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Hyperosmotic Stress Signaling to the Nucleus Disrupts the Ran Gradient and the Production of RanGTP[D]

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The RanGTP gradient depends on nucleocytoplasmic shuttling of Ran and its nucleotide exchange in the nucleus. Here we show that hyperosmotic stress signaling induced by sorbitol disrupts the Ran protein gradient and reduces the production of RanGTP. Ran gradient disruption is rapid and is followed by early (10–20 min) and late (30–60 min) phases of recovery. Results from SB203580 and siRNA experiments suggest the stress kinase p38 is important for Ran gradient recovery. NTF2 and Mog1, which are transport factors that regulate the nuclear localization of Ran, showed kinetics of delocalization and recovery similar to Ran. Microinjection of a nuclear localization signal reporter protein revealed that sorbitol stress decreases the rate of nuclear import. Sorbitol stress also slowed RCC1 mobility in the nucleus, which is predicted to reduce RCC1 dissociation from chromatin and RanGTP production. This was tested using a FRET biosensor that registers nuclear RanGTP levels, which were reduced in response to sorbitol stress. Although sorbitol alters nucleotide levels, we show that inverting the GTP/GDP ratio in cells is not sufficient to disrupt the Ran gradient. Thus, the Ran system is a target of hyperosmotic stress signaling, and cells use protein localization–based mechanisms as part of a rapid stress response.

INTRODUCTION

Cells subjected to UV radiation or oxidative, mechanical, or aniso-osmotic stress undergo both short- and long-term adaptation. Hyperosmotic stress induces rapid dehydration that drives an increase in intracellular salt concentration and a decrease in cell volume, placing physical strain on both the cytoskeleton and the plasma membrane (Lang *et al.*, 1998). In response to these changes, cells rapidly initiate a stress signaling cascade and attempt to restore iso-osmolarity through transport of inorganic ions and initiate an accompanying reorganization of the actin cytoskeleton (Haussinger, 1996; Di Ciano *et al.*, 2002). Short-term recovery of cell volume is facilitated by increasing intracellular concentrations of inorganic ions; however, elevated levels of intracellular salts can be detrimental to protein structure and function (Yancey *et al.*, 1982; Russo *et al.*, 2003). Long-term adaptation to hyperosmotic stress is achieved through signal transduction pathways that communicate with the metabolic and transcriptional machinery, resulting in the production or accumulation of organic osmolytes that increase intracellular osmolarity without adversely affecting protein structure or function (O'Neill, 1999).

One of the most thoroughly studied stress kinases is the mitogen-activated protein kinase (MAPK) p38. p38 and its yeast homologue Hog1p are activated in response to a wide variety of adverse environmental conditions. These include osmotic, UV, and mechanical stress. Cytokines, such as interleukins and tumor necrosis factor α are also known to activate p38 (Ono and Han, 2000). Hog1p and p38 are both phosphorylated on a threonine, glycine, tyrosine (TGY) motif within the kinase activation loop (Thr 180 and Tyr 182 in humans). Activation in response to hyperosmotic stress occurs in the cytoplasm, but the specific mechanism of activation varies among organisms (Brewster *et al.*, 1993; Han *et al.*, 1994; Raingeaud *et al.*, 1995). MAPK cascades usually involve three kinases, each successively phosphorylating the next kinase in the series, the MAPKKK phosphorylates the MAPKK, which, in turn, phosphorylates the MAPK. In higher eukaryotes, the MAPKKK MEKK3, in association with Rac and OSM, activates the MAPKK MKK3, which then activates p38 MAPK (Uhlik *et al.*, 2003). The osmotic sensor that triggers assembly of the Rac-OSM-MEKK3 complex remains undefined. In yeast, the osmosensors are Sln1 and Sho, which regulate the MAPKKKs Ssk2 and Ste11, respectively. Both Ssk2 and Ste11 activate the MAPKK, Pbs2, which then phosphorylates Hog1 (O'Rourke *et al.*, 2002). In yeast, Hog1 translocates to the nucleus in response to osmotic stress–induced phosphorylation (Ferrigno *et al.*, 1998). The regulation of p38 nuclear transport is not well understood in higher eukaryotes. p38 is found in both the cytosol and nucleus before activation and is responsible for activation of transcription under stress conditions (Ben-Levy *et al.*, 1998).

Nuclear import of Hog1p is mediated by the importin-β family member, NMD5. NMD5 binds to activated Hog1p in

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Abbreviations used: FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; YIC, yellow fluorescent protein, importin-β–binding domain, cyan fluorescent protein; GGNLS, GST-GFP-NLS; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; IBB, importin-β–binding domain; N/C, ratio of mean nuclear fluorescence to mean cytoplasmic fluorescence.

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the cytoplasm and then translocates through the nuclear pore complex (NPC) to the nucleus where the GTP bound form of Ran (GSP1) binds to NMD5 and releases Hog1p into the nucleoplasm (Ferrigno *et al.,* 1998). Hog1 export is exportin 1–dependent and is predicted to be exported by forming a trimeric complex with exportin 1 and RanGTP, which translocates to the cytoplasm. On reaching the cytoplasm, the Ran GTPase-activating protein, RanGAP, induces Ran catalyzed hydrolysis of GTP to GDP, resulting in disassembly of the export complex (Bischoff *et al.,* 1994). In *Saccharomyces cerevisiae*, Hog1 is responsible for the activation or repression of 579 genes in response to osmotic stress (O'Rourke and Herskowitz, 2004). Nuclear transport of Hog1p/p38 MAPK is a key aspect of the cellular response to osmotic stress, because multiple nuclear targets of p38 are important for mounting a long-term response to stress (Ko *et al.,* 2002).

An essential feature of most nuclear transport pathways is a dependence on the nucleocytoplasmic gradient of RanGTP (Izaurralde *et al.,* 1997). As mentioned above, RanGTP is required for disassociation of import complexes and is a stoichiometric component of nuclear export complexes. The Ran guanine nucleotide exchange factor (GEF), RCC1 is localized in the nucleus, whereas RanGAP is cytoplasmic. Because of the asymmetric localization of RanGAP and RCC1, Ran in the nucleus is predominantly in the GTP bound state, whereas cytoplasmic Ran is predominantly in the GDP bound state, leading to what is known as the RanGTP gradient. RanGTP is constantly exported to the cytoplasm in association with export complexes. Cytoplasmic RanGDP is imported into the nucleus by its transport receptor, NTF2 (Ribbeck *et al.,* 1998; Smith *et al.,* 1998). Once in the nucleus, RCC1 binds RanGDP and catalyzes exchange of GDP for GTP. With this constant recycling, at steady state, there is both a Ran protein gradient as well as a RanGTP gradient, both of which are concentrated in the nucleus. The Ran-binding protein Mog1 has been linked genetically to the Ran-recycling pathway, between NTF2 import of Ran and the RCC1-catalyzed exchange of nucleotide on Ran (Oki and Nishimoto, 1998; Baker *et al.,* 2001). In *S. cerevisiae*, deletion of Mog1 leads to temperature-sensitive growth and nuclear transport. This can be overcome by over expressing either NTF2 or Ran (GSP1; Oki and Nishimoto, 1998). A temperature-sensitive mutant of RCC1 in yeast is synthetically lethal when combined with deletion of Mog1 (Baker *et al.,* 2001). Mog1 has also been implicated in a role in the osmo-sensing Sln1 signal transduction pathway (Lu *et al.,* 2004). However, there are no known links between Mog1 function in the Ran gradient and its apparent links to the Sln1 pathway in yeast.

The link between Mog1 and the Sln1 pathway is part of the emerging body of evidence that there is cross-talk between the pathways for stress signaling and nuclear transport. In yeast, the arrest of secretion response (ASR), which is brought about through the use of certain Sec mutants, has been shown to result in the delocalization of some nuclear proteins. (Nanduri and Tartakoff, 2001a). Overexpression of Hog1p protects the proper nuclear localization of these proteins during the ASR. In addition, hyper-osmotic stress of yeast has been shown to cause delocalization of nuclear proteins. This occurs in a Pkc1-dependent manner and can be inhibited by overexpression of Hog1 (Nanduri and Tartakoff, 2001b).

In the course of analyzing the effect of stress signaling on nuclear transport, we found that the Ran protein gradient was disrupted in response to osmotic stress. To characterize the mechanism responsible for disruption of the Ran gradient, a battery of assays were used to define how the localization and activity of the nuclear transport machinery is affected by sorbitol stress signaling. This included using assays that measure fluorescence recovery after photobleaching (FRAP) of RCC1 and a RanGTP-sensitive biosensor that registers RanGTP production in the nucleus. We also used high-performance liquid chromatography (HPLC) and an inhibitor that inverts the GTP/GDP ratios, to define the relationship between the Ran gradient and nucleotide levels in the cell. Our results suggest that stress signaling inhibits nuclear transport in mammalian cells by reducing the production of RanGTP in the nucleus.

MATERIALS AND METHODS

Tissue Culture

HeLa cells were grown in DMEM supplemented with 5% newborn calf serum and 5% fetal bovine serum (Invitrogen, Carlsbad, CA). For stress experiments, sorbitol (S3889, Sigma, St. Louis, MO) was diluted from a 2 M stock solution into growth media to a final concentration of 0.4 M. Inhibition of p38 MAPK with SB203580 (Calbiochem, La Jolla, CA) was carried out by preincubating the cells in SB203580 for 1 h before the experiment and maintaining SB203580 during the experiment as well.

Small Interfering RNA

HeLa cells were transfected with siGENOME SMARTpool small interfering RNA (siRNA) against human p38α MAPK (cat. no. M-003512-05-0005, Dharmacon, Boulder, CO) using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's suggestions. The sequences of the sense strand for the siRNAs are as follows: CAAGGUCUCUGGAGGAAUUUU, GUCAGAAGCUUACAGAUGAUU, CCGCUUAUCUCAUUAACAGUU, and GUCCAUCAUUCAUGCGAAAUU. After 24 h, the cells were plated on glass coverslips, and 36 h after transfection the cells were stressed with sorbitol for the appropriate time and processed for immunofluorescence microscopy.

Immunofluorescence Microscopy

HeLa cells were grown on glass coverslips. After experimental treatment, cells were fixed in 3.75% formaldehyde for 30 min and permeabilized in 0.2% Triton X-100 for 5 min. Blocking and antibody incubations were done using a buffer containing 1× PBS, 2% bovine serum albumin, and 2% newborn calf serum.

The following primary antibodies were used: Ran (BD Transduction Laboratories, Lexington, KY), Mog1 (Steggerda and Paschal, 2001), NTF2 (Steggerda *et al.,* 2000), RCC1 (Santa Cruz Biotechnology, Santa Cruz, CA), importin-α (BD Transduction Laboratories), importin-β mAb 3e9 (Chi *et al.,* 1995), and RanGAP, pan- and phospho- p38 (Cell Signaling Technology, Beverly, MA). Secondary antibodies used included Cy3-labeled goat anti-rabbit and fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse (Jackson ImmunoResearch, West Grove, PA).

Immunofluorescence images were taken using Openlab (Improvision, Lexington, MA) on a Nikon Eclipse E800 upright microscope (Melville, NY) with a Hamamatsu C4742–95 CCD and a 60× magnification objective with an NA of 1.40 (Bridgewater, NJ).

Fluorescence Resonance Energy Transfer

The YFP IBB CFP (YIC) fluorescence resonance energy transfer (FRET) sensor (Kalab *et al.,* 2002; Li and Zheng, 2004), which contains the first 104 amino acids of Mouse Rch1 was transfected into HeLa cells, which were then stressed with 0.4 M sorbitol for the indicated time. Cells were washed with phosphate-buffered saline and fixed, and acceptor photobleaching was performed on a Zeiss LSM 510 Meta (Thornwood, NY). Fixation of the cells before FRET was found to have an effect on the absolute values of FRET efficiency, but did not change the relative behavior of the FRET sensor in response to changes in RanGTP concentration (Supplementary Figure S3).

Live Cell Analysis by Fluorescence Recovery after Photobleaching and Microinjection

For live cell imaging, HeLa cells were grown on Delta T dishes (Fisher Scientific, Pittsburgh, PA) and mounted on a Bioptechs (Butler, PA) stage warmer. For fluorescence recovery after photobleaching (FRAP) experiments, the cells were transfected with RCC1-GFP (Li *et al.,* 2003) 24 h before the experiment. In microinjection experiments, the cells were microinjected using an Eppendorf Femtojet/Injectman NI 2 microinjector (Fremont, CA) mounted on a Zeiss 510 Meta laser scanning microscope. GST-GFP-NLS (GGNLS; Welch *et al.,* 1999) in injection buffer (10 mM $NaPO_4$, 70 mM KCl, and 1 mM $MgCl_2$) was clarified and injected with an Eppendorf Femtotip. Several cells within a field were injected, the microscope was switched from wide-field mode to laser scanning confocal mode, and image collection was started. Twenty images were taken over the course of 132.7 s.

Image Quantitation

Immunofluorescence images were quantitated using Openlab (Improvision). Mean nuclear fluorescence (N) and mean cytoplasmic fluorescence (C) were measured for each field. After background subtraction, the ratio of mean nuclear fluorescence (N) to mean cytoplasmic fluorescence (C) was calculated for each field and then averages and SDs were calculated for each time point. Experiments were done at least twice, and quantitation represents a minimum of 50 cells per time point.

ImageJ (http://rsb.info.nih.gov/ij/) was used to quantitate GGNLS import, FRET, and FRAP images taken by the Zeiss LSM 510 Meta confocal laser scanning microscope with a 40× oil immersion objective with an NA of 0.55.

Mean nuclear fluorescence of GGNLS was plotted versus time after injection. Initial rate was calculated by determining the slope of the line through the linear region of the concentration versus time plot, and as such, initial rate is in fluorescence units per second. Mean fluorescence of the cytoplasm was used as the initial concentration. Each cell was then plotted as initial rate versus initial concentration. A line was fit to the data from each time point of sorbitol, utilizing all of the cells for that time point. Using the equation of the line for each sorbitol time point and assuming an ideal initial concentration of 150 fluorescence units, an initial rate of import was calculated for each time point of sorbitol stress in order to plot the changes in initial rate of import during sorbitol stress. The data shown is cumulative data from three separate experiments.

FRET efficiency was calculated as $(I_6 - I_5/I_6) \times 100$ in terms of I_n, where n is the image number. The bleach of the yellow fluorescent protein (YFP) was performed between images 5 and 6, such that FRET efficiency is an expression of the percent dequenching of the cyan fluorescent protein (CFP) acceptor (Karpova *et al.*, 2003). Each time point is representative of 10 cells.

FRAP data were calculated as $(X_n/Y_n)/(X_{prebleach}/Y_{prebleach})$, where X is the bleached area, Y is an unbleached area in the same nucleus, and n is the image number. By normalizing to the prebleach ratio, loss of fluorescence in the nonbleached area is accounted for, and equilibration is represented by a value of 1. The experiment shown contains data from more than 50 cells.

Statistical significance in the form of p values was determined using the *t* test: two-sample assuming equal variances in the data analysis package in Excel (Microsoft, Redmond, WA).

Nucleotide Separation by HPLC

Cells were lysed in 0.6 M perchloric acid, neutralized with 3 M $KHCO_3$, and separated by HPLC using a Partisil SAX (strong anion exchanger) column. The areas under the peaks of each nucleotide species were used to compare the ratio of ATP to ADP and the ratio of GTP to GDP.

Nucleotide Exchange and GAP assays

HeLa cells were stressed for 0, 5, and 30 min with 0.4 M sorbitol. A cytoplasmic extract containing RanGAP was made using 0.5% Triton X-100. The remaining nuclear material was then treated with 0.5% Triton X-100 and 500 mM NaCl to release RCC1. The extracts were dialyzed into 1× transport buffer (20 mM HEPES, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, and 0.5 mM EGTA) + 1 mM dithiothreitol + 2 mM sodium vanadate.

The RanGAP assay was carried out using ^{32}P-α–labeled GTP loaded on recombinant Ran (Bischoff *et al.*, 1994). The RanGTP was incubated with 100 ng of recombinant RanGAP or 5 μg of cell extract from the 0-, 5-, or 30-min sorbitol treatment. The assay was carried out for 30 min and stopped with SDS, and the nucleotide was resolved using thin-layer chromatography (TLC). TLC was performed with TLC tank buffer (1 M formic acid, 0.5 M LiCl) using cellulose PEI (polyethylenemine) plates from J. T. Baker Chemical (4475-00; Phillipsburg, NJ).

Ran loaded with ^{32}P-α–labeled GDP was incubated with RCC1 containing extract in the presence of excess cold GTP. An aliquot was removed at 2-min intervals for 18 min, bound to a nitrocellulose filter, and washed. Radioactivity was measured with a scintillation counter.

RESULTS

Sorbitol Stress Induces Relocalization of Nuclear Transport Factors

We set out to characterize the effect of stress signaling on the Ran GTPase using sorbitol as a stimulus for hyper-osmolarity–induced stress. HeLa cells were treated with 0.4 M sorbitol for 30 min, and immunofluorescence microscopy was used to determine the localization of endogenous Ran (Figure 1A). Mean nuclear fluorescence (N) and mean cytoplasmic fluorescence (C) were measured for multiple fields of cells and the average N/C ratio for 50–100 cells per condition was calculated. In response to sorbitol, the level of Ran in the nucleus decreased, while the level in the cytoplasm increased. This resulted in reduction of the Ran N/C ratio from 2.36 to 1.71, which corresponds to a 28% decrease in the N/C ratio ($p < 0.005$; Figure 1B).

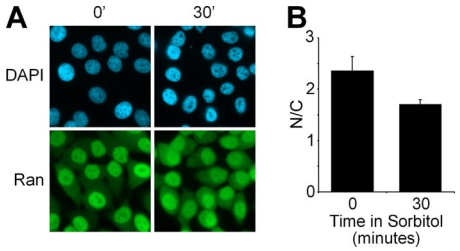

Figure 1. Osmotic stress causes a decrease in the Ran gradient. (A) HeLa cells were stressed with 0.4 M sorbitol for 0 and 30 min. DAPI was used to detect DNA and endogenous Ran was detected by indirect immunofluorescence. (B) Quantitation of the nuclear-to-cytoplasmic (N/C) ratio of Ran based on microscopy from A. Error bars, SD. $p < 0.005$ between 0 and 30 min.

We addressed the kinetics of sorbitol-induced changes in protein localization by analyzing samples at six time points (0, 5, 10, 20, 30, and 60 min). HeLa cells were exposed to 0.4 M sorbitol, fixed, and processed for indirect immunofluorescence using antibodies to nuclear transport factors (Figure 2). Quantitation of the data from the expanded time course made it clear that the changes in the localization of Ran and other transport factors were more pronounced early in the stress response. At later time points, many of the transport factors had partially re-established a prestress, steady-state localization (Figure 3). Several trends were noted for individual transport factors. Ran, Mog1, and NTF2 all showed a decrease in their N/C ratio in the first 5–10 min. This was followed by a partial recovery of the N/C ratio at 20 min, followed by a second reduction at 30 min. Although the RCC1 N/C ratio undergoes very small changes, the kinetics are similar to Ran, Mog1, and NTF2. From 30 to 60 min, Mog1, NTF2, and RCC1 show a slight drop, whereas Ran continues to recover during this period. Importin-β begins to become more nuclear between 5 and 10 min and then gradually accumulates in the nucleus. The N/C ratio of importin-α fluctuates for the first 30 min of stress, but maintains a predominantly cytoplasmic localization. From 30 to 60 min, however, importin-α also accumulates in the nucleus. Overall, Ran and its regulatory proteins appear to undergo similar changes in N/C ratio and accumulate in the cytoplasm in response to sorbitol. Proteins regulated by Ran, namely the importins, accumulate in the nucleus in response to the sorbitol stress, most likely due to the mislocalization of Ran.

Sorbitol Stress Causes a Defect in Nuclear Import of a Fluorescent NLS-Cargo

Mislocalization of components of the nuclear transport machinery suggested that sorbitol stress may negatively influence nuclear transport. We microinjected an NLS-tagged fluorescent reporter protein, GST-GFP-NLS (GGNLS), into HeLa cells during the aforementioned time course of sorbitol stress to measure import rates (Figure 4A). Injections were recorded by time-lapse confocal microscopy, and the rate of import into the nucleus (fluorescent units/second) was plotted versus the initial concentration of injected GGNLS (fluorescent units; Figure 4B). A line was fit to the data for each time point. To compare the import rate across multiple sorbitol time points, the initial rate of import was determined for each time point of sorbitol stress, using an initial concentration of 150 fluorescence units (Figure 4C).

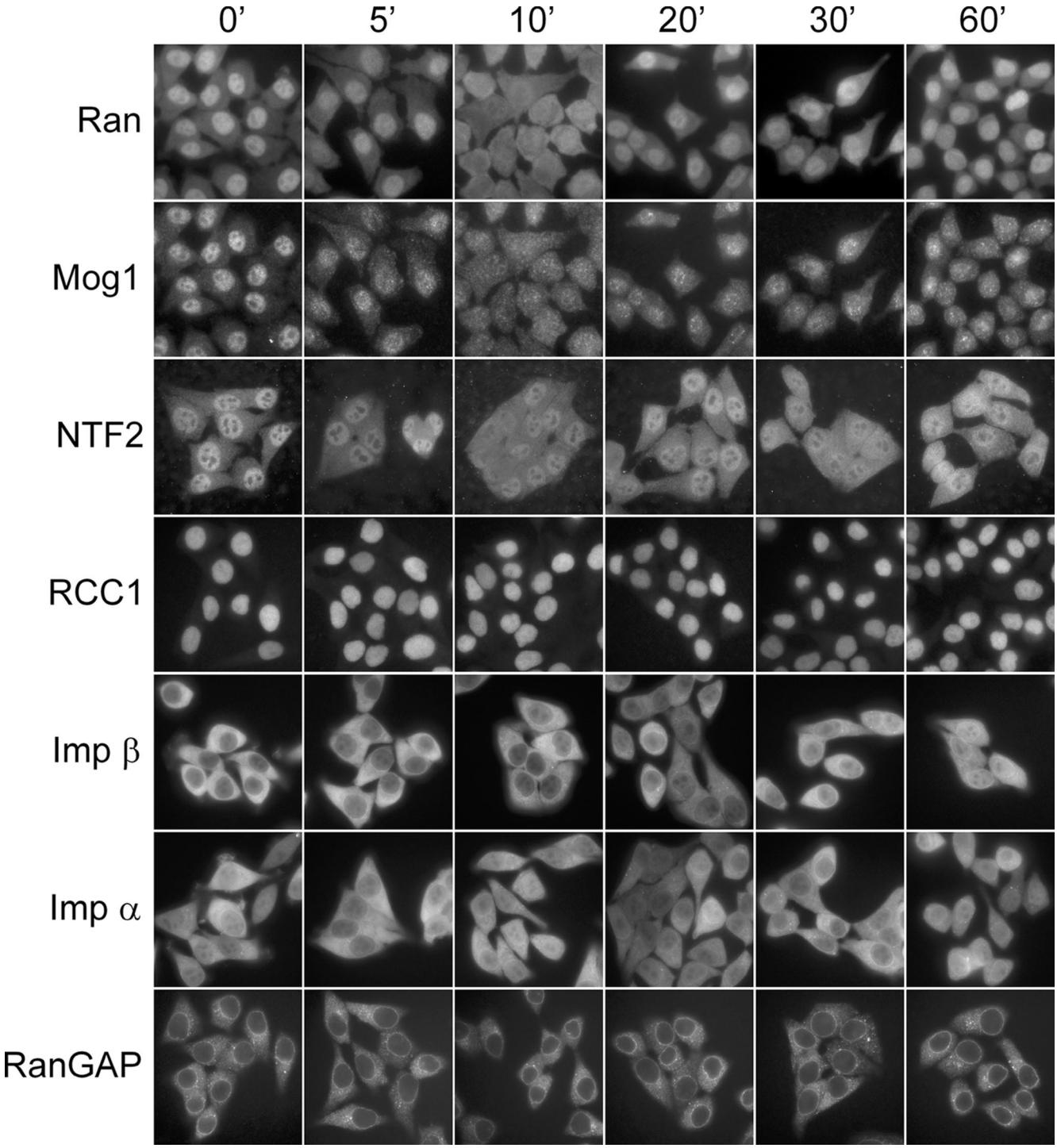

Figure 2. Nuclear transport factors undergo relocalization during osmotic stress. Hela cells were stressed with 0.4 M sorbitol for 0, 5, 10, 20, 30, and 60 min. Indirect immunofluorescence was used to detect endogenous Ran, Mog1, NTF2, RCC1, importin-α and -β, and RanGAP.

Similar to the Ran N/C ratio, the import rate of GGNLS shows a rapid decrease early in the response to sorbitol stress. A slight recovery is followed by a decline and then a second phase of recovery. The fact that import rate reaches a minimum at 30 min, whereas Ran often reaches a minimum between 5 and 10 min, may be due to the cumulative effect of the protein localization changes in Ran, Ran regulators, and importin proteins.

The reduced rate of import (Figure 4C) and the accumulation of import factors in the nucleus in response to sorbitol (Figures 2 and 3) may be due to decreased levels of RanGTP. FRET assay that registers the RanGTP-sensitive interaction between importin-β and -α. The FRET sensor consists of YFP and CFP separated by the importin-β–binding domain (IBB) of importin-α and is denoted YIC (Kalab *et al.*, 2002; Li and Zheng, 2004). Importin-β binding to the IBB in YIC prevents

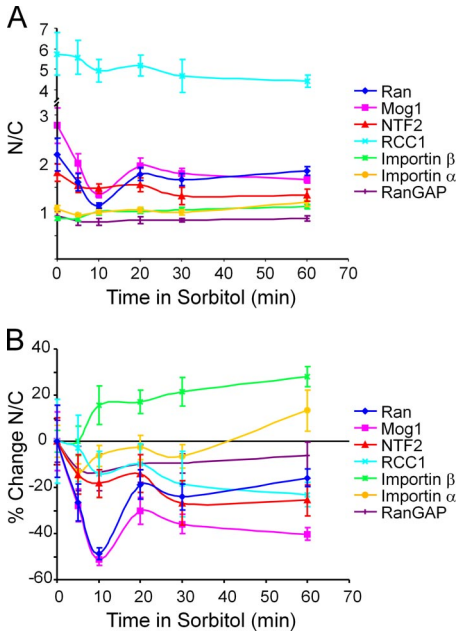

Figure 3. Quantitation of the subcellular localization of nuclear transport factors. (A) Mean nuclear fluorescence divided by the mean cytoplasmic fluorescence (N/C) plotted versus time in sorbitol (min). Calculations are based on immunofluorescence data from Figure 2. The scale is modified for RCC1. An N/C ratio of 1 represents equilibration between the nucleus and cytoplasm. Less than 1 is predominantly cytoplasmic, and greater than 1 is predominantly nuclear. Error bars, SEM. (B) Percent change in N/C ratio plotted versus time in sorbitol. A positive percent change represents movement into the nucleus, whereas a negative percent change represents movement into the cytoplasm. Error bars, SEM as a percent of the N/C ratio at 0 min of stress.

YFP from being in close proximity to CFP and reduces the FRET signal. In a test tube, β binds to YIC but the complex is rapidly dissociated by RanGTP, resulting in a FRET signal increase. Thus, an increase in FRET signal is predicted to occur if importin-β becomes limiting in the nucleus. A decrease in RanGTP levels would be predicted to decrease FRET in a single compartment, because importin-α and YIC would compete for β binding. However, in the context of an intact cell, α and β cycle between compartments, forming import complexes in the cytoplasm, whereas YIC is only found in the nucleus. If RanGTP production is reduced, import complexes formed in the cytoplasm will fail to dissociate in the nucleus, because there is insufficient RanGTP. Undissociated importin-α/β complexes in the nucleus would, therefore, decrease the available importin-β and increase FRET.

FRET was measured in HeLa cells expressing YIC using the acceptor photobleaching method (Karpova *et al.*, 2003). Bleaching of the YFP acceptor causes dequenching of the CFP donor, which allows a measurement of FRET efficiency (Supplementary Figure S1). The FRET efficiency increases slightly from 0 to 10 min of sorbitol stress (Figure 4D). From 10 to 20 min, FRET efficiency decreased to near steady-state levels, followed by a sharp increase in FRET efficiency from 20 to 30 min of stress, which was maintained through 60 min of sorbitol stress. The greatly increased FRET efficiency at later stress time points represents an accumulation of importin-α/β complexes in the nucleus, which are unable to bind YIC because endogenous importin-α has not been released by RanGTP. These data show that RanGTP production is reduced in response to sorbitol stress.

RCC1 and RanGAP Activities Are Not Inhibited by Sorbitol Stress

We tested whether the mechanism that underlies sorbitol-dependant disruption of the Ran protein gradient involves changes in activity of the components that regulate the nucleotide state of Ran, RanGAP, and RCC1. After exposing HeLa cells to sorbitol for 0, 5, or 30 min, nuclear and cytosolic extracts were prepared as sources of RCC1 and RanGAP, respectively. RCC1 activity in nuclear extract was measured in a guanine nucleotide exchange assay whereby α-^{32}P-GDP bound to Ran is exchanged with unlabeled GTP. The nuclear extracts from control and sorbitol-treated cells showed the same levels of RCC1 activity, suggesting that the sorbitol-induced changes in the Ran protein gradient are not due to a biochemical defect in RCC1 activity (Figure 5A).

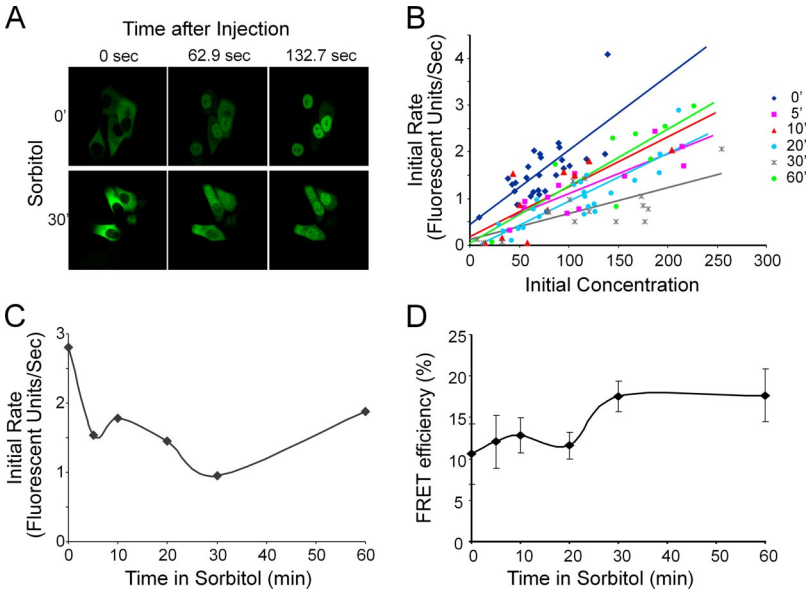

Figure 4. HeLa cells were injected with GST-GFP-NLS (GGNLS), at 0, 5, 10, 20, 30, and 60 min of sorbitol stress. (A) GGNLS-injected HeLa cells at 0 and 30 min of sorbitol stress shown at 0, 62.9, and 132.7 s after injection. (B) The initial rate of import of the GGNLS was plotted versus the initial concentration upon injection. Lines were fitted to the data for each time point of sorbitol stress. (C) The equations for the lines in A were solved for an initial concentration of 150 fluorescent units. This initial rate was then plotted versus time of sorbitol stress. (D) FRET efficiency of the YIC construct in HeLa cells during sorbitol stress. Increased FRET efficiency means less importin-α is available to bind the YIC construct. Error bars, SD. The 30- and 60-min time points have a p value < 0.0005 when compared with the 0-min time point.

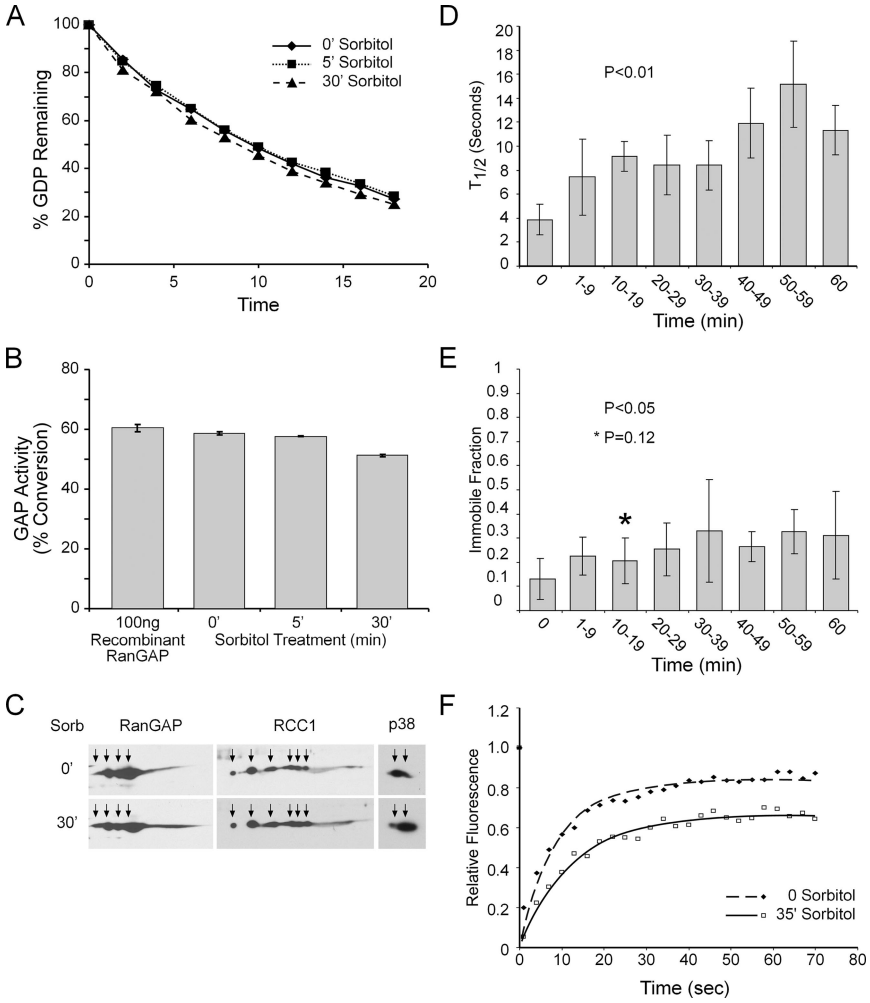

Figure 5. RanGAP and RCC1 activities are unchanged by sorbitol stress. HeLa cells were treated for 0, 5, and 30 min with 0.4 M sorbitol. Cytoplasmic and nuclear extracts were made. (A) To test RCC1 (RanGEF) activity, the nuclear extract was incubated with Ran loaded with ^{32}P-α–labeled GDP. Remaining radiolabeled nucleotide on Ran was measured over time. (B) RanGAP activity was tested by incubating the cytoplasmic extract with Ran preloaded with ^{32}P-α–labeled GTP. The nucleotide was separated by TLC chromatography, and the percent of GTP hydrolysis was graphed. Error bars, SD. (C) 2D gel analysis of RanGAP and RCC1 shows no difference in phosphorylation between untreated cells and HeLa treated with sorbitol for 30 min. p38 MAPK shows a rightward shift on the 2D gel upon sorbitol stress. (D) FRAP of RCC1-GFP in HeLa nuclei. Half-times of recovery were calculated for each cell, and these halftimes were averaged in 10-min bins. All data points have a $p < 0.01$ compared with the 0-min sorbitol time point. Error bars, SD. (E) Graph of the average immobile fraction of RCC1 from the same cells as D. All data points have a $p < 0.05$ when compared with the 0-min time point, except 10–19 min, which has a p value of 0.12. (F) Example of FRAP results from individual cells from 0 and 35 min of sorbitol stress. Data are graphed as relative fluorescence versus time in seconds.

Similarly, we found that RanGAP-mediated conversion of Ran-α-^{32}P-GTP to Ran-α-^{32}P-GDP assayed by TLC was comparable in cytosolic extracts from control and sorbitol-treated cells (Figure 5B). Sorbitol stress activates multiple signal transduction pathways (Kultz and Burg, 1998); therefore we also used two-dimensional (2D) gel electrophoresis and immunoblotting to probe for stress-induced posttranslational modifications in RCC1 and RanGAP. The isoform diversity of RCC1 and RanGAP did not appear to change in response to sorbitol stress. Under these conditions, the stress kinase p38 underwent a rightward shift reflective of its phosphorylation (Figure 5C). Though RCC1 and RanGAP are known to undergo multisite phosphorylation (Li and Zheng, 2004; Swaminathan *et al.*, 2004), sorbitol stress affects neither the biochemical activity nor the relative levels of RCC1 and RanGAP isoforms resolved by 2D gel electrophoresis.

RCC1 binds to chromatin through an interaction with histones H2A and H2B, and this interaction is known to promote RCC1-mediated catalysis of nucleotide exchange on Ran (Nemergut *et al.*, 2001; Li *et al.*, 2003). Photobleaching studies have shown that the interaction between RCC1 and chromatin is highly dynamic in vivo and is dependent on the ability of the RCC1/Ran complex to bind guanine nucleotide (Li *et al.*, 2003). RCC1 dissociation from chromatin is reduced significantly if RCC1 forms complexes with Ran T24N, a mutant defective for nucleotide binding (Klebe *et al.*, 1995). Therefore, RCC1 mobility measured by FRAP provides a sensitive readout of RCC1 interactions with chromatin, which positively regulate its catalytic function and production of RanGTP. We utilized FRAP to monitor the mobility of transiently transfected RCC1-GFP during sorbitol-induced osmotic stress (Figure 5F). A half time of recovery ($T_{1/2}$) was calculated for each cell, and values were binned in 10-min intervals (Figure 5D). RCC1 mobility decreased after sorbitol stress, as did the immobile fraction of RCC1 within the nucleus (Figure 5E). This decrease in RCC1 mobility suggests that stress signaling may decrease the rate of chromatin-dependent nucleotide exchange and consequently decrease production of RanGTP. This could be due to a defect upstream of RCC1 and the exchange reaction, or it could be indicative of stress-induced changes in chromatin that lead to a decreased ability for RCC1 to properly dissociate during the exchange reaction. RCC1 mobility is not decreased in response to energy depletion by sodium azide and deoxyglucose, conditions that also lead to a loss of the Ran protein gradient (Supplementary Figure S2). Therefore, the mobility changes in response to sorbitol cannot be accounted for by changes in the nucleotide levels or loss of Ran from the nucleus.

Energy Levels Are Affected by Sorbitol Stress

RCC1 is not specific in its catalysis of nucleotide exchange; it is capable of loading either GTP or GDP on to Ran with

equal efficiency (Bischoff and Ponstingl, 1991). It is believed that the loading of GTP onto Ran by RCC1 is determined by the ratio of GTP to GDP present in the cell and that GTP is loaded because it is the more abundant guanine nucleotide. Additionally, proper localization of Ran to the nucleus is dependent on the ability of RCC1 to load Ran with GTP (Ren *et al.*, 1993). Given these observations, it is predicted that if sorbitol stress caused a decrease in the ratio of GTP to GDP, it could cause Ran to be loaded with GDP, thereby diminishing the Ran protein gradient. To investigate this possibility, we analyzed the nucleotide levels in sorbitol-stressed cells by HPLC separation of ATP, ADP, GTP, and GDP. We found that the triphosphate forms of both adenine nucleotides and guanine nucleotides decreased in relation to the diphosphate form in response to sorbitol stress (Figure 6). Interestingly, the ATP/ADP and GTP/GDP ratios show clear evidence of reduction and recovery through the sorbitol stress time course, reminiscent of the Ran N/C ratios. Immediately upon stress, Ran N/C, Mog1 N/C, ATP/ADP, and GTP/GDP ratios all decline. From 10 to 20 min of stress, the ratios of all but Mog1 undergo a partial recovery. However, from 20 to 30 min, whereas the Ran N/C ratio remains relatively constant, the ATP/ADP and GTP/GDP ratios decrease again, reaching levels similar to the 10-min time point. From 30 to 60 min Ran N/C remains relatively unchanged, whereas ATP/ADP and GTP/GDP ratios again recover toward steady-state levels. The initial drop in Ran N/C may be a result of the decrease in nucleotide triphosphate to diphosphate ratios, or available GTP, but the second drop in GTP/GDP ratios obviously does not correlate with changes in Ran nucleocytoplasmic localization.

The Ran Protein Gradient Is Not Strictly Dependent on Guanine Nucleotide Levels

There were parallel reductions in the Ran N/C ratio and GTP levels during the first 10 min of sorbitol treatment. To determine if reduced levels of GTP are causal to the reductions in Ran N/C ratio, we made use of a compound that specifically reduces cellular GTP. Ribavirin is an antiviral drug that reduces cellular levels of GTP by inhibiting inosine monophosphate (IMP) dehydrogenase (Leyssen *et al.*, 2005). HeLa cells were treated with ribavirin (0, 10, 100, and 250 μM) overnight and subsequently analyzed by HPLC to assess changes in nucleotide levels. In parallel, HeLa cells were treated with ribavirin and processed for immunofluorescence microscopy. Ribavirin had a strong effect on GTP levels in cells, as 250 μM ribavirin produced a 75.9% reduction in cellular GTP (Figure 7A). Ribavirin had no effect on Ran and Mog1 localization at 10 μM; however, higher concentration of ribavirin actually increased N/C ratio slightly from 2.22–2.57 ($p < 0.005$) and 2.49–2.86 ($p < 0.05$) for Ran and Mog1, respectively, at 100 μM ribavirin (Figure 7, B and C). Because ribavirin had a stronger effect on the levels of GTP than on GDP, there was an inversion of the GTP/GDP ratio. In untreated cells we observed a GTP/GDP ratio of 1.88, whereas in cells treated with 250 μM ribavirin, the ratio was 0.15. These data show that neither reducing the level of GTP, nor altering the GTP/GDP ratio is sufficient to disrupt the Ran protein gradient in cells.

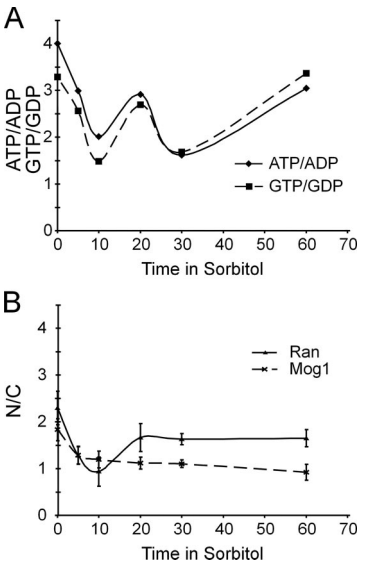

Figure 6. Nucleotide levels change in response to sorbitol stress. (A) Nucleotides were extracted and separated by HPLC after stress with 0.4 M sorbitol for the designated amount of time. Ratio of ATP to ADP and GTP to GDP were plotted versus time. (B) Ran and Mog1 N/C ratios from the same set of cells are plotted versus time in sorbitol. Error bars, SD.

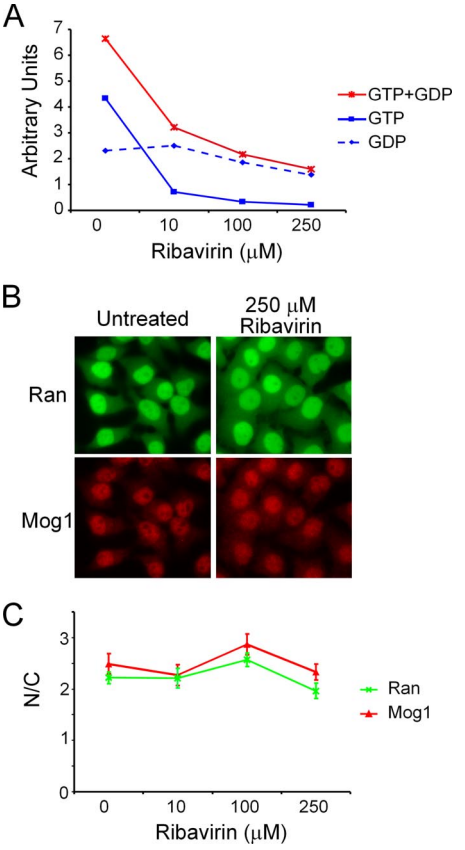

Figure 7. Depletion of cellular GTP is not sufficient to cause Ran relocalization. The antiviral drug ribavirin was used to deplete the guanine nucleotide levels in HeLa cells. Cells were treated with 0, 10, 100, and 250 μM ribavirin. (A) Relative levels of GTP and GDP in cells during ribavirin treatment as determined by HPLC separation of nucleotides. (B) Immunofluorescence of endogenous Ran and Mog1 in untreated (0 μM) and 250 μM ribavirin. (C) The N/C ratios of Ran and Mog1 were calculated from immunofluorescence microscopy and plotted versus the concentration of ribavirin. Error bars, SD.

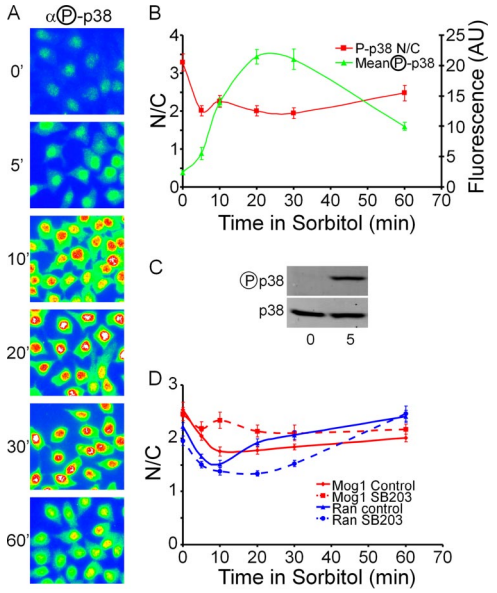

Figure 8. p38 MAPK is involved in the osmotic stress response. p38 MAPK is phosphorylated in response to sorbitol stress. (A) Phospho-p38 MAPK staining in HeLa cells during osmotic stress. Images are pseudocolored to emphasize changes in intensity. (B) Quantitation of total phosphorylation as well as N/C ratio of phospho p38 based on images collected for A. Green line, mean fluorescence of the cells as a function of time of osmotic stress; red line, the nuclear to cytoplasmic ratio of phospho-p38 MAPK during the osmotic stress. Error bars, SD. (C) Western blot of p38 at 0 and 5 min of sorbitol stress. Top, phospho p38,bottom, total p38. This demonstrates the phospho-p38 antibody specificity for activated p38. (D) Ran relocalization in response to sorbitol stress, in the presence and absence of the p38 MAPK inhibitor, SB203580. Addition of SB20350 changes the recovery pattern of Ran and Mog1. Error bars, SEM.

p38 MAPK Is Involved in the Recovery of Ran Distribution during Osmotic Stress

We assessed the role of the stress-activated kinase p38 MAPK in our system because it is known to be phosphorylated and activated in response to 0.4 M sorbitol. Phosphoblotting of p38 during osmotic stress showed rapid phosphorylation of p38, reaching peak intensity at 20 min of stress (Figure 8C). By immunofluorescence microscopy, sorbitol treatment resulted in the appearance of phosphorylated p38 in the nucleus of HeLa cells (Figure 8A). This was evident as early as 5 min after sorbitol stress and reached a maximum at 20 min. The N/C ratio of phospho-p38 displayed a similar profile to the changes in Ran and Mog1 localization, implying that p38 is changing localization as well. Because phosphorylation of p38 is being measured; however, the changes may represent a stress-induced differential in the cytoplasmic or nuclear regulation of the p38 phosphorylation state (Figure 8B). When the cells are pretreated with the p38 MAPK inhibitor SB203580, the recovery of the Ran protein gradient at 20 and 30 min of stress is suppressed (Figure 8D).

We also tested whether p38 plays a role in the Ran protein gradient by reducing p38 levels in the cell. siRNA was used to knock down the p38 α isoform by ~80% as assessed by immunoblotting with a pan-p38 antibody (data not shown). Because the pan-p38 antibody did not work well for immunofluorescence microscopy, phospho-p38 antibody was used in immunofluorescence experiments. We used the phospho-p38 antibody to quantify the levels of p38 and N/C ratios of Ran in the same cells over a 60-min time course of 0.4 M sorbitol treatment. Sample images from the 0- and 10-min time points indicated that cells expressing higher levels of p38 contained higher nuclear concentrations of Ran (Figure 9A). We measured the phospho-p38 and Ran levels in three sets of cell: untransfected cells (labeled Untransfected), p38 siRNA transfected cells in which p38 staining was reduced (labeled Reduced p38), and cells from the p38 siRNA transfection in which p38 staining was comparable to the untransfected culture (labeled Normal p38). Similar levels of p38 phosphorylation were induced by sorbitol in the untransfected and normal p38 cells, whereas the phospho-p38 levels in detected cells subject to knockdown remained at near-background levels throughout the time course (Figure 9B). Reducing the p38 levels in HeLa cells resulted in a reduction of the p38-dependent protection and/or recovery of the Ran protein gradient (Figure 9C). The SB203580 results and the p38 knockdown data suggest that p38 plays a role in regulating the Ran protein gradient in response to hyperosmotic stress.

We have shown that energy levels in the cell decrease rapidly upon sorbitol stress. While our previous experiments have demonstrated that the ATP/ADP and GTP/GDP ratios do not display correlation with Ran N/C ratio throughout the sorbitol stress, there is still the possibility that the initiating event for Ran delocalization is the early decrease in energy levels. In the course of studying p38 MAPK, we found that SB203580 inverted the triphosphate to diphosphate levels for both adenine and guanine nucleotides, and we exploited this effect to examine the relationship between energy levels, Ran, and Mog1 in the early response to sorbitol stress. Cells were pretreated with SB203580 for 1 h and then subjected to sorbitol stress in the presence of SB203580. Within 5 min of sorbitol stress, energy levels in the drug-treated cells increase, whereas energy levels in untreated cells drop (Figure 10). However, in both cases, Ran and Mog1 N/C ratios decrease within 5 min of sorbitol stress. This serves to further demonstrate that energy levels are not the determining factor in stress-induced relocalization of Ran and Mog1.

DISCUSSION

We have shown that osmotic stress–induced by sorbitol causes a rapid disruption of the Ran protein gradient, delocalization of nuclear transport factors, and slowing of nuclear transport. The RanGEF, RCC1, displayed decreased nuclear mobility in response to sorbitol, which is predicted to result in a decreased rate of nucleotide exchange on Ran. The osmotic stress–induced delocalization was found to be reversible even in the continuous presence of sorbitol. Maximum delocalization occurs within 10 min, after which the transport factors begin recovering a prestress distribution. The recovery occurs in two phases, a rapid initial phase of recovery from 10 to 20 min, followed by a longer, slower recovery to 60 min. We found that MAP kinase p38, a key component of the osmotic stress response in cells, is required for the initial phase of recovery of Ran localization during osmotic stress; however, p38 activity was not required for the second phase of recovery. We also found that sorbitol caused a decrease in ATP/ADP and GTP/GDP ratios but that these changes did not correlate with changes in nuclear transport factor delocalization throughout the sorbitol time course. Additionally, specific depletion of guanine nucleotide as well as inversion of the GTP/GDP ratio is not sufficient to disrupt the Ran protein gradient. Therefore disruption of the Ran protein and RanGTP gradients in response to

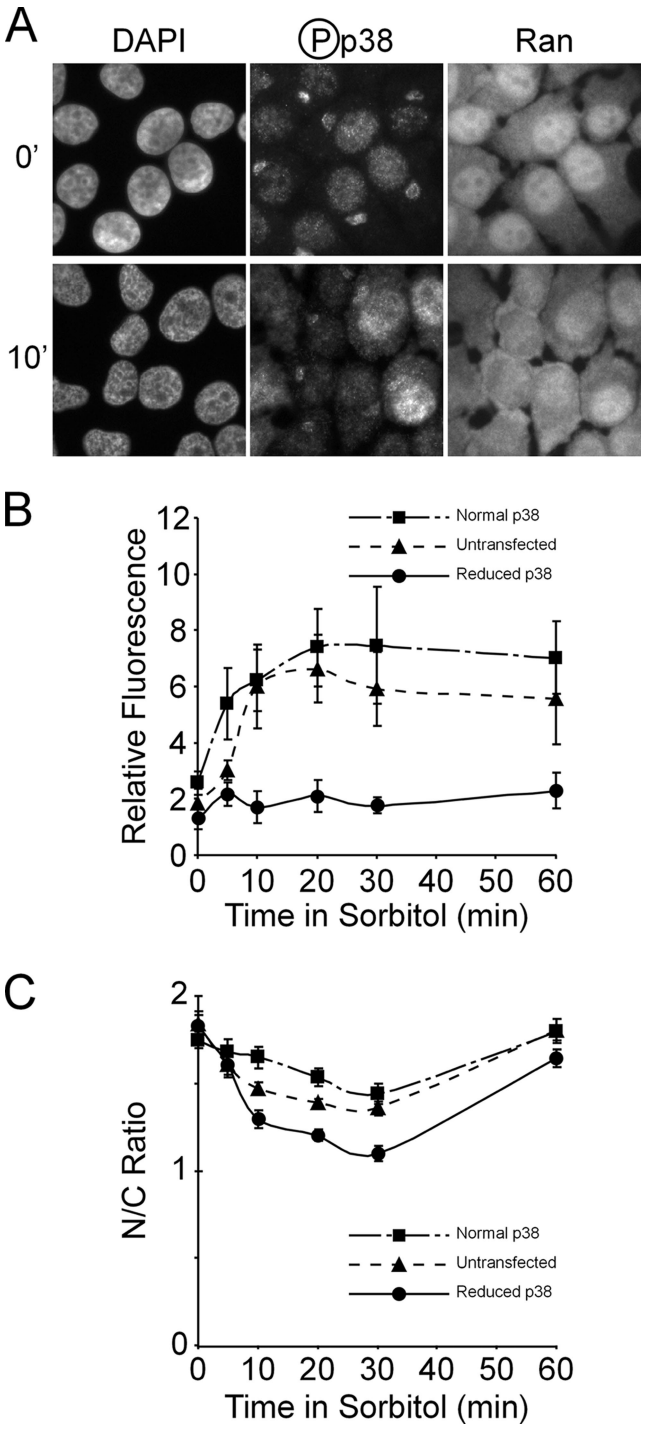

Figure 9. The protective effect of p38 on the Ran gradient. siRNA was used to knock down the α-isoform of p38 in HeLa cells, which were subsequently exposed to sorbitol for up to 60 min. (A) Immunofluorescence localization of phospho-p38 and Ran at the 0- and 10-min time points. (B) Quantitation showing the relative changes in phospho-p38 during the time course of sorbitol treatment. Quantitation of total phospho-p38 was performed in untransfected cells (▲). Coverslips from cultures transfected with p38 siRNA were used to select cells with reduced and normal p38 levels. The plot labeled reduced p38 (●) refers to cells transfected with p38 siRNA that showed reduced phospho-p38 staining. The plot labeled normal p38 (■) refers to cells transfected with p38 siRNA that showed approximately the same level of phospho-p38 staining as untransfected cells. Error bars, SD. (C) N/C ratios of Ran in the same cells in which phospho-p38 levels were determined. Error bars, SEM.

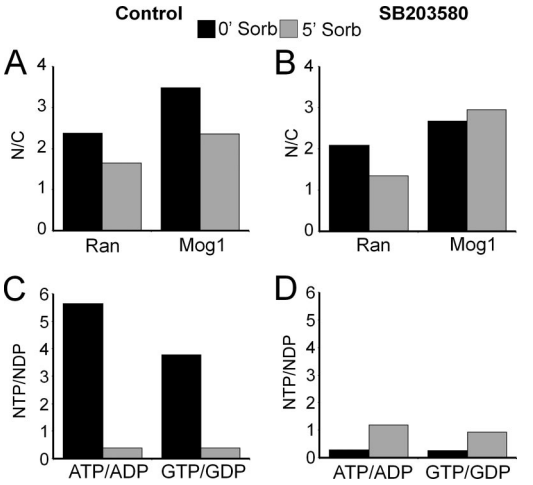

Figure 10. The effects of SB203580 on nucleotide levels during osmotic stress. (A) The N/C ratio of Ran and Mog1 at 0 and 5 min of sorbitol stress. P value for the change in N/C ratio from 0 to 5 min are $p < 0.00005$. (B) The N/C ratio of Ran and Mog1 in the presence of the p38 inhibitor SB203580 at 0 and 5 min of sorbitol stress. P values for the change in N/C ratio from 0 to 5 min are $p < 0.00005$ and $p < 0.20$ for Ran and Mog1, respectively. (C) ATP/ADP and GTP/GDP ratios at 0 and 5 min of sorbitol stress. (D) ATP/ADP and GTP/GDP ratios in the presence of the p38 inhibitor SB203580 at 0 and 5 of sorbitol stress.

osmotic stress cannot be explained simply by a decrease in the availability of GTP.

Nuclear Transport and the Stress Response

Nuclear transport is critical for the cellular response to stress, which requires both MAP kinases and transcription factors. The nuclear transport and transcriptional activity of NFAT5/TonEBP is regulated by osmotic stress. Under isotonic conditions TonEBP is predominantly cytoplasmic (Tong *et al.*, 2006). On hypertonic stress, it accumulates in the nucleus where it up-regulates the expression of several genes required for the accumulation of organic osmolytes: aldose reductase, the enzyme responsible for production of sorbitol; the sodium/myo-inositol transporter; and the betaine γ-butyric acid transporter (Ho, 2003). In hypotonic solutions, TonEBP is exported and has a predominantly cytoplasmic localization (Woo *et al.*, 2000). In addition to TonEBP, p38 MAPK, Erk 1/2, Erk 5, and Jnk are activated and must also import into the nucleus upon sorbitol stress (Itoh *et al.*, 1994; Rosette and Karin, 1996; Kato *et al.*, 1997; Ferrigno *et al.*, 1998; Yan *et al.*, 1999; Cyert, 2001). Thus, changes that result in diminished capacity for nuclear import will diminish the cellular capacity to deal with stress.

Several links have been established between stress and nuclear transport. Oxidative stress, UV stress, heat shock, and osmotic stress have all been linked to defects in transport, predominantly through the mislocalization of nuclear transport factors or the cytoplasmic localization of nuclear proteins. UV stress and oxidative stress induced by hydrogen peroxide have been shown to cause Ran relocalization to the cytoplasm and importin-α accumulation in the nucleus (Czubryt *et al.*, 2000; Miyamoto *et al.*, 2004). In yeast, oxidative stress has been shown to reduce NLS-dependent import (Stochaj *et al.*, 2000). Also, osmotic stress and certain Sec mutants, which induce the ASR, are capable of delocalizing nuclear and nucleolar proteins (Nanduri and Tartakoff, 2001a,b). However, recovery of nuclear transport or nuclear

transport factor localization has not been previously shown, either due to experimental design or the model system used.

The stress kinase p38 MAPK is activated by sorbitol-induced osmotic stress (Brewster *et al.*, 1993; Han *et al.*, 1994). When yeast experience hyperosmotic stress, the nucleolar protein Fpr3p relocalizes to the cytoplasm; however, over expression of Hog1 in this setting has a protective effect on Fpr3p nuclear localization (Nanduri and Tartakoff, 2001b). We examined the role of p38 MAPK in the nuclear transport factor response to osmotic stress using the p38 MAPK inhibitor, SB203580. In the presence of SB203580, after the addition of sorbitol, there is a drop in Ran N/C ratio, followed by a steady recovery to 60 min, bypassing the initial recovery usually seen at 20 min, but maintaining the second recovery. In yeast, Hog1 has been shown to have protective effects on nuclear transport pathways during both osmotic stress and the ASR, which is a stress brought about through the use of certain Sec mutants (Nanduri *et al.*, 1999; Nanduri and Tartakoff, 2001a). Recovery of the Ran protein gradient in response to sorbitol stress was slowed by SB203580 and by p38 knockdown, whereas the initial delocalization of Ran was unaffected under these conditions. We therefore conclude that p38 MAPK does not have a protective role in HeLa cells, but does play a role in recovery from sorbitol stress.

The Ran Protein Gradient Is Not Strictly Dependent on Guanine Nucleotide

It is widely held that the maintenance of the Ran protein gradient is dependent on energy levels in the cell (Schwoebel *et al.*, 2002). RCC1 will catalyze exchange of either GDP or GTP on Ran with equal efficiency (Bischoff and Ponstingl, 1991). Therefore RanGTP generation is dependent on GTP being the predominant species of guanine nucleotide present. In tsBN2 cells, RCC1 is temperature sensitive, and when grown at the nonpermissive temperature, these cells lose the Ran protein gradient, presumably due to a requirement for Ran to be in the GTP-bound form in order to concentrate in the nucleus under steady-state conditions (Ren *et al.*, 1993). When cells are treated with sodium azide and deoxyglucose, Ran rapidly delocalizes, again presumably because of the loss of ATP and due to the nucleotide diphosphate kinase GTP levels (Schwoebel *et al.*, 2002).

Oxidative and UV stress have both been shown to cause Ran delocalization (Czubryt *et al.*, 2000; Kodiha *et al.*, 2004; Miyamoto *et al.*, 2004). It has recently been reported that the Ran delocalization in response to oxidative stress induced by hydrogen peroxide is due to a stress-induced drop in ATP levels (Yasuda *et al.*, 2006) However, our measurements of nucleotide levels during osmotic stress showed that Ran relocalization does not strictly correlate with changes in energy levels in the cell. Although sorbitol stress caused a decrease in both ATP/ADP and GTP/GDP ratios within 10 min, mimicking the changes in Ran N/C ratios, later time points showed little correlation between Ran localization and ATP/ADP or GTP/GDP levels. Use of ribavirin to deplete guanine nucleotide levels resulted in a large drop in GTP and GDP, as well as an inversion of the GTP/GDP ratio. However, there was no significant decrease in Ran N/C ratio, contrary to what would be predicted based on tsBN2 cells. Treatment of cells with SB203580 to inhibit p38 MAPK decreased prestress nucleotide ratios without changing the N/C ratio of Ran. On sorbitol stress, the control cells showed normal decrease in Ran N/C, GTP/GDP, and ATP/ADP ratios. However, in the presence of SB203580, the Ran N/C ratio decreased similarly to the control cells, whereas the GTP/GDP and ATP/ADP ratios both increased from their lower than normal starting levels, demonstrating that the delocalization of Ran is not due to the sudden drop in nucleotide levels, because the drop in Ran nuclear levels occurs whether ATP/ADP and GTP/GDP ratios are decreasing or increasing. Collectively, these data argue for a more complex mechanism of Ran delocalization upon sorbitol stress, not explained simply by changes in energy levels.

Stress Signaling to the Nucleus

Biochemical, genetic, and cell biological data provide compelling evidence for the involvement of Mog1, NTF2, and RCC1 in the maintenance of the Ran gradient (Bischoff and Ponstingl, 1991; Tachibana *et al.*, 1994; Paschal *et al.*, 1997; Oki and Nishimoto, 1998; Ribbeck *et al.*, 1998; Smith *et al.*, 1998; Steggerda and Paschal, 2000; Baker *et al.*, 2001). Our data support an interdependence of the localization and function of these proteins during sorbitol stress–induced disruption and recovery of the Ran protein gradient as well. The kinetics of delocalization of Mog1, NTF2, and Ran as well as the concomitant decrease in RCC1 mobility suggests that all four of these proteins are targets of stress signaling (Figure 11). Reduced function of all of these proteins in response to sorbitol could contribute to loss of the Ran protein gradient, in which case a single initiating event for disruption of the Ran protein gradient might not be apparent.

Because a number of kinases are activated by sorbitol-induced stress, phosphorylation-dependent inhibition of Ran regulators would be a logical means of disrupting the Ran protein gradient. However, the fact that sorbitol treatment does not change the isoelectric point of Ran regulators suggests that phosphorylation of these factors is not the explanation. This leads us to propose an alternative model, which is that there is a nuclear target of stress signaling whose function is intimately related to the localization and function of Mog1, NTF2, and Ran. Our data point to RCC1 as a target whose function is reduced during stress signaling (Figure 11). Because RCC1 protein modification does not change under conditions where its mobility in the nucleus increases, we speculate that stress signaling alters RCC1 mobility and function by altering its binding sites on chromatin. This results in reduced interactions with chromatin

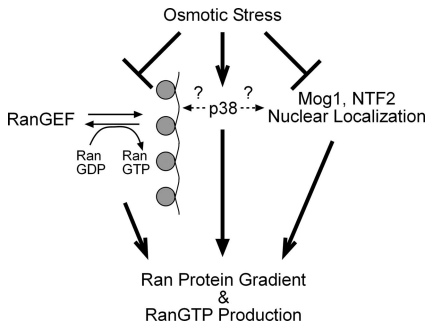

Figure 11. Model summarizing the effects of osmotic stress on the Ran protein gradient and on the production of RanGTP. Osmotic stress disrupts the proper nuclear localization of Mog1 and NTF2, proteins that are necessary for establishing the Ran protein gradient. Osmotic stress also reduces RanGTP production, presumably by affecting RCC1 activity through alteration of its interactions with chromatin. Osmotic stress activation of p38 MAPK is necessary for the protection of the Ran gradient. This is predicted to involve modulation of Mog1, NTF2, or RCC1 activity.

and a reduction in RanGTP production, an interpretation that is consistent with our FRET results. Reducing the level of RanGTP results in the nuclear accumulation of importin-α and -β in the form of undissociated complexes, causing unoccupied YIC reporter to display FRET (Figure 4). Disruption of the Ran protein gradient would also be predicted to occur under conditions where GTP is limiting, but surprisingly, this is not the case. Cells treated with the drug ribavirin have an inverted ratio of GTP/GDP; however, this condition does not disrupt the Ran gradient. Thus, the changes in nucleotide levels that occur during stress signaling are predicted to be insufficient to cause a breakdown in the Ran protein gradient.

The response of the FRET sensor under conditions of osmotic stress may appear to be contradictory to data generated with the same construct by the Zheng lab, as well as the work done with a similar construct by the Weis lab (Kalab *et al.*, 2002, 2006; Li and Zheng, 2004). The YIC sensor detects free importin-β, and, as such does not measure RanGTP directly. Nonetheless, through a variety of controls these groups showed that a high FRET signal is correlated with high RanGTP levels and a low FRET signal is correlated with low RanGTP levels. Our data, in contrast, shows there is an increase in FRET signal under conditions where we predict there is a decrease in RanGTP levels. So why is there a discrepancy? Previous applications of the YIC sensor have been primarily in mitotic cells. In the absence of a nuclear envelope, the YIC sensor and importin-α compete equally for binding to importin-β. In the presence of a nuclear envelope, however, there is an unequal competition for binding to the YIC sensor. Why? 1) The YIC sensor is nuclear in interphase cells. 2) Importin-α and -β are shuttling, form complexes in the cytoplasm, and enter the nucleus. 3) Sorbitol stress increases the nuclear concentration of importin-α and -β. The reason why the YIC FRET signal increases in response to sorbitol relates to a decrease in the size of the pool of importin-β available for binding YIC. The explanation that we have offered, that there is less importin-β available to bind YIC because reduced RanGTP production is conducive to importin-α/β complex formation (a high-affinity complex), is plausible. The alternative explanation is that sorbitol actually increases RanGTP production. However, it is difficult to reconcile such an interpretation with the fact that sodium azide and deoxyglucose addition to cells, a condition that reduces RanGTP levels, increases the YIC FRET signal (Supplementary Figure S3). It does deserve mention that the YIC signal decreases in tsBN2 cells after several hours of temperature shift (Li and Zheng, 2004), but it is possible that a gradual loss of RanGTP in that setting is not directly comparable to the acute stress signaling in our system.

Mog1 remains a mysterious player in the Ran cycle. The synthetic lethality of Δ*mog1* with a temperature-sensitive RCC1 allele together with the fact that Mog1 binds directly to Ran both point to a function proximal to nucleotide exchange (Oki and Nishimoto, 1998; Baker *et al.*, 2001). We found that Mog1 undergoes delocalization from the nucleus in response to sorbitol-induced stress. This may reflect a stress-induced uncoupling of Mog1 from the Ran cycle, possibly in response to the changes in binding to and dissociating from chromatin. Defining how sorbitol-induced signaling alters the interaction of RCC1 with chromatin may hold the key to understanding how signal transduction is used to disrupt and re-establish the Ran protein gradient in cells.

ACKNOWLEDGMENTS

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