Specific cellular stresses, including hyperosmotic stress, caused a dramatic but reversible cytoplasmic accumulation of the otherwise nuclear 45-kDa variant of the protein-tyrosine phosphatase TCPTP (TC45). In the cytoplasm, TC45 dephosphorylated the epidermal growth factor receptor and down-regulated the hyperosmotic stress-induced activation of the c-Jun N-terminal kinase. The hyperosmotic stress-induced nuclear exit of TC45 was not inhibited by leptomycin B, indicating that TC45 nuclear exit was independent of the exportin CRM-1. Moreover, hyperosmotic stress did not induce the cytoplasmic accumulation of a green fluorescent protein-TC45 fusion protein that was too large to diffuse across the nuclear pore. Our results indicate that TC45 nuclear exit may occur by passive diffusion and that cellular stress may induce the cytoplasmic accumulation of TC45 by inhibiting nuclear import. Neither p42ERK2 nor the stress-activated c-Jun N-terminal kinase or p38 mediated the stress-induced redistribution of TC45. We found that only those stresses that stimulated the metabolic stress-sensing enzyme AMPK (AMP-activated protein kinase) induced the redistribution of TC45.

In addition, specific pharmacological activation of the AMPK was sufficient to cause the accumulation of TC45 in the cytoplasm. Our studies indicate that specific stress-activated signaling pathways that involve the AMPK can alter the nucleocytoplasmic distribution of TC45 and thus regulate TC45 function in vivo.

Protein-tyrosine phosphatases (PTPs) are a large and structurally diverse family of enzymes that catalyze the dephosphorylation of tyrosyl phosphorylated proteins (1, 2). In doing so PTPs either antagonize or potentiate tyrosine phosphorylation-dependent signal transduction events and thus regulate fundamental cellular processes such as cellular growth and proliferation, differentiation, migration, and survival (1–3).

Despite the importance of PTPs in a wide range of physiological and pathological processes, comparatively little is known about the regulation of individual PTP family members.

Cellular Stress Regulates the Nucleocytoplasmic Distribution of the Protein-tyrosine Phosphatase TCPTP*

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‡‡‡ The abbreviations used are: PTP, protein-tyrosine phosphatase; TCPTP, T-cell PTP; NLS, nuclear localization sequence; EGF, epidermal growth factor; PTK, protein-tyrosine kinase; AMPK, 5′-AMP-activated protein kinase; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis; NES, nuclear export sequence; HIV, human immunodeficiency virus; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; AICAR, 5′-aminoimidazole-4-carboxamide ribonucleoside.

2 M. T. Fodero-Tavoletti, M. Klingler-Hoffmann, B. E. Kemp, N. K. Tonks, and T. Tiganis, unpublished observations.
cellular stresses that activate the AMPK cause a dramatic accumulation of TC45 in the cytosol. We report that TC45 nuclear exit occurs by passive diffusion and that TC45 localization may be regulated at the level of nuclear import. Our studies indicate that specific signaling events involving the AMPK can alter TC45 nucleocytoplasmic distribution to regulate TC45 access to substrates and therefore function.

EXPERIMENTAL PROCEDURES

Materials—SB203580 was purchased from Toecis (Ballwin, MO); Hoechst 33258, TOTO-3 iodide, and rhodamine-phalloidin were from Molecular Probes (Eugene, OR); 5-aminolevulinic acid, formylketopentane, chloramphenicol, amphotericin B, 13-15-myristate-13-sulfate, cytochalasin D, genistein, bovine serum albumin (BSA) (radioimmunoassay grade, fraction V), poly-t-lysine, polyethylene glycol 8000, and anisomycin were from Sigma; and nucodazol, calyculin A, okadaic acid, and PD98059 were from Calbiochem Oncogene Research Products (Cambridge, MA). Polyephal GFR receptor (sc-60) antibody used for immunohosting was from Santa Cruz Biotechnology (Santa Cruz, CA), polyclonal phospho-P38 antibody from Promega (Madison, WI), and FLAG M2 antibody was from Eastman Kodak Company (New Haven, CT). The following constructs and reagents were generously provided by colleagues: FLAG-tagged JNK pCMV construct by L. Van Aelst (Cold Spring Harbor Laboratory, NY); Rev 1.4 NES-GFP construct by B. Henderson (Westmead Institute, for Cancer Research, Sydney, Australia); monoclonal p42/p44 MAPK antibody by J. Darnell (University of Virginia, Charlottesville, VA), monoclonal anti-TCPTP antibody CF4 by D. Thompson (St Vincent’s Institute of Medical Research, Melbourne, Australia), and leptomycin B by M. Yoshida (University of Tokyo, Japan). The following antibodies have been described previously: monoclonal anti-phospho-tyrosine (4G10 antibody (13, 16), anti-phosphoThr172-AMPK α1, and anti-AMPK α1 antibodies (17).

Cell Culture, Transfections, and Immunofluorescence—COS1, COS7, HeLa, and NIH3T3 cells were cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Where indicated, the cells were serum-starved for 24 h in DMEM containing 0.1% FBS, plus antibiotics.

The cells were transfected by the calcium phosphate precipitation method as described previously (13). Transfected cells were either collected at 36–48 h post-transfection or were washed once with phosphate-buffered saline (PBS) at 24 h post-transfection and serum-starved prior to collection. Where indicated in the figure legends, the serum-starved cells were exposed to various stimuli in DMEM containing 0.1% FBS, plus antibiotics.

For immunofluorescence studies, cells on glass coverslips were fixed with 3.2% paraformaldehyde in PBS and processed as described previously (13) using the monoclonal anti-TCPTP antibody CF4 and the Alexa Fluor 488 goat anti-mouse IgG (H + L) secondary antibody (Molecular Probes, Eugene, OR). For confocal microscopy, after incubation with the appropriate secondary antibody, the cells were washed first with 0.2% (w/v) Triton X-100 in PBS, washed in PBS, and then incubated with 5 μg/ml RNase (Roche Molecular Biochemicals) in PBS for 30 min. The cells were then incubated with 1:4000 TOTO-3 in PBS containing RNase for 15 min, washed extensively with PBS, and mounted on glass slides in Dako fluorescent mounting medium (Dako Corporation, Carpinteria, CA). Immunofluorescence was visualized on an OLYMPUS BX60 microscope or a Bio-Rad MRC 1024 confocal microscope.

Plasmid Constructs—Site-directed mutagenesis of the TC45 cDNA-pBluescript II KS construct (10) to generate TC45-D182A and 3′ oligonucleotide used was 5′-GGCTCCCAAGTACTAGTGGGCCCAAGCTGCGAGG-3′, and the 3′ oligonucleotide was 5′-CCACGGTGCATAGGTCCCTAGTTGCTGTAATTTG-3′. SpeI/BamHI-digested polymerase chain reaction products were cloned into the XbaI/BamHI site of the mammalian expression vector pCG. To generate the TC45-pEGFP construct the 5′ oligonucleotide incorporated a BglII site immediately 5′ to the initiating codon, and the 3′ oligonucleotide was based on sequence from the TCPTP 3′-untranslated region containing a unique HindIII restriction site. The 5′ oligonucleotide was 5′-GGTTCCCGAGATCATCGCCACACATCGGGGAG-3′, and the 3′ oligonucleotide was 5′-GCGGCAGAAGCTTGCGTTGGCTAAATTG-3′. BglII/HindIII-digested polymerase chain reaction products were cloned into the same sites in pEGFP-C1 for the expression of a GFP-TC45 fusion protein in mammalian cells. To generate constructs for the expression of the GFP-TC45(242–249) or GFP-TC45(318–325) fusion proteins, sense and antisense oligonucleotides with a BglII restriction site at the 5′ end were annealed and cloned into the BglII and SmaI restriction sites of pEGFP-C1. The structures of the recombinant plasmids generated were confirmed by restriction endonuclease analysis, and the fidelity of the cloned cDNA was confirmed by sequencing.

Heterokaryon Assays—HeLa cells (3.5 × 106) were electroporated at 260 V and 960 microfarads with 10 μg of TC45 pCG plasmid DNA plus 10 μg of salmon sperm DNA. Electroporated cells were seeded onto glass coverslips in 60-mm dishes and incubated for 12 h in DMEM containing 10% FBS. A 5-fold excess of untransfected NIH3T3 cells was added, and the HeLa-bearing coverslips were washed once with 0.2% (w/v) Triton X-100, PBS and then resuspended in 50 mM Tris-HCl, pH 7.5, and cycloheximide. Medium was then aspirated, the attached cells were supplemented with fresh medium containing 100 μg/ml cycloheximide, and incubation continued for further 30 min. The coverslips were then washed with PBS and inverted for 2 min onto a drop of 50% polyethylene glycol 8000 that had been prepared in PBS and prewarmed to 37 °C. The cells were then incubated for 2 h in medium containing 100 μg/ml-cycloheximide, rinsed five times with PBS, fixed with 3.2% paraformaldehyde and processed for immunofluorescence. Overexpressed TC45 was detected using the antibody CF4, and nuclei were stained with Hoechst 33258.

Cell Detachment Studies—COS1 cells electroporated with the TC45 pMT2 construct as described previously (12) were serum-starved for 24 h, washed in PBS, and then harvested by limited trypsin-EDTA treatment as described previously (12). Detached cells were resuspended in DMEM (minus phenol red) containing 0.25% (w/v) BSA. The cells were pelleted by centrifugation and washed twice with DMEM (minus phenol red) containing 0.25% (w/v) BSA and resuspended in 1 ml of DMEM (minus phenol red) containing 0.1% (v/v) BSA. The cells were held in suspension at 37 °C for 30 min by gentle agitation, collected on glass coverslips for 15–30 min at 37 °C, and processed for immunofluorescence.

Protein Kinase Assays—JNK assays were performed as described previously (12). For AMPK assays, COS1 cells were serum-starved and either left untreated or treated with 600 nm sorbitol or 2 mM AICAR for 30 or 60 min, respectively. The cells were then washed twice with PBS and harvested on ice in 0.4 ml of lysis buffer (20 mM Hepes, pH 7.4, 1% (w/v) Nonidet P-40, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM dithiothreitol, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation (20,000 × g, 5 min at 4 °C), and AMPK was immuno precipitated with polyclonal anti-AMPK α1 bound to protein A-Sepharose CL-4B (Amersham Pharmacia Biotech). The precipitates were washed with PBS, PBS containing 2% (w/v) Triton X-100, PBS and then resuspended in 50 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol, 10% (v/v) glycerol, and 0.1% (w/v) Triton X-100. AMPK activity was assayed in duplicate as described previously (12). In brief, AMPK activity was detected by the phosphorylation of the SAMS peptide substrate (100 μM) in the presence or absence of AMP (100 μM) with 250 μCi [γ-32P]ATP (500 cpm/pmol) in kinase assay buffer (50 mM Hepes, pH 7.5, 10 mM MgCl2, 5% glycerol, 1 mM dithiothreitol, 0.05% Triton X-100). Aliquots of the precipitates used for the kinase reactions were resolved by SDS-PAGE and immobiloblotted with anti-phospho-AMPK α1 antibodies to detect the activated catalytic subunit of AMPK phosphorylated on Thr-172 within the activation loop. The same blot was stripped and reprobed for total AMPK α1 using specific antibodies (17), and the relative phosphorylation was determined by comparative densitometry.
RESULTS

Specific Cellular Stresses Alter the Nucleocytoplasmic Distribution of TC45—Despite its apparently exclusive nuclear localization in resting cells, TC45 readily shuttles between the nucleus and the cytoplasm and therefore has access to both nuclear and cytoplasmic substrates. This shuttling can be seen readily in heterokaryon assays that are used to characterize nucleocytoplasmic transport (20, 21). When HeLa cells overexpressing TC45 were fused with untransfected NIH3T3 cells to form heterokaryons (where HeLa and 3T3 nuclei share a common cytoplasm), the overexpressed TC45 evenly distributed between the nuclei from the two different cell lines (Fig. 1). We postulated that the predominant nuclear localization of TC45 may result from a higher rate of nuclear import than exit and that the activation of certain signaling cascades may alter this import/exit balance to increase TC45 cytoplasmic localization. Accordingly, we found that specific stress-inducing agents altered the balance between TC45 nuclear import and exit. In cells transiently overexpressing TC45, cellular stresses such as hyperosmolarity (600 mM sorbitol, 10–30 min), oxidative stress (0.1–1 mM H2O2, 10–30 min), and cold shock (18 °C, 2 h) induced a dramatic accumulation of TC45 in the cytoplasm (Fig. 2, A and B). The fraction of cells in which TC45 was undergoing this change in localization varied but was always in the order of 50–90%. Importantly, nuclear integrity (as determined by Hoechst or TO-TO-3 staining) was maintained in response to these stress stimuli (Fig. 2A), and the change in localization was reversed within 5 min of removal of stress stimuli such as hyperosmotic shock (Fig. 2C). Moreover, the stress-induced cytoplasmic accumulation of TC45 was observed in variety of mammalian cell types including African green monkey COS1 fibroblasts (Fig. 2), Chinese hamster ovary cells, and murine 3T3-L1 adipocytes (data not shown), indicating that this is not a cell type-dependent event. Other stress-inducing agents including anisomycin (100 μg/ml, 30 min), exposure to heat shock (42 °C, 30 min) (Fig. 2B), or ultraviolet irradiation (100 J/m2) (data not shown) had no effect on TC45 localization. These results demonstrate that specific cellular stresses can alter the nucleocytoplasmic distribution of TC45.

TC45 Subcellular Localization Is Independent of Cell Shape—The common feature of each of the stresses that caused a change in the nucleocytoplasmic distribution of TC45 was their ability to perturb cell shape (22). To evaluate the effects of cell shape on TC45 subcellular localization, we detached COS1 cells by trypsinization and maintained them in DMEM (without phenol red). We found that although the cells underwent a dramatic change in cell shape (progressing from a planar morphology with an organized actin cytoskeleton to one that was

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**Fig. 1.** Heterokaryon analysis of TC45 nucleocytoplasmic shuttling. HeLa cells overexpressing TC45 were fused with untransfected NIH3T3 cells to form heterokaryons in which mouse and human nuclei share a common cytoplasm. Fusions were allowed to proceed in the presence of cycloheximide for 2 h, and heterokaryons were processed for immunofluorescence using TC45 specific antibodies. Nuclei were visualized with Hoechst stain. NIH3T3 nuclei have a characteristic punctate staining.

**Fig. 2.** Specific cellular stresses induce the reversible cytoplasmic accumulation of TC45. COS1 cells transiently overexpressing TC45 were serum-starved and either left untreated or exposed to several different stress stimuli including hyperosmolarity (600 mM sorbitol (Sorb), 30 min) (A) and anisomycin (100 μg/ml, 30 min), oxidative stress (0.1 mM H2O2, 15 min), cold shock (18 °C for 2 h), and heat shock (42 °C, 30 min) (B). The cells were then processed for confocal microscopy using TC45 specific antibodies. Where indicated nuclei were visualized with TOTO-3 dye. C, TC45 expressing cells were stimulated with 600 mM sorbitol for 30 min, washed with DMEM containing 0.1% FBS, and incubated at 37 °C for the indicated times. The cells were then processed for confocal microscopy using TC45 specific antibodies.

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Cellular Stress Regulates TCPTP Localization

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**Fig. 2.** Specific cellular stresses induce the reversible cytoplasmic accumulation of TC45. COS1 cells transiently overexpressing TC45 were serum-starved and either left untreated or exposed to several different stress stimuli including hyperosmolarity (600 mM sorbitol (Sorb), 30 min) (A) and anisomycin (100 μg/ml, 30 min), oxidative stress (0.1 mM H2O2, 15 min), cold shock (18 °C for 2 h), and heat shock (42 °C, 30 min) (B). The cells were then processed for confocal microscopy using TC45 specific antibodies. Where indicated nuclei were visualized with TOTO-3 dye. C, TC45 expressing cells were stimulated with 600 mM sorbitol for 30 min, washed with DMEM containing 0.1% FBS, and incubated at 37 °C for the indicated times. The cells were then processed for confocal microscopy using TC45 specific antibodies.
recognized by the exportin protein CRM1 (otherwise known as exportin-1) (23–27). We identified two sequences, 242INIKGVLL249 and 318IGLEEKL325, present in the C-terminal segment that displayed similarity to the classical NESs from heat stable inhibitor of the cAMP-dependent protein kinase PKI (LALKAGLDI) and the HIV accessory protein Rev (LPLLERLTL) (28). These sequences were fused to the C terminus of GFP, and their localization in COS1 cells was assessed by immunofluorescence (Fig. 4A). In both cases the GFP-TC45 fusion proteins were not excluded from the nucleus, indicating that they are not functional NESs (Fig. 4A). In contrast the REV(1.4)NES-GFP control protein containing a functional REV NES (28) was readily excluded from the nucleus (Fig. 4B). Given that no other sequences in TC45 resemble the classical CRM-1-dependent NES, our results suggest that TC45 nuclear exit is independent of CRM-1. However, to exclude the possibility that TC45 may be “piggy-backed” by another classical NES-containing protein, we tested the effects of leptomycin B, an antibiotic that inhibits specifically the CRM-1 nuclear export pathway (29, 30). Whereas leptomycin B inhibited effectively the nuclear export of the REV(1.4)NES-GFP fusion protein and resulted in its nuclear accumulation (Fig. 4B), pretreatment of TC45-expressing cells with leptomycin B (10–40 ng/ml, 1–16 h) had no effect on the cytoplasmic accumulation of TC45 in response to hyperosmolarity (600 mM sorbitol) (Fig. 4C). Therefore the data suggest that the hyperosmotic stress-induced cytoplasmic accumulation of TC45 is independent of the CRM-1 export pathway.

Proteins with relative molecular masses of less than 40–60 kDa can diffuse freely across the nuclear pore complex (31, 32). To assess whether TC45 exits the nucleus by passive diffusion, we generated a GFP-TC45 fusion protein that has a molecular

**Fig. 3.** TC45 subcellular localization is independent of cell shape. A, COS7 cells transiently overexpressing TC45 were detached and resuspended in DMEM containing BSA. The cells were held in suspension at 37 °C for 30 min prior to collection on glass coverslips. The cells were processed for confocal microscopy using and TC45-specific antibodies and TOTO-3 to visualize nuclei. B, COS1 cells transiently overexpressing TC45 were serum-starved and incubated with the actin polymerization inhibitor cytochalasin D (2 μM, 30 min). The cells were then either left untreated or treated for 30 min with 600 mM sorbitol (Sorb) in the presence of cytochalasin D. The cells were then processed for immunofluorescence using TC45-specific antibodies, rhodamine-phalloidin (Phalloidin) to stain actin and Hoechst to stain nuclei.

**Fig. 4.** The hyperosmotic stress-induced nuclear exit of TC45 is CRM1-independent and occurs by passive diffusion. A, COS1 cells were transfected by the calcium phosphate precipitation method with constructs expressing GFP control, GFP-TC45(242–249) (TC(242–249)), or GFP-TC45(318–325) (TC(318–325)) were processed for immunofluorescence. B, COS1 cells were transfected by the calcium phosphate precipitation method with pREV(1.4)NES-GFP (28) to express a REV-GFP fusion protein with a functional NES (REV1.4/NES-GFP). REV1.4/NES-GFP expressing cells were either left untreated or treated with 0.2 ng/ml leptomycin B (LMB) for 3 h and then processed for immunofluorescence. The nuclei were visualized with Hoechst stain. C, COS1 cells transiently overexpressing TC45 were serum-starved and pretreated with 10 ng/ml LMB for 2.5 h. The cells were then either left untreated or treated for 30 min with 600 mM sorbitol (Sorb) containing 10 ng/ml LMB. The cells were then processed for immunofluorescence using TC45-specific antibodies. D, COS1 cells transiently overexpressing the GFP-TC45 fusion protein were serum-starved and either left untreated or treated for 30 min with 600 mM sorbitol (Sorb). The cells were then processed for immunofluorescence. The nuclei were visualized with Hoechst stain.
mass of ~72 kDa, thus impairing exit from or entry into the nucleus by passive diffusion. The fusion protein was transiently overexpressed in COS1 cells, and the subcellular localization of GFP-TC45 was assessed following hyperosmotic shock. GFP-TC45 maintained an apparently exclusive nuclear localization both in serum-starved and in hyperosmotic stressed cells (Fig. 4D), confirming that nuclear import of TC45 is active and demonstrating that hyperosmotic stress does not in general alter nuclear integrity or nuclear permeability. Moreover, these results demonstrate that cellular stress does not induce the active export of TC45 but that exit of TC45 from the nucleus may be passive and that the control of its subcellular localization may occur at the level of nuclear import.

The Hyperosmotic Stress-induced Accumulation of TC45 in the Cytosol Is Independent of EGF Receptor and MAPK Activation—Recent studies have indicated that the activation of serine/threonine kinases can inhibit the nuclear import of the SV40 large T-antigen NLS (mediated by importin α/β) and the M9 sequence of hnRNP A1 (mediated by the importin β family member transportin) but not CRM1-mediated export of NFAT (nuclear factor of activated T-cells) (33). More specifically, Czubryt et al. (34) have shown that pathological levels of H2O2 can inhibit the nuclear import of the SV40 large T-antigen NLS and that this inhibition was blocked by general PTK inhibitors and mediated by the MAPK p420x2. In this study we sought to dissect the signaling pathways involved in the stress-induced cytoplasmic accumulation of TC45. We assessed the localization of TC45 in response to 600 mM sorbitol (hyperosmotic stress) in the presence or absence of a panel of pharmacological agents known to inhibit defined signaling molecules. Initially we tested whether tyrosine phosphorylation in general was necessary for the change in TC45 localization. We found that the nonspecific PTK inhibitor genistein inhibited the sorbitol-induced tyrosine phosphorylation of proteins but had no significant effect on TC45 localization (Fig. 5). These results indicate that tyrosine phosphorylation induced by cellular stress does not mediate the stress-induced change in TC45 localization. However, we cannot exclude the involvement of specific PTKs that may not be inhibited effectively by genistein. Nevertheless, consistent with the effects of genistein, we found that specific inhibition of the sorbitol-induced activation of the EGF receptor PTK with AG1478 (2 μM, IC50 3 nm) did not prevent the cytoplasmic accumulation of TC45 (Fig. 5). The MAPKs have been implicated in the stress-induced inhibition of classical nuclear import and the MAPKs p420x2, p38, and JNK are potently stimulated in response hyperosmotic stress (Ref. 22 and Figs. 5 and 7). We found that complete inhibition of ERK1/2 with PD98059 (50 μM, IC50 2 μM) or almost complete inhibition of p38 with SB203580 (50 μM, IC50 600 nm) did not significantly alter the sorbitol-induced cytoplasmic accumulation of TC45 (Fig. 5). Similarly, we found that treatment of cells with anisomycin, a potent activator of JNK (22), did not alter TC45 localization (Fig. 2). Our results indicate that neither genistein-sensitive PTKs such as the EGF receptor nor the MAPKs ERK1/2, p38, or JNK are responsible for the stress-induced TC45 cytoplasmic localization.

Activation of the AMP-dependent Protein Kinase Increases the Cytosolic Localization of TC45—Cellular stress results in increased intracellular 5’-AMP and concomitant activation of the AMPK (14, 15). Hyperosmotic stress (600 mM sorbitol, 30 min), H2O2 (1 mM, 15 min), and cold shock (18 °C, 2 h) were potent activators of the AMPK (Fig. 6A). Activation of the AMPK was monitored with phosphorylation site-specific antibodies that detect phosphorylation of the AMPK catalytic subunit on Thr-172 (Fig. 6A) or the phosphorylation of an AMPK-specific peptide substrate (data not shown). Importantly sorbitol, H2O2, and cold shock also induced the cytoplasmic accumulation of TC45 (Fig. 2), whereas agents such as heat shock (42 °C, 30 min) (data not shown), EGF (100 ng/ml, 30 min), or anisomycin (100 μg/ml, 30 min) that had no measurable effect on TC45 localization (13) (Fig. 2) also failed to stimulate AMPK activity (Fig. 6A). Thus the AMPK may play a role in mediating the stress-induced signaling that leads to TC45 cytoplasmic accumulation. Consistent with this supposition we found that pharmacological activation of the AMPK by 5-aminomidazole-4-carboxamide ribonucleoside (AICAR) induced the cytoplasmic accumulation of TC45 (Fig. 6B). AICAR is a compound that is taken up by intact cells and phosphorylated to form 5-aminomidazole-4-carboxamide riboside monophosphate that mimics the activating effects of AMP on AMPK (35). AICAR stimulated AMPK activity by up to 2-fold (Fig. 6C), and hyperosmotic stress activated the AMPK by ~5-fold (Fig. 6D), as measured by the phosphorylation of AMPK peptide substrate. The mechanism by which the AMPK may exert its effects on TC45 localization is not currently understood because AMPK was not able to phosphorylate recombinant TC45.
in vitro, and hypertonic stress did not alter the phosphorylation state of TC45 in a cellular context (data not shown). Nevertheless, in contrast to the effects of AICAR, pharmacological activation of the cAMP-dependent protein kinase with forskolin (30 μM, 30 min) and then processed for immunofluorescence using TCPTP-specific antibodies. Magnifications are indicated. In parallel, serum-starved COS1 cells were stimulated with AICAR (2 μM, 30 min) and the AMPK was immunoprecipitated and assayed in vitro. AMPK activity was detected by the AMP-activated phosphorylation of the SAMS substrate peptide.

**FIG. 6.** Activation of the AMPK induces the cytoplasmic accumulation of TC45. A, serum-starved COS1 cells were left untreated, stimulated with EGF (100 ng/ml), or exposed to several different stress stimuli including high osmolarity (600 mM sorbitol (Sorb), 30 min), anisomycin (Aniso) (100 μg/ml, 30 min), H₂O₂ (0.5 mM, 15 min), and cold (18 °C, 2 h). The AMPK was immunoprecipitated from cell lysates, and the precipitates were resolved by SDS-PAGE and immunoblotted with antibodies specific for the phosphorylated and activated AMPK α1 catalytic subunit (Phos-AMPK) and then stripped and reprobed for total AMPK α1 catalytic subunit. Right panel, the phosphorylation of the AMPK α1 catalytic subunit on Thr-172 relative to untreated cells was determined by comparative densitometry. B, COS1 cells transiently overexpressing TC45 were serum-starved and either left unstimulated or stimulated with either sorbitol (600 mM, 30 min) or AICAR (2 μM, 60 min) and then processed for immunofluorescence using TCPTP-specific antibodies. Magnifications are indicated. In parallel, serum-starved COS1 cells were stimulated with AICAR (2 μM) for the indicated times (C) or sorbitol (600 mM, 30 min) (D), and the AMPK was immunoprecipitated and assayed in vitro. AMPK activity was detected by the AMP-activated phosphorylation of the SAMS substrate peptide.

**FIG. 7.** TC45 down-regulates the hypertonic stress-induced activation of the EGF receptor and the MAPK JNK. A, serum-starved COS1 cells were either left unstimulated or stimulated with 600 mM sorbitol (Sorb) for 30 min. Where indicated cells were pre-treated for 30 min with the EGF receptor inhibitor AG1478 (2 μM). The cells were collected in 3× hot Laemmli sample buffer, and the proteins were resolved by SDS-PAGE and immunoblotted with antibodies specific for the EGF receptor. B, COS1 cells were electroporated as described previously (12) with pMT2 vector control, TC45, or TC45-D182A (TC45D), serum-starved, and either left unstimulated or stimulated with 600 mM sorbitol for 30 min. The cells were collected in Laemmli buffer, and the cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies specific for phosphotyrosine (pTyr). The immunoblot was stripped and reprobed with antibodies specific for the EGF receptor. C, COS1 cells were co-transfected with plasmids expressing FLAG-tagged JNK and either vector control, TC45, or TC45D. The cells were serum-starved and either left unstimulated or stimulated with 600 mM sorbitol (Sorb) for 30 min. The cells were then lysed and FLAG-tagged JNK immunoprecipitated and assayed using GST-Jun. Activity was normalized for FLAG-tagged JNK protein. Shown is a representative experiment of three independent experiments involving duplicate transfections.
the tyrosine phosphorylation status of the EGF receptor in response to sorbitol in lysates of cells expressing TC45 (Fig. 7B). Compared with vector control, the EGF receptor was dephosphorylated by wild type TC45 but protected from dephosphorylation by the TC45-D182A substrate-trapping mutant (Fig. 7B). These results are consistent with the EGF receptor being a direct substrate of TC45, as we have demonstrated previously for EGF- and integrin-induced activation of the receptor (13). In COS1 cells, sorbitol stimulation induced the phosphatidylinositol 3’-kinase-independent activation of JNK (data not shown). We determined the effects of TC45 on the sorbitol-induced activation of JNK by co-expressing FLAG-tagged JNK with either TC45 or TC45-D182A in COS1 cells. JNK activity in FLAG immunoprecipitates was measured in vitro using GST-Jun as described previously (12). Wild type TC45 but not the TC45-D182A mutant inhibited the sorbitol-induced activation of JNK by ~50% (Fig. 7C). These results indicate that TC45 can down-regulate signal transduction events initiated by hyperosmotic stress.

**DISCUSSION**

TC45 can regulate signal transduction events induced by receptor PTKs such as the EGF receptor at the cell surface. Hyperactivation of the EGF receptor is known to contribute to transformation and malignant progression of many human tumors. Therefore maintenance of TC45 in the cytoplasm may provide a means by which to suppress EGF receptor signaling in transformed cells. To this end we have focused on elucidating the processes involved in the control of TC45 subcellular localization. We have demonstrated that specific cellular stresses lead to the cytoplasmic accumulation of TC45. This change in localization of the phosphatase occurred in various cell types and was reversed when cellular stress was alleviated. As such, the events triggered by cellular stress can be regulated and may provide an important means for manipulating TC45 localization reversibly in vivo.

Cellular stress may either stimulate nuclear export or inhibit nuclear import. A recent study has indicated that cellular stresses such as osmotic shock and H2O2 can induce the cytoplasmic accumulation of HIV Rev, whereas UV irradiation and heat shock have no effect (37). In the case of HIV Rev, it was concluded that cellular stress activated the CRM-1-mediated export of Rev (37). Our studies indicate that the TC45 nuclear import is an active process, as manifested by the apparently exclusive nuclear localization of the GFP-TC45 fusion protein that was too large to diffuse into the nucleus. In contrast, TC45 nuclear exit was independent of CRM-1 and occurred instead by passive diffusion. Accordingly, we propose that the stress-induced cytoplasmic accumulation of TC45 may result from an inhibition of TC45 nuclear import.

Our previous studies indicate that TC45 nuclear import may occur via a novel mechanism (13). Whereas classical nuclear import is mediated by the direct interaction of importin α with SV40 T-antigen-like NLS motifs (31, 32, 38), the nuclear import of TC45 is conferred by an atypical bipartite NLS (10). Like the NLS motifs of ribosomal protein L23a (39), parathyroid hormone-related protein (40), and histone H1 (41), which bind directly to importin β family members, the TC45 NLS binds directly to importin β1 (10). In addition, the TC45 NLS also binds an unidentified 116-kDa protein and also interacts with lower affinity to importin α (10). In the case of L23a, parathyroid hormone-related protein, or histone H1, binding of importin α family members is sufficient for nuclear import independent of importin α. Moreover, in the case of histone H1, optimal nuclear import is conferred by a heterodimer of importin β1 and the importin β family member importin 7 (41). In the case of TC45, importin β1 alone is not sufficient for nuclear import, and the nuclear import mechanism of TC45 may require additional factors such as the unidentified 116-kDa protein (10). Because the exact proteins mediating TC45 nuclear import remain unknown, we cannot ascertain directly whether cellular stress does indeed inhibit TC45 nuclear import using in vitro nuclear import assays.

A recent study by Czubryt et al. (34) has indicated that in digitonin-permeabilized cells, H2O2-induced oxidative stress can inhibit the nuclear import of the SV40 T-antigen NLS. Moreover, this H2O2-induced inhibition of nuclear import is mediated by the MAPK p42/p44 (34). In the case of TC45, we showed that neither p42/p44 nor the MAPKs JNK, or p38 were involved in the stress-induced cytoplasmic accumulation. These results are again consistent with distinct mechanisms for the nuclear import of TC45 and the SV40 T-antigen. In another recent study, Kehlenbach and Gerace (33) have reported that in digitonin-permeabilized cells the activation of Ser/Thr kinases with Ser/Thr phosphatase inhibitors can suppress the nuclear import of the SV40 T-antigen NLS (mediated by importin α/β) and the import of M9 sequence of hnRNP A1 (mediated by the importin β family member transportin). In this latter study, a role for the MAPK p42/p44 in the inhibition of nuclear import was excluded (33). As such it would seem that multiple Ser/Thr kinases might play important roles in regulating the nuclear import of proteins. It is conceivable that distinct phosphorylation-dependent signaling cascades have evolved to regulate the import processes conferred by an ever growing number of importin family members (42).

In this study we found a correlation between the cellular stresses that activated the Ser/Thr protein kinase AMPK and the induced cytoplasmic accumulation of TC45. Agents such as ansisomycin, heat shock, and EGF that did not significantly activate the AMPK had no measurable effect on TC45 localization. We found that Ser/Thr phosphatase inhibitors such as okadaic acid, which lead to the hyperphosphorylation of proteins in vivo but do not activate the AMPK in intact cells (43), also had no effect on TC45 localization. Moreover, specific pharmacological activation of the AMPK with AICAR induced the cytoplasmic accumulation of TC45, providing compelling evidence that the AMPK may be a critical regulator of the subcellular distribution of TC45. Cellular stress and the AMPK exerted their effects on TC45 subcellular localization without altering the phosphorylation state of the phosphatase. Similarly, the activation of Ser/Thr kinases can lead to the inhibition of nuclear import of the M9 sequence of hnRNP A1 or SV40 large T-antigen NLS without altering the phosphorylation of these proteins (33, 34). Therefore cellular stress and the AMPK may exert their effects by altering the phosphorylation state of proteins involved in the subcellular targeting of TC45 rather than the phosphatase itself. As has been previously postulated for the import of the SV40 T-antigen NLS and the M9 sequence of hnRNP A1 (33, 34), this may involve the phosphorylation and inhibition of proteins involved in TC45 nuclear import.

In this study we have shown that specific cellular stresses that activate the AMPK can induce the cytoplasmic accumulation of TC45. In the cytoplasm, TC45 down-regulates stress-induced signaling events including the activation of the EGF receptor and the MAPK JNK. Our results indicate that TC45 nuclear exit occurs by passive diffusion and that cellular stress-induced signaling pathways may act to inhibit TC45 nuclear import. The control of such signaling pathways would provide a means for achieving an acute regulation of TC45 localization and function in vivo.

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Cellular Stress Regulates the Nucleocytoplasmic Distribution of the Protein-tyrosine Phosphatase TCPTP

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