically the most dynamic phase of the cell cycle, and a large number of events are co-ordinated temporally and spatially by reversible protein phosphorylation [31,34]. In the present study we have identified Ser-304 as a major phosphorylation site in the ubiquitous tyrosine phosphatase TCPTP. We have generated phosphorylation-specific antibodies and demonstrated that Ser-304 phosphorylation occurs in mitosis and is mediated by CDKs.

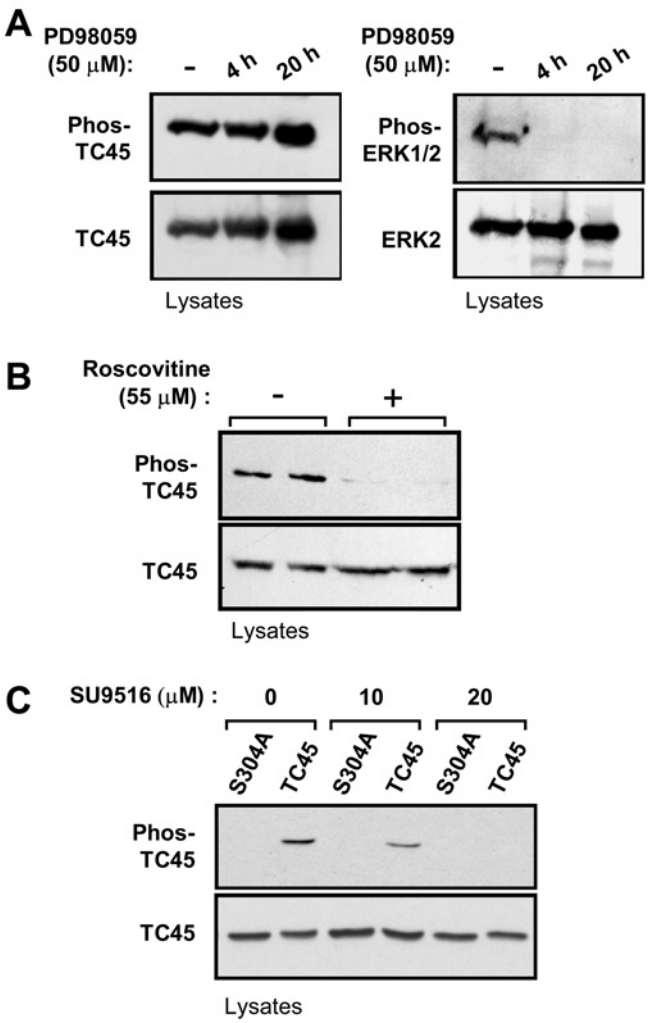

Figure 7 Phosphorylation of Ser-304 is inhibited by the CDK inhibitors roscovitine and SU9516, but not by the MEK (MAPK/ERK kinase) inhibitor PD98059

(**A**) COS1 cells were transfected with constructs for the expression of TC45. Cells were treated with 50 μM PD98059 for the inhibition of ERK1/2 for 4 or 20 h as indicated. Cells were collected in hot 3 × Laemmli sample buffer containing 6 % (v/v) 2-mercaptoethanol, resolved by SDS/PAGE and immunoblotted with polyclonal antibodies specific for phosphorylated and activated ERK1/2 (Phos-ERK1/2), or for TCPTP phosphorylated on Ser-304 to detect phosphorylated TC45 (Phos-TC45). Immunoblots were re-probed with the anti-ERK2 antibody 1B3B9 or the anti-TCPTP antibody CF4 to detect ERK2 and TC45 respectively. (**B**) HEK293 cells were transfected with constructs for the expression of TC45. Cells were treated with vehicle alone or 55 μM roscovitine for 20 h. Cells then were collected in hot 3 × Laemmli sample buffer containing 6 % (v/v) 2-mercaptoethanol, resolved by SDS/PAGE and immunoblotted with Phos-TC45 or TC45 CF4 antibodies. (**C**) HEK293 cells were transfected with constructs for the expression of TC45 or TC45-S304A and treated with vehicle alone or the indicated concentrations of SU9516 for 20 h. Cells were collected, resolved by SDS/PAGE and immunoblotted with Phos-TC45 or TC45 (CF4) antibodies.

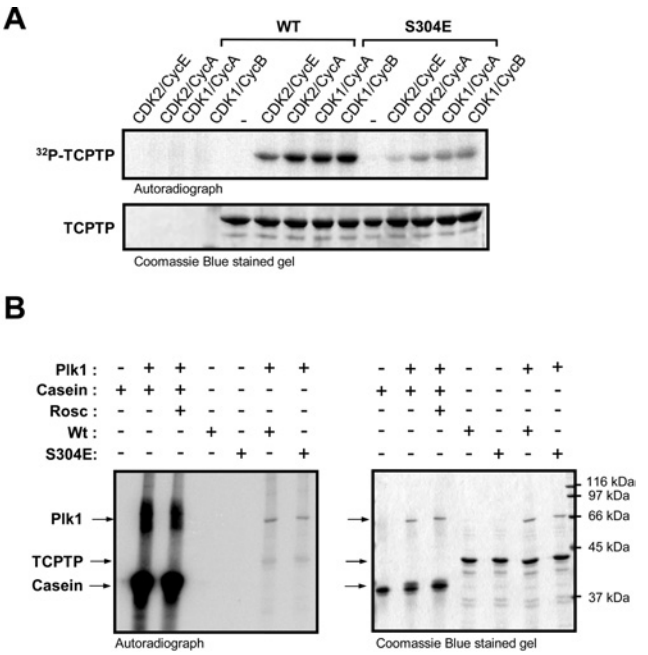

Figure 8 CDKs, but not Plk1, phosphorylate Ser-304 *in vitro*

(**A**) Recombinant TCPTP-(1–349) (WT) or TCPTP-(1–349)S304E (S304E) proteins were phosphorylated with the indicated CDK/cyclin proteins prepared and purified as described previously [28] in the presence of 100 μM ATP (plus 5 μCi of [γ-^{32}P]ATP) as described in the Materials and methods section. Reactions were terminated by the addition of 3 × Laemmli sample buffer, and resolved by SDS/PAGE and analysed by Coomassie Blue staining and autoradiography. (**B**) Recombinant TCPTP-(1–349) (Wt) or TCPTP-(1–349)S304E proteins or dephosphorylated α-casein were incubated with purified recombinant Plk1 in the presence of 25 μM ATP (plus 10 μM [γ-^{32}P]ATP) with or without 50 μM roscovitine (Rosc) for 30 min at 30 °C. Reactions were terminated by the addition of 3 × Laemmli sample buffer, and resolved by SDS/PAGE and analysed by Coomassie Blue staining and autoradiography. Indicated are casein, Plk1 and the TCPTP proteins.

CDKs phosphorylate a wide array of substrates regulating cell cycle progression [31,34,40]. Different CDKs act to regulate different stages of the cell cycle, with CDK4/cyclin D activity being optimal in G_1, CDK2/cyclin E at the G_1/S transition, CDK2/cyclin A at the G_2/M transition and CDK1/cyclin B1 in mitosis [31,34]. Although all CDKs tested phosphorylated Ser-304 efficiently *in vitro*, phosphorylation of Ser-304 was optimal as cells progressed into mitosis, and subsided upon mitotic exit, consistent with phosphorylation being mediated by either CDK1/cyclin B1 or CDK2/cyclin A *in vivo*. Whereas CDK2/cyclin A is important for S-phase progression and the G_2/M transition, CDK1/cyclin B1 is essential for mitotic entry and regulates mitotic processes such as nuclear envelope breakdown, centrosome separation, spindle assembly, chromosome separation and mitotic exit [34]. CDK2/cyclin A activity increases steadily from S phase and peaks at the G_2/M transition, but decreases as cells proceed into mitosis. In contrast, CDK1/cyclin B is activated at the G_2/M transition and peaks in mitosis [31,34], but induction of the G_2/M checkpoint prevents CDK1 activation and mitotic entry [31,34]. Since Ser-304 phosphorylation did not occur in response to G_2/M checkpoint activation by adriamycin, our studies indicate that Ser-304 may be phosphorylated by CDK1/cyclin-B1 *in vivo*.

Whereas the phosphorylation of dual-specificity Cdc25 phosphatases by CDKs to regulate mitotic progression is well documented [41,42], evidence for phosphorylation of tyrosine-specific PTPs by CDKs *in vivo* has been somewhat scant. TCPTP and PTP1B are highly related phosphatases and share an overall sequence identity of 65 % (72 % identity and 86 % similarity within the catalytic domain) [1]. PTP1B is phosphorylated in mitosis on two Ser-Pro sites [43] that are not present in TCPTP. Only one of these two PTP1B Ser-Pro sites can serve as an efficient site for phosphorylation by CDKs *in vitro* [43]. Moreover, the phosphorylation of PTP1B on both sites also occurs in response to cellular stresses, in particular osmotic shock, independent of cell cycle stage and MAPK activation [44]. In contrast, phosphorylation of TCPTP on Ser-304 was not induced by cellular stresses such as osmotic shock, indicating that TCPTP and PTP1B may be phosphorylated by distinct protein kinases *in vivo*.

In contrast with the Cdc25 phosphatases, where phosphorylation on different sites by CDKs and other protein kinases can regulate subcellular localization and activity [31,41,42,45], phosphorylation of TCPTP on Ser-304 had no apparent effect on TC45 activity. In addition, we observed no apparent role for Ser-304 phosphorylation in controlling TC45 localization (see Supplementary Figure 2 at http://www.BiochemJ.org/bj/380/bj3800939add.htm). Mutation of Ser-304 to alanine or glutamic acid did not alter the nucleocytoplasmic distribution of TC45 in response to mitogens such as EGF or to cellular stresses such as hyperosmotic shock (results not shown) that induce the cytoplasmic accumulation of TC45 [12], and we observed no significant difference in localization in G_1, G_2 or mitotic cells (see Supplementary Figure 2). In addition, in mitotic cells, where Ser-304 phosphorylation was optimal, the localization of TC45 phosphorylated on Ser-304, as monitored with our phosphorylation-specific antibodies, was similar to that of total TC45 monitored with the monoclonal anti-TCPTP antibody CF4 (results not shown). Furthermore, when nuclei from TC45-overexpressing HeLa cells were fractionated into soluble, chromatin and nuclear matrix-associated proteins, TC45 was detected in the soluble and nuclear matrix fractions, but not in chromatin fractions, and mutation of Ser-304 to Ala had no significant effect on this distribution (results not shown). Similarly, phosphorylation of Ser-304 did not alter TC45 protein stability, as assessed by [^{35}S]methionine pulse–chase experiments comparing wild-type TC45 and the Ser-304 phosphorylation site mutants (see Supplementary Figure 2). In the case of Cdc25, a recent paper by Bulavin et al. [42] has shown that dual phosphorylation of Ser-214 and Ser-216 controls localization and mitotic progression. Whereas phosphorylation of Cdc25C on Ser-216 allows for 14-13-3 binding and nuclear exclusion, preventing CDK1 activation prior to mitosis, phosphorylation on Ser-214 prevents phosphorylation on Ser-216 and 14-13-3 binding, making cells unresponsive to DNA damage and ensuring that CDK1 remains active once mitosis is initiated [42]. Our studies indicate that the Ser-304 site is the major site of phosphorylation in human TCPTP in randomly growing cells, and the major site of CDK-mediated phosphorylation *in vitro*. However, we cannot exclude the possibility that other steady-state phosphorylation sites may exist, or that other sites may be phosphorylated in response to specific stimuli, and that, together with Ser-304 phosphorylation, these may regulate TC45. In line with this possibility, we observed low levels of ^{32}P incorporation in the TC45-S304A mutant, but the physiological significance of such low-level incorporation in overexpressed TC45 remains unclear.

Although the Ser-304 site is conserved in the porcine orthologue of human TCPTP, rats lack the sequence corresponding to residues 296–314 and mice lack the proline residue immediately following Ser-304 [4]. Therefore the Ser-304 CDK site is not conserved in rodents. However, it is possible that other sites may be phosphorylated in place of Ser-304; consistent with this, a previous study has reported that rat TCPTP is phosphorylated in mitosis on a yet to be identified site, and that this phosphorylation event retards TC45's electrophoretic mobility [30]. Hence the functional outcome of phosphorylation on Ser-304 of human TCPTP in mitosis might be conserved in different species.

A recent study from the laboratory of David Shalloway [46] has indicated that PTPα-mediated c-Src activation in mitosis is regulated by protein kinase C-dependent phosphorylation of PTPα. Whereas the catalytic activity of PTPα towards non-specific substrates such as tyrosine-phosphorylated MBP is suppressed only slightly when the protein kinase C phosphorylation sites are mutated to Ala, activity towards the physiological substrate c-Src is abrogated completely, demonstrating that Ser/Thr phosphorylation of a PTP can regulate substrate specificity [46]. Therefore, although mutating Ser-304 to Ala had no significant effect on TC45 activity towards non-physiological substrates such as tyrosine-phosphorylated RCML and MBP, we cannot exclude the possibility that Ser-304 phosphorylation may regulate activity towards a physiologically relevant mitotic substrate. Although very early studies on TCPTP had indicated that expression of the TCPTP catalytic domain in baby hamster kidney cell lines could induce cytokinetic failure and multinucleation [47], mitotic substrates for TCPTP remain unknown.

In the present study we have observed differential mitotic phosphorylation of human TC48 and TC45 on Ser-304, suggesting that the TCPTP variants might have distinct functions during cell division. Our studies identify Ser-304 as a major TCPTP phosphorylation site and the TC45 variant as a potential mediator of CDK1/cyclin B1 processes in mitosis.

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