The c-Jun N-terminal kinases (JNKs) are serine/threonine kinases of the mitogen-activated protein kinase (MAPK) family that also includes the p38 MAPK and the extracellular signal-regulated kinase (ERK) subfamilies (1, 2). Of the three JNK isoforms, JNK1 and JNK2 exhibit broad tissue distribution, whereas JNK3 is found predominantly in neuronal tissue, testes, and the heart. The JNK pathway is activated in response to a broad range of stimuli, including the exposure to growth factors, cytokines, or various stresses such as oxidative and hyperosmotic stress. After its activation by upstream kinases, JNK can target multiple cytoplasmic and nuclear substrates and influence a wide range of biological processes (3). In particular, JNK has been shown to be a prominent regulator of cell death (4). Thus, the targeted inhibition of JNK by chemical or peptide inhibitors can reduce cell loss or tissue injury induced by stress (5). In other cellular contexts, JNK activity may conversely promote cell survival and proliferation (4). In the case of cancer cells, this can promote tumorigenesis (6). Thus, the role of JNK in regulating the dichotomy of cell death and survival is complex and requires closer examination.

To date, studies on JNK regulation of cell death have primarily focused on its role to enhance apoptosis by phosphorylation of key components of the apoptotic machinery (e.g. Bcl2 family members), signaling to AP-1 and p53 transcription factors and triggering the degradation of anti-apoptotic proteins (e.g. Mcl-1, cFLIP). However, JNK-mediated processes in response to detrimental stimuli do not always promote cell death but may also confer protection. For example, the targeted activation of JNK in the heart is protective against ischemia/reperfusion injury (7), a protective cardiac pre-conditioning response that has been reported to require JNK (8), and JNK activation can delay cardiac myocyte cell death in response to oxidative stress (9, 10). Similar protective functions for JNK have been reported in other cell types such as B and T lymphocytes (11–13). Importantly, JNK activation in the absence of a detrimental stimulus is not sufficient to trigger cell death (14). Although JNK isoform-specific functions and activation kinetics will influence the ultimate consequences of JNK activation, the downstream JNK substrates involved in promoting cell survival have not been fully determined.

In this study we have identified a key microtubule regulatory factor, stathmin (STMN,2 also known as oncoprotein 18), as a novel JNK substrate. STMN is a ubiquitous, highly conserved 18-kDa cytoplasmic protein (15). Initial studies identified STMN as a protein that was differentially phosphorylated during mitosis and highly overexpressed in rapidly proliferating cells (15). Subsequently, STMN was shown to be a key microtubule-regulatory protein through its capacity to bind tubulin and trigger the depolymerization of microtubules into short tubulin oligomers (16). The microtubule-stabilizing activity of STMN contributes substantially to the dynamic nature of the microtubule array. The activity of STMN is exquisitely regulated by multisite serine phosphorylation on four highly conserved serine residues (Ser–16, -25, -38, and -63), which negatively impacts on STMN/tubulin association and, therefore, promotes microtubule stabilization (17, 18). The kinases involved in phosphorylating STMN Ser–16 and Ser–63 include cAMP-dependent protein kinase (PKA) and PAK1 (17, 19), whereas STMN Ser–25 and Ser–38 have been shown to be targets for proline-directed serine/threonine kinases such as

The c-Jun N-terminal kinase (JNK) is a stress-activated kinase that can promote either cell survival or death in response to detrimental stimuli, the JNK-regulated mechanisms involved in survival are not fully characterized. Here we show that in response to hyperosmotic stress, JNK phosphorylates a key cytoplasmic microtubule regulatory protein, stathmin (STMN), on conserved Ser–25 and Ser–38 residues. In in vitro biochemical studies, we identified STMN Ser–38 as the critical residue required for efficient phosphorylation by JNK and identified a novel kinase interaction domain in STMN required for recognition by JNK. We revealed that JNK was required for microtubule stabilization in response to hyperosmotic stress. Importantly, we also demonstrated a novel cytoprotective function for STMN, as the knockdown of STMN levels by siRNA was sufficient to augment viability in response to hyperosmotic stress. Our findings show that JNK targeting of STMN represents a novel stress-activated cytoprotective mechanism involving microtubule network changes.

Protection against Cellular Stress* □

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

1 Recipient of National Heart Foundation of Australia/National Health and Medical Research Council Project Grants 628335 and 566804.
2 The abbreviations used are: STMN, stathmin; JBD, JNK binding domain; MEF, murine embryonic fibroblast; ca-, constitutively active.
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cyclin-dependent kinases, ERK1/2, and members of the p38 MAPK subfamily (18, 20, 21). STMN has been shown to be a critical regulator of cell migration and cell cycle through its control of interphase microtubule dynamics and the assembly of the mitotic spindle. Although STMN can also be phosphorylated in response to cellular stress (22), the consequence of STMN phosphorylation in response to stress stimulation and the regulatory mechanisms involved remain relatively uncharacterized. Here we show that stress-activated JNK-mediated phosphorylation of STMN confers a cytoprotective effect. These studies point to a JNK/STMN signaling mechanism involved in regulating cellular responses to deleterious stress stimuli.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibodies: STMN, α-tubulin (Sigma); p-JNK, p-p38, (Ser(P)-73)-c-Jun, c-Jun, and p-ERK (Cell Signaling); (Ser(P)-16)-STMN, ERK1, p38, GAPDH (Santa Cruz Biotechnology), (Ser(P)-25)-STMN, (Ser(P)-38)-STMN (Abcam); JNK (BD Biosciences Australia). Chemical inhibitors, U0126, SP600125, JNK inhibitor VIII, and SB203580 were from Calbiochem. TI-JIP peptide was from Proteomics International. Recombinant protein kinases, JNK1α1, JNK2α2, JNK3α1, p38α, and ERK1 were from Millipore. Cell culture serum, media, and supplements were obtained from Invitrogen. Nerve growth factor (NGF), sorbitol, trypsin blue, and routine laboratory chemicals were from Sigma.

Cell Lysate Preparation and Immunoblotting—Cell lysates were prepared in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 0.1 mM EDTA, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.2% (w/v) NaF, and 100 µM Na3VO4) supplemented with protease inhibitors. After 10 min on ice, cell debris was removed by centrifugation (14,000 x g, 10 min). Protein concentrations were then determined by Bradford assay. Protein lysates were resolved by SDS-PAGE transferred onto polyvinylidene fluoride membranes and immunoblotted (23). Protein bands were quantitated using Image Gauge v4.23 (FUJIFILM) software.

Immunofluorescence—Cells were grown on glass coverslips overnight in serum media. After cell treatments, cells were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.2% (v/v) Triton X-100, and blocked with 10% (v/v) fetal calf serum in PBS. Cells were then stained with primary antibodies diluted (1:250) in 1% horse serum and immunoblotted as described above.

In Vitro Kinase Assay—Purified recombinant protein substrate (10 µg) was incubated with active kinase (10 ng) and [γ-32P]-radiolabeled ATP (1 µCi/reaction) in a kinase reaction buffer (20 mM HEPES, pH 7.6, 20 mM MgCl2·6H2O, 20 µM ATP, 20 mM β-glycerophosphate and supplemented with 25 µM Na3VO4 and 100 µM DTT) over a 120-min time course at 30 °C. Reactions were stopped with the addition of Laemmli sample buffer. Samples were then resolved by SDS-PAGE, stained with Gelcode Blue Stain reagent (Thermo Scientific), and analyzed by autoradiography and Cerenkov Counting. For analysis of in vitro kinase assays by immunoblotting, [γ-32P]ATP was omitted from the reaction. After kinase assays were stopped, 50 ng of substrate was resolved by SDS-PAGE and immunoblotted as described above.

Plasmids—STMN mutant constructs (STMN-S16A, STMN-S25A, STMN-S38A, STMN-S63A, and STMN-AXA) were made by site-directed mutagenesis. Mutant constructs were then cloned into pGEX-6p-1 and transformed into competent BL21 Codon Plus Escherichia coli for protein expression. Myc-JNK1 and Myc-JNK2 constructs were made by subcloning PCR-amplified Jnk1α1 and Jnk2α2 isoforms into pX140-myc. All constructs were subjected to restriction digestion and sequencing analysis before further use.

Cell Culture, Transfection, and Treatments—JNK1/2+/+ and JNK1/2−/− murine embryonic fibroblasts (MEFs, kindly provided by K. Sabapathy) were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 100 units/ml penicillin/streptomycin. PC12 cells were maintained in DMEM supplemented with 10% horse serum and 5% fetal calf serum and 100 units/ml penicillin/streptomycin. All cells were cultured in a humidified 5% CO2 environment. Liposomal-mediated transfection was performed with Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Cells were pretreated with JNK inhibitor VIII, U0126, or SB203580 for 30 min before stress stimulation/cell treatments.

Cell Viability—Dye exclusion, indicating an intact cellular plasma membrane, was used as a reporter of cell viability and performed by the trypan blue staining method. Cells were trypsinized and collected by centrifugation (5 min, 100 g) and resuspended in cold PBS. An aliquot of cells was then diluted with an equal volume of 0.4% (w/v) trypan blue and mixed at room temperature (5 min). Counting chambers of a hemocytometer were then filled, and the unstained (live) and blue-stained (dead) cells were counted under phase contrast microscopy. Cell viability was determined from the number of live cells divided by the total cell number and expressed as a percentage.

siRNA—Expression vectors encoding STMN siRNA were constructed by ligating annealed oligonucleotides into pSuper basic (Oligoengine). The double-stranded oligonucleotide sequences targeting STMN were: 5’-GATCCCCAGGCTAAACAAAAAGTTGAGAGACTCTTTGTTAGCC-GTGTTTGCTTTT-3’ and 5’-TCGAGAAAAAAAATGGAGGCTAACAAGAGTCTCTTGAACTCTTTTGTAGCCTCATTG-3’. A non-targeting sequence (5’-GATTCCTCATTGCAGGAAAAATTAGGTTGAGACTCTTTTGTAGCCTCATTG-3’) was used as a control. siRNA vectors were transfected into cells using Lipofectamine™ 2000 following the manufacturer’s protocol.

In Vivo Polymerized Tubulin Assay—Measurement of polymerized tubulin was performed as previously described (24). In brief, MEFs were scraped into a microtubule-stabilizing buffer (100 mM Pipes, pH 6.9, 2 mM glycerol, 5 mM MgCl2·2 mM EGTA, 0.5% (v/v) Triton X-100, and protease inhibitors) supplemented with 4 µM taxol to maintain microtubule stability during isolation. The supernatant containing solubilized tubulin was clarified by centrifugation (20,000 g for 45 min) and separated from the pellet containing sedimented polymerized tubu-
lin. The pellet was washed once in microtubule-stabilizing buffer before being denatured in Laemmli buffer.

RESULTS

Hyperosmotic Stress Is a Potent Trigger for Phosphorylation of STMN Regulatory Sites

The microtubule-destabilizing protein STMN can be phosphorylated on key negative regulatory sites in response to a range of extracellular stimuli such as growth factors, pro-inflammatory cytokines, chemical toxicity, and heat stress (22, 25). Whereas the protein kinases targeting STMN in growth factor-stimulated signaling have now been defined (e.g. ERK, PKA, and cyclin-dependent kinase), less is known about the stress-activated protein kinase(s) that drives STMN phosphorylation and inactivation.

Using MEFs, we first evaluated the phosphorylation of key regulatory serine residues of STMN under conditions of hyperosmotic stress and compared this to the kinetics of phosphorylation/activation of several MAPK family members. Immunoblot analysis with site-specific phospho-STMN antibodies revealed an increase in STMN Ser-38 phosphorylation at 15 min that peaked at 1–2 h in response to 0.5M sorbitol treatment (Fig. 1A). STMN Ser-25 phosphorylation was more rapid, detected at 5 min, and reached maximal levels by 15 min. STMN Ser-16 phosphorylation showed similar kinetics (i.e. plateau at 15 min sorbitol treatment and appearing as multiple bands) (Fig. 1A). Immunoblot analysis with a pan-STMN antibody indicated that STMN levels were not markedly changed during exposure to sorbitol (Fig. 1A).

When we investigated the changes in the MAPKs, the phosphorylation of all three MAPK subfamilies was increased in response to hyperosmotic stress (Fig. 1B). JNK phosphorylation, indicative of its activation, was evident at 5 min and peaked at 1–2 h after sorbitol treatment (Fig. 1B). In contrast, p38 MAPK phosphorylation levels did not plateau even at the latest time point investigated (8 h), and ERK phosphorylation peaked at 15 min and returned to basal levels by 8 h of sorbitol stimulation (Fig. 1B). Our results indicate that the kinetics of STMN Ser-25 and Ser-38 phosphorylation correlated with the time courses of ERK and JNK activation, respectively (Fig. 1, C and D). This suggests the possibility that different MAPKs phosphorylate the two different proline-directed serine phosphorylation sites of STMN (STMN Ser-25 and STMN Ser-38).

FIGURE 1. STMN is phosphorylated in response to hyperosmotic stress. A, MEFs were treated with sorbitol (0.5 M) for the indicated times before protein lysates were prepared and blotted with site-specific phosphoserine STMN antibodies. Immunoblotting with a pan-STMN antibody showed equivalent total STMN levels. B, lysates from MEFs treated with sorbitol (0.5 M) over a time course were immunoblotted for levels of phosphorylated and total JNK, p38, and ERK MAPKs. C, phosphorylated STMN Ser-25 and ERK were quantitated and expressed as fold increase over unstimulated controls. D, phosphorylated STMN Ser-38 and JNK were quantitated and expressed as fold increase over unstimulated controls. E, PC12 cells were treated with sorbitol (0.5 M), NGF (50 ng/ml), or 0.1% BSA (w/v) in PBS as a control for the indicated times. Protein lysates were then prepared, and levels of STMN Ser-25 and Ser-38 phosphorylation as well as phosphorylated JNK and ERK were determined by immunoblotting. Total protein levels were also revealed with pan-STMN, JNK, and ERK antibodies. F, phosphorylated STMN Ser-25 and Ser-38 bands were quantitated by densitometric analysis and expressed as fold increase over unstimulated control. Values are the mean ± S.E. (n = 3, ns, not statistically different, Student’s t test).
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FIGURE 2. **JNK inhibition blocks osmotic stress-stimulated STMN phosphorylation.** A, MEFs were pretreated with JNK inhibitor VIII (20 μM, 30 min), SB203580 (20 μM, 30 min), U0126 (10 μM, 30 min), or DMSO (0.1% v/v) before sorbitol treatment (0.5 M, 15 or 60 min) or left unstimulated (0 min). Protein lysates were then blotted for STMN Ser-25 and Ser-38 phosphorylation and STMN levels. B, MEFs were pretreated with either JNK inhibitor VIII (inh, 20 μM, 30 min) or U0126 (10 μM, 30 min) alone or JNK inhibitor VIII and U0126 in combination. DMSO (0.1% v/v) was used as a vehicle control. MEFs were then stimulated with sorbitol (0.5 M, 15 or 60 min) or left un-stimulated as a control (0 min). STMN Ser-25 and Ser-38 phosphorylation and STMN levels were then determined by immunoblot analysis. Sorbitol-stimulated (60 min) STMN Ser-38 phosphorylation (C) and STMN Ser-25 phosphorylation (D) in the presence of kinase inhibitors were quantified by densitometric analysis and expressed as -fold change over unstimulated DMSO-treated controls. Values are the mean ± S.E. (n = 3; *, statistically significant compared with sorbitol-stimulated cells pre-treated with DMSO; ns, not statistically different to DMSO pretreatment, Student’s t test). E, protein lysates from PC12 cells pretreated with JNK inhibitor VIII (20 μM, 30 min) or DMSO (0.1% v/v) followed by sorbitol treatment (0.5 M) for the indicated times were blotted for phospho-Ser-25 or phospho-Ser-38 STMN. Equivalent protein loading in each lane was demonstrated by blotting for GAPDH.

We compared these stress-stimulated changes with those elicited by growth factor stimulation by examining rat pheochromocytoma cells, PC12, treated either with 0.5 M sorbitol or with NGF that has been shown to stimulate STMN phosphorylation (26). This revealed NGF-stimulated phosphorylation levels of STMN Ser-25 and Ser-38 that were comparable with those observed after hyperosmotic stress (Fig. 1E). The levels of growth factor-stimulated STMN Ser-25 or Ser-38 phosphorylation were not significantly different to that stimulated by osmotic stress (Fig. 1F). In contrast, the phospho-MAPK profiles differed; 0.5 M sorbitol potently stimulated JNK phosphorylation with only marginal increases in ERK phosphorylation, whereas NGF stimulated phosphorylation of ERK but not JNK (Fig. 1E). Thus, despite comparable levels of STMN phosphorylation after different forms of stimulation, the profiles of MAPK phosphorylation differed markedly.

**JNK Is a Major Mediator of STMN Phosphorylation during Hyperosmotic Stress**—To address the contribution of MAPKs to hyperosmotic stress-stimulated STMN Ser-25 and Ser-38 phosphorylation, we used JNK inhibitor VIII, SB203580, and U0126 as chemical inhibitors of JNK, p38, and MEK/ERK, respectively. The efficacies of the JNK or MEK/ERK inhibitors were directly shown under our experimental conditions with JNK inhibitor VIII pretreatment sufficient to block sorbitol-stimulated c-Jun phosphorylation (supplemental Fig. 1A), and U0126 substantially reduced ERK phosphorylation in response to 0.5 M sorbitol (supplemental Fig. 1B). In addition, JNK or p38 inhibition did not perturb sorbitol-stimulated ERK phosphorylation (supplemental Fig. 1B).

In extending this analysis to include evaluation of STMN during hyperosmolar stress, we observed that the pretreatment of MEFs with JNK inhibitor VIII blocked sorbitol-stimulated STMN Ser-38 phosphorylation, whereas U0126 or SB203580 pretreatment were each without effect (Fig. 2A). Conversely, pretreatment with each of these three inhibitors partially blocked sorbitol-stimulated increases in STMN Ser-25 phosphorylation, suggesting a contribution by these different kinases in stress-stimulated STMN Ser-25 phosphorylation (Fig. 2A). Indeed, our pretreatment of MEFs with a combination of both JNK inhibitor VIII and U0126 was sufficient to abolish STMN Ser-25 phosphorylation in response to sorbitol, highlighting the combined roles of JNK and ERK in regulation of sorbitol-stimulated STMN Ser-25 phosphorylation (Fig. 2B). Conversely, the effect of inhibiting both JNK and ERK simultaneously on STMN Ser-38 phosphorylation levels was comparable with the effect of JNK inhibition alone (Fig. 2B). Quantitative densitometric analysis confirmed that MEK and p38 MAPK inhibition did not significantly decrease sorbitol-stimulated STMN Ser-38 phosphorylation, whereas JNK inhibition was sufficient to substantially attenuate STMN Ser-38 phosphorylation (Fig. 2C). In contrast, either JNK or MEK inhibition partially reduced sorbitol-stimulated STMN Ser-25 phosphorylation (Fig. 2D). Pretreatment with SB203580 did not significantly inhibit STMN Ser-25 phosphorylation (Fig. 2D). Furthermore, co-treatment with both JNK and MEK inhibitors further reduced sorbitol-stimulated STMN Ser-25 phosphorylation to near basal levels (Fig. 2D). Thus, JNK...
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To consider whether the responses to hyperosmotic stress were relevant in other cellular contexts, we further examined the effects of the JNK inhibitor VIII in PC12 cells. We showed the striking effects of JNK inhibitor VIII to inhibit phosphorylation of both STMN Ser-38 and STMN Ser-25 (Fig. 2E). Furthermore, in agreement with a previous report (26), MEK/ERK inhibition was sufficient to block NGF-stimulated phosphorylation of both STMN Ser-25 and Ser-38 in PC12 cells (supplemental Fig. 1C). In contrast, JNK inhibition did not attenuate NGF-stimulated STMN Ser-25 or Ser-38 phosphorylation, indicating that ERK, not JNK, was the predominant kinase targeting STMN during neurotrophic stimulation in PC12 cells. Taken together, these results indicate that an important link between JNK activation and STMN phosphorylation was not restricted to fibroblasts during their response to osmotic stress but also that JNK and ERK make different contributions to STMN phosphorylation under conditions of stress and growth factor stimulation.

We capitalized on the availability of JNK-deficient (JNK1/2−/−) MEFs (27) to evaluate the contribution of JNK1 and JNK2 to stress-stimulated changes in STMN phosphorylation independent of the use of chemical inhibitors. After immunoblot analysis confirming the absence of JNK expression in JNK1/2−/− MEFs, we found that sorbitol treatment did not stimulate STMN Ser-38 phosphorylation to the same level as wild-type (WT) MEFs (Fig. 3A). We confirmed that sorbitol-stimulated STMN Ser-38 phosphorylation in JNK1/2−/− MEFs was significantly lower when compared with identically treated WT MEFs (Fig. 3B).

With the reconstitution of JNK expression in JNK1/2−/− MEFs with transient expression of Myc-tagged constructs of both JNK1 and JNK2, sorbitol-stimulated STMN Ser-38 phosphorylation was markedly increased (Fig. 3C). To consider the possibility of JNK isoform-specific contributions, we further examined STMN phosphorylation in response to sorbitol exposure upon transient expression of equivalent levels of either Myc-JNK1 or Myc-JNK2 in JNK1/2−/− MEFs.

The presence of JNK1 or JNK2 alone allowed maximum STMN Ser-38 phosphorylation in response to hyperosmotic stress (Fig. 3D). We showed that sorbitol-stimulated STMN Ser-38 phosphorylation in JNK1/2−/− MEFs was similarly restored by reconstituting with JNK1-Myc, JNK2-Myc, or JNK1/2-Myc (Fig. 3E). These findings indicated that either JNK isoform was capable of mediating STMN phosphorylation in response to stress.

JNK-dependent STMN Phosphorylation under a Range of Stress Conditions; JNK Activation Is Sufficient for STMN Phosphorylation—Our finding of JNK/STMN signaling during osmotic stress raised the question of the broader relevance of this mechanism as part of a cell stress response. We, therefore,
investigated JNK-mediated STMN phosphorylation stimulated by stresses other than hyperosmolarity. In PC12 cells we observed phosphorylation of STMN Ser-38 and JNK in response to cytotoxic arsenite treatment and heat stress (Fig. 4A). Arsenite and heat shock–stimulated increases in STMN Ser-38 phosphorylation were inhibited by JNK inhibitor VIII but not U0126 (Fig. 4B). In contrast, the oxidative stress stimuli, H2O2, did not stimulate JNK or STMN phosphorylation in PC12 cells (Fig. 4A). These studies indicate STMN can be targeted for phosphorylation in response to a range of extracellular stress stimuli when JNK is activated.

We extended our analysis to determine whether the specific activation of JNK in the absence of stress stimulation was sufficient to trigger robust STMN phosphorylation in the absence of a stress stimulus.

In Vitro Characterization of STMN Targeting by JNK—Previous reports have indicated that STMN may act as a substrate for several MAPK family members (21, 28). We next characterized STMN as a direct phosphorylation target of JNK and made comparisons with the phosphorylation of STMN by the related MAPKs, ERK1, and p38α. In our in vitro kinase assays using purified recombinant proteins, GST-STMN was readily phosphorylated by recombinant JNK1 and ERK1 but not p38α, as determined by 32P incorporation from [γ-32P]ATP into GST-STMN (Fig. 5A). Indeed, under our assay conditions, the extent of GST-STMN phosphorylation by JNK1 was comparable with that mediated by ERK1 (Fig. 5A), suggesting effective targeting by either protein kinase.

To determine the STMN residues targeted by JNK, we analyzed our in vitro kinase assays by immunoblot analysis with site-specific phospho-STMN antibodies. Our results indicated that co-incubation of JNK1, JNK2, or JNK3 with GST-STMN resulted in robust STMN Ser-25 and Ser-38 phosphorylation (Fig. 5B). In contrast, ERK1 predominantly phosphorylated STMN Ser-25 and did not phosphorylate STMN Ser-38 (Fig. 5B). These in vitro findings are in agreement with our findings utilizing chemical inhibitors in cells (Fig. 2) that showed a preference for ERK in targeting STMN S25 but that JNK can phosphorylate STMN on both residues Ser-25 and Ser-38.

When we compared JNK-mediated phosphorylation of GST-STMN with a STMN mutant in which all conserved Ser residues were changed to Ala (GST-STMNtetraA), no 32P incorporation was detected for this STMN mutant (Fig. 5C). Quantitation from three independent experiments showed linear incorporation of 32P into GST-STMN over the time points investigated (Fig. 5D). In contrast, JNK1-mediated phosphorylation of GST-STMNtetraA was completely abolished (Fig. 5D). Thus, these results highlight that JNK phosphorylation of STMN was restricted to one or more of the conserved STMN Ser residues in vitro, and so we effectively also eliminated the possibility that JNK mediated phosphorylation of additional non-regulatory residues of STMN under in vitro conditions.

To determine the STMN sites phosphorylated by JNK and to more specifically probe the relative stoichiometries of phosphorylation at the different regulatory sites of STMN, we generated single Ser → Ala mutants of each conserved Ser residue and tested these recombinant protein substrates in our in vitro kinase assays. We found that the JNK-catalyzed 32P incorporation into GST-STMN S25A mutant was reduced to 41 ± 5% (n = 3) of WT GST-STMN phosphorylation levels (Fig. 6A). Furthermore, recombinant JNK1-mediated phosphorylation of the GST-STMN S38A mutant was reduced to 13 ± 1% (n = 3) of GST-STMN WT levels (Fig. 6A). In contrast, the JNK1-mediated phosphorylation of GST-STMN S16A or S63A was not markedly different from the JNK1-mediated phosphorylation of GST-STMN WT (Fig. 6A). Representative autoradiographs show the partial loss of JNK1-mediated 32P incorporation into the GST-STMN S25A mutant and the near total loss of phosphorylation of GST-STMN S38A (Fig. 6B), highlighting the substrate preferences of JNK1 toward STMN Ser-38 but also the JNK-mediated phosphorylation of STMN Ser-25.

**FIGURE 4. JNK-dependent STMN phosphorylation in response to arsenite and heat shock.** A, PC12 cells were treated with H2O2 (1 mM), arsenite (300 μM), or heat shock (43 °C) for the indicated times before blotting for STMN Ser-38 phosphorylation, JNK phosphorylation, and total STMN and JNK levels. B, PC12 cells were pretreated with JNK inhibitor VIII (inh, 20 μM, 30 min) before stress treatments with arsenite (300 μM, 1 h), heat shock (43 °C, 1 h), or sorbitol (0.5 M, 1 h). Protein lysates were then prepared and blotted for STMN Ser-38 phosphorylation, total STMN, c-Jun, and JNK.
To establish the substrate preferences of both JNK and ERK, we, therefore, compared our JNK-mediated phosphorylation results with the ERK1-mediated phosphorylation of this series of STMN Ser → Ala mutants. As shown in Fig. 6C, no ERK-mediated phosphorylation of GST-STMN S25A was observed (1 ± 0.6% of GST-STMN WT, n = 3). In contrast, GST-STMN S38A phosphorylation levels by ERK1 were 87% of GST-STMN WT levels. Statistical evaluation of these results indicated that the levels of 32P incorporated in GST-STMN S38A and GST-STMN WT by ERK1 were not significantly different (p = 0.21, n = 3; Fig. 6C). ERK1-mediated phosphorylation of GST-STMN S16A and S63A did not differ from GST-STMN WT (Fig. 6C). Representative autoradiographs showed the complete loss of phosphorylation in the GST-STMN S25A mutant, but other GST-STMN Ser → Ala mutants were unchanged compared with GST-STMN WT (Fig. 6D). In summary, our *in vitro* assays revealed that JNK targeted STMN Ser-25 and Ser-38, whereas ERK1 phosphorylated STMN Ser-25 predominantly. These *in vitro* results are again in accord with our results in cellular studies where STMN serine residues are preferentially phosphorylated by these different MAPKs (Fig. 2).

Our observation that JNK1-mediated phosphorylation of GST-STMN S38A was greatly reduced compared with WT STMN highlighted STMN Ser-38 as the predominant JNK target site. Because we also found that JNK phosphorylation of GST-STMN S25A was reduced by ~50%, we explored the possible links between JNK targeting of STMN Ser-25 and Ser-38. To examine an unprecedented link between JNK-mediated STMN Ser-25 phosphorylation and STMN Ser-38 phosphorylation, we thus performed immunoblot analysis of *in vitro* kinase reactions with the site-specific phospho-STMN antibodies and showed that the S38A mutation resulted in a substantial reduction in Ser-25 phosphorylation by JNK1 (Fig. 6F). In contrast, JNK1 phosphorylation of Ser-38 was unaffected in the S25A STMN mutant (Fig. 6E).

Further evidence of a requirement for STMN Ser-38 site phosphorylation for efficient JNK-mediated phosphorylation on Ser-25 was demonstrated in COS-1 cells by transiently expressing Myc-tagged STMN WT and Ser → Ala mutants. Myc-tagged STMN constructs were distinguishable by size from endogenous STMN, expressed to comparable levels, and basally phosphorylated (example lane 4, Fig. 6F). Immunoblot analysis with phospho-Ser-25 STMN antibody indicated that 0.5 M sorbitol treatment increased the phosphorylation of Myc-STMN Ser-25 and Ser-38, whereas ERK1 phosphorylated STMN Ser-25 predominantly.

Identification of a STMN JNK Binding Domain—Kinase docking sites can facilitate MAPK-dependent substrate phosphorylation through physical interaction that improves sub-
Our results indicate that JNK phosphorylation of STMN may regulate microtubule organization during cellular responses to stress. We, therefore, investigated microtubule changes in response to stress by immunoblotting for a post-translationally modified form of tubulin, detyrosinated tubulin (also named Glu-tubulin for the penultimate glutamate revealed by reversible removal of tyrosine from the α-tubulin C terminus) (32). Tubulin detyrosination occurs on microtubules with slow-turnover kinetics (32) and, therefore, is a marker of stable, “longer-lived” microtubules. Immunoblot analysis revealed that

<ref>FIGURE 6. STMN Ser-38 is the predominant target site for JNK-mediated phosphorylation. A, GST-STMN with Ser → Ala mutations on Ser-16, Ser-25, Ser-38, or Ser-63 (10 μg) were incubated with active recombinant JNK1 (10 ng), and the reactions were stopped at the time points indicated. Wild-type GST-STMN (WT) and GST-STMN tetraA were used as positive and negative controls, respectively. 32P incorporation (pmol) was calculated from Cerenkov counts, with values representing mean ± S.E. 32P incorporation (pmol) was from $n = 3$. B, GST-STMN with Ser → Ala mutations on Ser-16, Ser-25, Ser-38, or Ser-63 (10 μg) were incubated with active recombinant ERK1 (10 ng), and the reactions were stopped at the time points indicated. Wild-type GST-STMN (WT) and GST-STMN tetraA were used as positive and negative controls, respectively. 32P incorporation (pmol) was calculated from Cerenkov counts, with values representing mean ± S.E. C, GST-STMN with Ser → Ala mutations on Ser-16, Ser-25, Ser-38, or Ser-63 (10 μg) were incubated with active recombinant JNK1 (10 ng), and the reactions were stopped at the time points indicated. GST-STMN WT and GST-STMN tetraA were used as positive and negative controls, respectively. Values represent the mean ± S.E. 32P incorporation (pmol) was from $n = 3$. D, GST-STMN S25A and GST-STMN S38A were phosphorylated with JNK1 in a non-radioactive kinase reaction and immunoblotted with site-specific phospho-STMN antibodies. Blotting with a pan-STMN antibody was used to show equivalent protein loading between lanes. F, COS-1 cells were transiently transfected with Myc-tagged GST-STMN WT, GST-STMN S25A, GST-STMN S38A, or GST-STMN S25A/S38A or a Myc-tagging vector as control. After transfection, cells were stimulated with sorbitol (0.5 M, 30 min), sorbitol along with JNK inhibitor VIII (20 μM) treatment, or left untreated as a negative control. STMN Ser-25 phosphorylation and STMN protein expression were then assessed by immunoblotting. Inh, inhibitor.</ref>
Glu-tubulin levels were elevated in MEFs in response to hyperosmotic stress (Fig. 8A). An increased level of Glu-tubulin was evident within 2 h and was increased substantially during 8 h of sorbitol treatment (Fig. 8A). In addition, an analysis of tubulin polymer levels by partitioning into free and polymerized fractions indicated that the levels of polymerized tubulin in MEFs were elevated under osmotic stress conditions (Fig. 8B). These results indicate that the microtubule array in fibroblasts is stabilized in direct response to osmotic stress.

To determine whether stress-induced microtubule stabilization was JNK-dependent, we investigated the levels of deytrosine-ated microtubules in JNK1/2−/− MEFs. Immunofluorescence staining with a Glu-tubulin-specific antibody revealed that the levels of post-translationally modified tubulin in WT MEFs were increased in response to osmotic stress (Fig. 8C), which was consistent with our biochemical analysis (Fig. 8A). However, treatment of JNK1/2−/− MEFs with sorbitol did not induce an increase in Glu-modified microtubules (Fig. 8C). Immunoblot analysis with the Glu-tubulin antibody was also in agreement with this finding. Whereas Glu-tubulin levels were increased in WT MEFs in response to 2 h sorbitol treatment, Glu-tubulin levels were unchanged in response to osmotic stress (Fig. 8D).

These studies indicate that stress-induced stabilization of microtubules in fibroblasts is mediated by JNK and highlights a role for JNK/STMN signaling in regulating microtubule organization during cell stress.

**JNK Signaling to STMN Regulates Protection against Cellular Stress**—With our studies revealing STMN as a novel substrate of JNK in response to stress-signaling, we next investigated the function of this mechanism in regulating cell viability in response to stress stimulation. We first showed that, when challenged with hyperosmotic stress (sorbitol, 0.5 M, 6 h), the viability of JNK1/2−/− MEFs was substantially reduced (29 ± 5% viable JNK1/2−/− MEFs) compared with control WT MEFs (81 ± 4%) (Fig. 9A). Similarly, chemical inhibition of JNK activity reduced viability (14 ± 2 versus 34 ± 4% when pretreated with the DMSO vehicle) in PC12 cells treated with sorbitol (0.5 M) for 8 h (Fig. 9B). These studies highlight a function for JNK in maintaining viability in MEFs and PC12 cells during hyperosmolar stress.

The depolymerization of the microtubule network is also recognized as an early event in apoptosis execution, and loss of microtubules can initiate cell death (33, 34). Thus, we investigated the contribution of microtubule stabilization to cell responses to stress.

We first evaluated the effect of taxol-induced microtubule stabilization on cytotoxicity induced by hyperosmolarity. In Fig. 9C, we showed that in WT MEFs pretreated with a DMSO control, cell viability was reduced by 57 ± 10% in response to sorbitol treatment. In contrast, cell loss was prevented by taxol pretreatment, with greater than 86 ± 6% (n = 3, p < 0.05) of cells remaining viable after identical sorbitol treatment (Fig. 9C).
STMN siRNA may be underestimated, as only a non-targeting siRNA control in response to osmotic stress response to hyperosmolarity treatment (supplemental Fig. 3). This has previously been shown to result in the expansion of interphase microtubules (35). We demonstrated in PC12 cells that transient transfection of STMN siRNA significantly improved cell viability (49 ± 8% viable versus 31 ± 2% with a non-targeting siRNA control) in response to osmotic stress (Fig. 9D). In these experiments the cytorepression conferred by STMN siRNA may be underestimated, as only ~40% of PC12 cells were transfected as estimated with a FITC-tagged non-targeting siRNA control. Similar STMN down-regulation in WT MEFs improved the viability of WT MEFs in response to hyperosmolarity treatment (supplemental Fig. 3).

As we had demonstrated JNK-dependent STMN Ser-38 phosphorylation in response to heat shock and arsenite treatment (Fig. 8). Thus, consistent with a previous report (34), chemically-stabilized microtubules protected against detrimental stress stimuli.

To evaluate cytoprotection by STMN-regulated microtubules, we down-regulated STMN levels using siRNA (Fig. 9D). This has previously been shown to result in the expansion of interphase microtubules (35). We demonstrated in PC12 cells that transient transfection of STMN siRNA significantly improved cell viability (49 ± 8% viable versus 31 ± 2% with a non-targeting siRNA control) in response to osmotic stress (Fig. 9D). In these experiments the cytorepression conferred by STMN siRNA may be underestimated, as only ~40% of PC12 cells were transfected as estimated with a FITC-tagged non-targeting siRNA (data not shown). Similarly STMN down-regulation in WT MEFs improved the viability of WT MEFs in response to hyperosmolarity treatment (supplemental Fig. 3).

As we had demonstrated JNK-dependent STMN Ser-38 phosphorylation in response to heat shock and arsenite treatment, we subsequently tested the effect of STMN down-regulation on the viability of PC12 cells in response to these stress stimuli. We found that transient transfection with STMN siRNA improved the viability of cells exposed to heat shock (Fig. 9E, 76 ± 5.5% viable versus 41 ± 3.3% with a non-targeting siRNA control) or chemotoxic arsenite treatment (Fig. 9F, 71 ± 6.8% viable versus 55 ± 5.6% with a non-targeting siRNA control). Thus, microtubule expansion and stabilization due to STMN loss can be cytoprotective against an acute stress insult. Taken together, our studies suggest that JNK phosphorylation of STMN to inhibit STMN signaling events result in robust phosphorylation of STMN

**FIGURE 8. Stress-stimulated microtubule stabilization is JNK-dependent. A, MEFs were stimulated with sorbitol (0.5 M) for the indicated times and blotted for post-translationally modified Glu-tubulin and α-tubulin. B, tubulin polymers (p) were separated from soluble tubulin (s) by differential centrifugation of protein extracts from sorbitol-stimulated (0.5 M, 0, 30, or 120 min) WT MEFs. Soluble and polymerized tubulin fractions were then blotted with α-tubulin. α-Tubulin bands were quantified by densitometric analysis, and tubulin polymers were calculated as a percentage of total tubulin. Values are the mean ± S.E. (n = 3). C, WT or JNK1/2−/− MEFs grown on glass coverslips were treated with sorbitol (0.5 M, 2 h) or left untreated (Control) before fixing and staining with a Glu-tubulin antibody and a Cy2 conjugated secondary antibody. Images of stable Glu-modified microtubules were then collected using confocal microscopy. The scale bar represents 20 μm. D, WT or JNK1/2−/− MEFs were treated with sorbitol (0.5 M, 2 h) or left untreated (control) before protein lysates were prepared and immunoblotted for Glu-tubulin and α-tubulin.

DISCUSSION

The cytosolic microtubule-destabilizing protein STMN has been previously shown to integrate inputs by protein kinases of different signaling pathways to modulate microtubule dynamics in the regulation of complex cellular processes such as differentiation and proliferation. During growth factor stimulation, both STMN Ser-25 and Ser-38 have been previously established as target sites for proline-directed kinases such as the cyclin-dependent kinases and members of the MAPK family that can, thus, regulate STMN activity in neuron of the brain and proliferating cancer cells (36–40). In contrast, the phosphorylation of STMN in response to stress stimuli and pro-inflammatory cytokines has been noted (22, 25, 41, 42), but the regulation of these events and specifically the protein kinases involved have been less intensively studied.

In the present study we demonstrated that JNK-dependent signaling events result in robust phosphorylation of STMN Ser-25 and Ser-38 in cells during stress conditions and that each of the three major JNK isoforms (JNK1, JNK2, and JNK3) can directly phosphorylate these residues in STMN in vitro in the absence of additional binding proteins or scaffolds. Previous considerations of the phosphorylation of STMN in response to stress have included the actions of various stress-activated p38 MAPK isoforms (21, 42). Under conditions of hyperosmotic stress and as judged by the lack of effects of SB203580, we found only a small contribution of p38-α/β to the phosphorylation of STMN Ser-25 and no contribution to the phosphorylation of STMN Ser-38. Furthermore, in in vitro assays in which p38-α...
FIGURE 9. **JNK signaling to STMN is cytoprotective.** A, WT or JNK1/2−/− MEFs were treated with sorbitol (0.5 M, 6 h) or left untreated as a control. Cell viability was then measured by trypan blue dye exclusion and cell counting. Viable cells are expressed as a percentage of total cell number. Values are the mean ± S.E. (n = 3). B, PC12 cells were pretreated with SP600125 (20 μM, 30 min) or an equivalent volume of DMSO (0.1% v/v) before sorbitol treatment (0.5 M, 8 h). Cell viability was then measured by trypan blue dye exclusion and cell counting. Values are the percentage of viable cells (mean ± S.E., n = 3). C, WT MEFs were pretreated with taxol (10 μM, 30 min) or an equivalent volume of DMSO (0.1% v/v) before treatment with sorbitol (0.5 M, 8 h). Cell viability was then determined by trypan blue staining (mean ± S.E., n = 3). D, E, and F, PC12 cells were transiently transfected with STMN siRNA or a non-targeting sequence (Control siRNA). Cell viability after sorbitol (0.5 M, 8 h) (D), heat shock (43 °C, 2 h) (E), or arsenite (300 μM, 8 h) (F) treatment was then measured by trypan blue staining. Percentage values of the viable cells are the mean ± S.E. (n = 3); STMN expression was determined by immunoblot analysis, and GAPDH blotted to demonstrate equivalent loading.

was directly incubated with recombinant STMN, we did not observe significant levels of STMN phosphorylation even over a prolonged time course of reaction. Previous studies have indicated a likely reason for this lies in a p38 isoform-specific targeting of STMN. Thus, the p38 family member, p38α, was previously shown to have potent activity toward STMN (42). The contributions by p38α would, therefore, be expected in tissues with significant expression of this p38 isoform and after appropriate stimulation of the cells to elicit robust p38α activation.

When a contribution by JNK in regulating STMN and STMN-like proteins has been previously considered by initial analyses in *in vitro* kinase assays, the highest activity of JNK was noted for the STMN-like protein, SCG10, but with lower activity of JNK toward STMN (28). Indeed, SCG10 was phosphorylated predominantly by the JNK1 isoform, and this has ramifications for the understanding of how JNK isoform-specific modulation impacts on the brain (28). Our studies substantially expand these observations by showing for the first time that in fibroblasts and PC12 cells STMN Ser-25 and Ser-38 can be effectively phosphorylated in response to osmotic stress; the extent of stress-induced STMN phosphorylation was comparable with that observed after growth factor (NGF) stimulation. In addition, STMN targeting by JNK was dependent on the short interaction motif in STMN. Thus, we found that a small peptide, TI-JIP, derived from a minimal JBD of JIP1 blocked JNK-mediated STMN phosphorylation. This revealed the requirement of a STMN JBD in JNK-mediated phosphorylation. We identified the 41KKKDLSEL48 sequence as a STMN JBD, with Leu-45 and Leu-47 representing critical residues required for JNK targeting of STMN. An identical motif can also be found in SCG10 (75KKKDLSEL83) that is phosphorylated by JNK, but the corresponding sequence in another SCG10-like protein, SCLIP, lacks a critical leucine residue (76KKDTSLE83). This lack of a JNK binding motif in SCLIP is consistent with previous biochemical studies where JNK failed to phosphorylate SCLIP (28). In addition, this region in another STMN family member, RB3 (85RRRDPSLE92), does not match a consensus JBD. Therefore, we hypothesize that RB3 cannot be directly phosphorylated by JNK, but this requires experimental verification.

In cell-based studies, we showed that hyperosmotic stress-induced STMN phosphorylation was largely dependent on JNK. Indeed, the inhibition of JNK activity or the loss of JNK expression largely abrogated STMN Ser-25 and Ser-38 phosphorylation in response to hyperosmotic stress, the residual Ser-25 phosphorylation being attributed to the activity of ERK that was also increased during sorbitol treatment. Furthermore, our reconstitution of individual JNK isoforms in a JNK-null background (JNK1/2−/− MEFs) has demonstrated that either JNK1 or JNK2 can rescue stress-induced STMN phosphorylation in a fibroblast context. Thus, consistent with our *in vitro* kinase assay data, the phosphorylation of STMN does not appear to be JNK isoform-specific, and this contrasts the observations that SCG10 was phosphorylated predominantly by the JNK1 isoform in the brain (28). Taken together, our studies have revealed STMN as a JNK substrate *in vitro* as well as *in vivo* under conditions of cellular stress. In contrast, ERK activity appears to be largely responsible for growth factor-stimulated STMN Ser-25 phosphorylation.
In addition to revealing the differential phosphorylation of STMN by the different MAPK family members, we also discovered an ordered phosphorylation of STMN serine residues by JNK. Specifically, we showed that JNK-mediated phosphorylation of STMN Ser-38 was required for further JNK-mediated phosphorylation of STMN Ser-25. This differed from the observations of ERK-mediated phosphorylation of STMN Ser-25 examined in parallel that did not require prior phosphorylation of STMN Ser-38. Previously, it has been reported in vitro assays of microtubule stability that the double phosphorylation of STMN Ser-25 and Ser-38 only caused a small impact on the ability of STMN to bind microtubules and induce catastrophe when compared with the double phosphorylation of STMN Ser-16 and Ser-63 that strongly inhibited STMN (43). However, the mitotic phosphorylation of STMN Ser-25 and Ser-38 sites by cyclin-dependent kinases appeared necessary for subsequent Ser-16 and Ser-63 phosphorylation by other protein kinases to permit G2/M transition during cell cycle progression (18). Furthermore, the complete phosphorylation of STMN on all four serine residues results in the greatest inhibition of the microtubule-destabilizing activity of STMN (44). Thus, JNK phosphorylation of STMN Ser-25 and Ser-38 has the potential to impact significantly on subsequent targeting by other kinases and serve an important role in microtubule network regulation during cellular stress.

Although our studies highlight a complex regulatory mechanism involved in stress-activated JNK targeting of STMN in mammalian cells, our results also raise the significance of JNK-mediated STMN phosphorylation. JNK regulation of microtubules has been shown to be required for proper neuronal development (45, 46). This may involve JNK targeting of kinesin to regulate axonal transport (47) and phosphorylation of STMN-like protein SCG10 in the control of neurite elongation (28). Although a recent report has also described a requirement for JNK in regulating microtubule dynamics during axon regeneration (48), the mechanism involved is still unknown. The negative regulation of STMN by JNK may play a key role, as it is unlikely that negative regulation of Golgi-restricted STMN-like proteins, such as SCG10, would be sufficient to initiate a global up-regulation of microtubule dynamics required for nerve regeneration. Our findings indicate that the role of JNK regulation of microtubules extends to the maintenance of cell viability in response to acute stress.

Finally, the use of chemical stabilizers of microtubules, such as taxol, has additionally provided functional evidence that microtubule stabilization is cytoprotective in the cell stress response. Taxol protects different cell types from a range of detrimental conditions, including cytoprotective effects for cardiac myocytes and neurons exposed to oxidative stress and cytotoxic proteins, respectively (34, 49, 50). These studies emphasize that rapid microtubule array stabilization can form part of a response that maintains cell survival and function in the defense against cytotoxic stress. Critically, the role of STMN as a regulator of microtubule stability and function in the response to stress has not been defined. In our study we have shown that STMN knockdown was sufficient to protect cells against osmotic stress, thus, indicating that negative regulation of STMN and the ensuing microtubule stabilization is cytoprotective. Our findings suggest that STMN acts as a novel regulator of cytoprotection downstream of stress-activated JNK signaling and provides a counterpoint to other established pro-apoptotic roles of JNK (4) that trigger proteasomal degradation of key anti-apoptotic proteins and target pro-apoptotic proteins directly to promote cell death (14, 51, 52). Our study now adds STMN to the list of microtubule regulatory proteins known to be directly targeted by JNK, a list that includes Tau, MAP2, and doublecortin (3). JNK regulation of microtubule-stabilizing proteins, such as MAP2, was initially characterized in context of neuronal development (45). Thus, our studies raise the possibility of important contributions by other microtubule-regulatory proteins in JNK-dependent microtubule changes linked to cell stress response that should be addressed in future studies.

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