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The Nucleolus Exhibits an Osmotically Regulated Gatekeeping Activity That Controls the Spatial Dynamics and Functions of Nucleolin*

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We demonstrate that physiologically relevant perturbations in the osmotic environment rheostatically regulate a gatekeeping function for the nucleolus that controls the spatial dynamics and functions of nucleolin. HeLa cells and U2-OS osteosarcoma cells were osmotically challenged with 100–200 mM sorbitol, and the intranuclear distribution of nucleolin was monitored by confocal microscopy. Nucleolin that normally resides in the innermost fibrillar core of the nucleolus, where it assists rDNA transcription and replication, was expelled within 30 min of sorbitol addition. The nucleolin was transferred into the nucleoplasm, but it distributed there non-uniformly; locally high levels accumulated in 4′,6-diamidino-2-phenylindole-negative zones containing euchromatic (transcriptionally active) DNA. Inositol pyrophosphates also responded within 30 min of hyperosmotic stress: levels of bisdiphosphoinositol tetrakisphosphate increased 6-fold, and this was matched by decreased levels of its precursor, diphosphoinositol pentakisphosphate. Such fluctuations in inositol pyrophosphate levels are of considerable interest, because, according to previously published *in vitro* data, they regulate the degree of phosphorylation of nucleolin through a novel kinase-independent phosphotransferase reaction (Saiardi, A., Bhandari, A., Resnick, R., Cain, A., Snowman, A. M., and Snyder, S. H. (2004) *Science* 306, 2101–2105). However, by pharmacologically intervening in inositol pyrophosphate metabolism, we found that it did not supervise the osmotically driven switch in the biological activities of nucleolin *in vivo*.

When subjected to a hyperosmotic environment, the cell faces perturbations to cellular hydration, cytoskeletal integrity, metabolic balance, and genomic stability (1, 2). If the cell fails to adapt to these stresses, it dies by apoptosis (2). Yet, surprisingly, it is only recently that hypertonicity has come to be recognized as a ubiquitous and biologically important challenge for cells in higher organisms. Instead, it has been a popular opinion for much of the previous 70 years (3, 4) that metazoan cells, with the exception of those of the renal medulla, avoid the potentially deleterious effects of anisosmotic gradients, by virtue of their being bathed in an osmotically stable extracellular fluid (280–300 mosM in most mammals). To illustrate that this must be an oversimplified concept, one need only point to the array of cellular responses to hyperosmotic stress that are conserved from yeast to mammals (5); why do cells from higher organisms retain these activities if they are not required? Indeed, several non-renal cell types are now accepted to routinely experience considerable changes in extracellular osmolarity, including airway epithelial cells, lymphocytes, and the various cell types in bones and cartilage (6–8). For example, lymphocyte development depends upon an ability to adapt to the hyperosmotic environment of the thymus (6). Bone cells also have a particular necessity to adapt to hyperosmotic conditions: chondrocytes embedded in cartilage are enveloped by a matrix containing a high concentration of fixed negative charge from proteoglycans, and hence also high levels of sodium (9). There are also fluctuating osmotic pressure gradients between osteocytes and their surrounding lacunocanalicular system, which are important for mechanochemical coupling and for driving bulk fluid flow through the bone tissue (8). Hyperosmotic stress can also occur in airway epithelial cells when the composition of the airway surface liquid layer is compromised by inadequate airway humidification, such as during rapid breathing (*e.g.* during exercise), breathing of dry/cold air, tracheotomy breathing, and in some airway diseases (10). Even in cell types that do not routinely experience these significant fluctuations in *extracellular* osmolarity, it is now accepted that they must also adapt to ongoing alterations in *intracellular* osmolarity. This is a situation that inevitably accompanies a range of normal cellular processes: changes in ion-transport across the plasma membrane, uptake and release of sugars and amino acids, and polymerization/depolymerization of macromolecules such as glycogen and proteins (11). An anisosmotic gradient in the order of 80 mosM can result from just these activities alone (12).

The wide-ranging effects of hyperosmotic stress upon cell function lead to numerous adaptive changes (2), and so establishing their molecular and functional interrelationships presents this field of research with one of its major difficulties. A recent addition to this repertoire of responses to hyperosmotic stress is the activation of inositol pyrophosphate metabolism (13–15). The inositol pyrophosphates are specialized members

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of the inositol phosphate cell-signaling family that are synthesized by "pyrophosphorylation" of $InsP_5$[3] and $InsP_6$ (15–18). The two most abundant inositol pyrophosphates, PP-$InsP_5$ and $[PP]_2$-$InsP_4$, are thought to participate in regulating a range of cellular activities, including DNA repair, vesicle trafficking, apoptosis, chemotaxis, and insulin secretion (19, 20). To regulate all of these diverse biological processes, it is possible that the inositol pyrophosphates have several different mechanisms of action, but it is their ability to phosphorylate certain proteins that is attracting particular attention from the cell signaling community (21–24). This is a new mechanism for the covalent modification of proteins. In a kinase-independent manner, inositol pyrophosphates can directly transfer a β-phosphate from their diphosphate groups to selected serine residues that reside in an appropriately acidic context (21–24). Thus, there is now considerable interest in the idea that stimulus-dependent fluctuations in the cellular levels of inositol pyrophosphates might act as a novel signaling mechanism, by directly altering the degree of phosphorylation of appropriate protein targets (25, 26). To date, however, there have not been any attempts to test whether, *in vivo*, the activities of any of the candidate target proteins are influenced by changes in inositol pyrophosphate concentrations *that are biologically relevant*. A hyperosmotic environment provides an appropriate context in which to examine the credentials of this novel signaling paradigm. No other biological stimulus is known to bring about more acute changes in inositol pyrophosphate turnover *in vivo* than those brought about by hypertonic stress (13–15, 27).

To pursue this problem, we reviewed the list of target proteins known to be phosphorylated by inositol pyrophosphates *in vitro* (21, 22). We initially tried to identify a candidate protein that is already known to have its activity modulated by hyperosmotic stress, so that we could then determine if this functional regulation might be driven by fluctuations in cellular levels of inositol pyrophosphates. However, we could not find any evidence in the literature that hyperosmotic stress regulated any of the proteins known to be phosphorylated by inositol pyrophosphates.

So instead, our attention was drawn to nucleolin. Both the human[4] and yeast (22) versions of this protein are phosphorylated by inositol pyrophosphates *in vitro*. Nucleolin is concentrated in the nucleolus, where it participates in all aspects of ribosome biogenesis (28): transcription, replication, and recombination of rDNA in the innermost fibrillar core of the nucleolus, followed by pre-rRNA processing, pre-rRNA modification, and ribosome assembly in the outer "granular" zones (28). The transcription of rRNA genes is sensitive to both general metabolic status and to certain environmental challenges (29). There is also evidence that nucleolin and some of the other proteins that regulate ribosomal biogenesis may leave the nucleolus when they become phosphorylated (29, 30). This intracellular protein relocation is analytically amenable, because it can be analyzed in intact cells by confocal microscopy; this can provide the *in vivo* data, which this aspect of inositol pyrophosphate signaling currently lacks (see above). In the current study we have investigated if the functions of nucleolin respond to hyperosmotic stress, and we have studied whether these events are regulated by changes in inositol pyrophosphate turnover.

EXPERIMENTAL PROCEDURES

Cell Culture—All cell lines were maintained at 37 °C in a 5% CO_2 atmosphere. U2-OS cells were cultured in McCoy's 5A medium (ATCC) with 10% fetal bovine serum. HeLa cells and H1299 cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum.

Assay of Inositol Polyphosphate Turnover—U2-OS cells were seeded on 60-mm Petri dishes (5×10^5 cells/dish) with 20 μCi/ml [^{3}H]inositol (American Radiolabeled Chemicals) in 4 ml of McCoy's 5A medium with 10% fetal bovine serum. Cells were cultured at 37 °C in a 5% CO_2 atmosphere for 6 days. The medium was replaced every 2 days. After completion of the experiments, cells were immediately quenched with 1.5 ml of ice-cold 0.6 M perchloric acid containing 0.2 mg/ml $InsP_6$. Samples were then neutralized, and individual ^{3}H-labeled inositol phosphates were resolved by anion-exchange HPLC using a 4.6 × 125 mm Partisphere SAX column (Krackeler) as previously described (15).

Expression of MINPP—cDNA encoding residues 31–487 of human MINPP (*i.e.* lacking the N-terminal ER targeting sequences (31)) was amplified by PCR, digested with KpnI and EcoRI, and ligated into the corresponding sites of pcDNA 3.1-Myc-His A (Invitrogen) driven by the cytomegalovirus promoter and containing the neomycin resistance gene. One day prior to transfection with MINPP, cells were seeded onto a 35-mm dish with a 14-mm microwell (MatTek) at a density of 8×10^5 cells/dish. They were transfected with 4.5 μg of the MINPP cDNA construct, using FuGENE 6 (Roche Molecular Biochemicals) at 70% confluency. After 24 h, cells was fixed and analyzed as described below.

For immunoblots, proteins were separated by NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electroblotted on polyvinylidene difluoride membranes (Invitrogen). The blots were subsequently blocked and probed with C-terminal Myc tag antibody (Invitrogen, dilution 1/500). Blots were then incubated with an anti-mouse IgG (Cell Signaling, dilution 1:7000) coupled to horseradish peroxidase for 1 h at 4 °C. Proteins were visualized using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Biosciences).

Fixation and Immunostaining—Cells for fixation were seeded (5×10^4 cells/dish) in 35-mm Glass Bottom Microwell dishes (MatTek Corp) for 48 h prior to the experiment. At the completion of the experiment, cells were rinsed with 2 ml of phosphate-buffered saline (PBS) and then fixed using the following procedures at room temperature: first, 1 ml of 3% paraformaldehyde in PBS was added for 15 min, followed by three rinses with PBS. Cells were then permeabilized using 0.25% Triton X-100 (in PBS) for 10 min, followed by three rinses with PBS. Cells were incubated with primary antibodies

[3] The abbreviations used are: $InsP_5$, inositol pentakisphosphate; PP-$InsP_5$, diphosphoinositol pentakisphosphate ($InsP_7$); bisdiphosphoinositol tetrakisphosphate ($InsP_8$)); $InsP_6$, inositol hexakisphosphate; DAPI, 4′,6-diamidino-2-phenylindole; HPLC, high-performance liquid chromatography; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PBS, phosphate-buffered saline.

[4] A. Saiardi, personal communication.

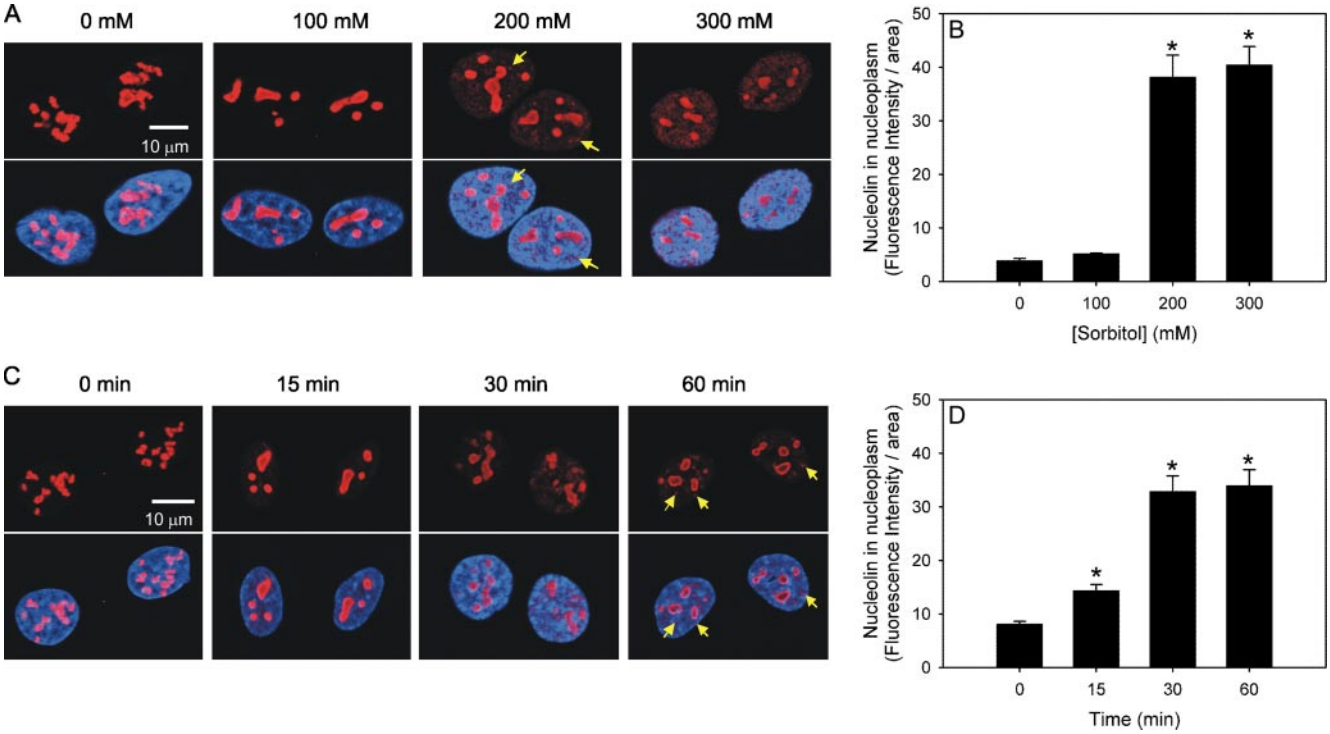

FIGURE 1. **Hyperosmotic stress causes nucleolin to be released from the nucleolus of U2-OS cells.** U2-OS cells were fixed and stained as described under "Experimental Procedures." Nucleolin staining is shown in *red*, whereas DAPI staining is shown in *blue*. *A*, cells were treated for 30 min with either vehicle or the concentration of sorbitol indicated in the figure. *B*, average intensity of nucleolin staining throughout the nucleoplasm (see "Experimental Procedures" for details). *C*, U2-OS cells were treated with 200 mM sorbitol for the various times indicated in the figure. *D*, average intensity of nucleolin staining in the nucleoplasm (see "Experimental Procedures" for details). *Yellow arrows* highlight areas that are poorly stained by DAPI (*i.e.* euchromatin), in which foci of nucleolin were observed. In *B* and *D*, data represent mean ± S.E. of 14–19 cells. *, $p < 0.01$.

(diluted as described below with 2% bovine serum albumin in PBS) for 2 h at room temperature and subsequently with secondary antibodies (all diluted 1:500 with 2% bovine serum albumin in PBS) for 1 h at room temperature. Cells were washed three times in PBS after each antibody incubation, and after the final wash coverslips were mounted with PBS buffer. Murine anti-nucleolin antibody (1:2000) was supplied by AbCam, and the secondary antibody was Alexa Fluor 594 goat anti-mouse IgG (Invitrogen). For MINPP-c-*myc* detection, the primary antibody was rat monoclonal anti-c-*myc* (GeneTex, 1:1000), and the secondary antibody was Alexa 564. After the antibody staining, the cells were washed again and then incubated with DAPI (1:15,000 dilution, Sigma) for 10 min. The fixed cells on chambers were then mounted in PBS buffer.

Confocal Imaging—A laser scanning confocal microscope (LSM 510 NLO mounted on Axiovert 100M microscope, Carl Zeiss, Inc.) was used to obtain the immunofluorescence and differential interference contrast (DIC) images. For imaging nucleolin fluorescence, the 543 nm HeNe laser was used for excitation and LP 560 filter for emission; for DAPI fluorescence, two-photon excitation was used with the Coherent Mira laser (Coherent Laser, Auburn, CA) tuned to 750 nm, and the BP 390–465 IR filter for emission. The fluorescence images were obtained sequentially, so that signal could be optimized and bleed-through minimized; DIC images were obtained simultaneously. The characteristics of the optics and digital sampling were chosen to acquire images at the highest possible resolution. The objective lens used was Plan-Apo 63×/1.4 Oil DIC. For the confocal channels the pinhole was set to 0.87 Airy units, corresponding to a z-resolution of 0.7 μm. The pixel size in the *x-y* plane was 0.2 μm, to match the lateral optical resolution. Acquisition slice was through the center of the nucleus. The acquisition software was Zeiss LSM 510, and for analysis Zeiss Image Examiner was used (version 3.2sp2).

Analysis of Cell Volume by Flow Cytometry—Cell volume was measured by electronic cell sizing using a Beckman Coulter Cell Lab Quanta SC flow cytometer equipped with a 488 nm laser. Calibration of the electronic volume channel was accomplished using a 6-μm AlignFlow Plus flow cytometry alignment beads for 488 nm excitation (Molecular Probes) by positioning the beads in channel 16 on the electronic volume scale. Cells (10^6) were pelleted by centrifugation and resuspended in 1 ml of either normal media, or media containing various concentrations of sorbitol. Then, 500 μl of each sample was immediately removed and examined on the Cell Lab Quanta SC for changes in mean cell volume (1-min time point). The remaining sample was incubated for 30 min at 37 °C, in a 5% CO_2 atmosphere, and then analyzed as described above. Five thousand cells were examined for each sample, and the data were analyzed by gating on the single cell population on an electronic volume histogram using Cell Lab Quanta SC software.

RESULTS AND DISCUSSION

Intranuclear Redistribution of Nucleolin in U2-OS Cells following Osmotic Stress—One of the goals of the current study was to investigate whether the intracellular compartmentalization of nucleolin responds to hyperosmotic stress. U2-OS osteosarcoma cells were used for this work because hypertonic-

ity is a physiologically relevant condition for bone cells (9). The extracellular fluid surrounding bone cells can be almost 200 mosM above isotonic (9). Additional intracellular osmotic stress can also arise from changes in ion-transport across the plasma membrane, uptake, and release of sugars and amino acids, and polymerization/depolymerization of macromolecules such as glycogen and proteins (11).

We analyzed the intracellular dynamics of nucleolin in U2-OS cells using confocal microscopy. The characteristics of the optics and the digital sampling were optimized for acquiring images at the highest possible resolution. We analyzed endogenous nucleolin (*i.e.* we did not overexpress the protein) in an effort to maintain a physiologically relevant context. In cells incubated in standard culture media, nucleolin was found to be concentrated in the nucleolus (Fig. 1*A*), in agreement with previous work with this cell line (32). There are reports that, in some cell types, nucleolin can be found in the cytoplasm under certain conditions (32, 33), but in our experiments no cytoplasmic nucleolin was detected (*e.g.* Fig. 1). We treated U2-OS cells with increasing concentrations of sorbitol to simulate the development of an anisosmotic gradient across the plasma membrane (Fig. 1, *A* and *B*). When only 100 mM sorbitol was added, there was no significant effect upon nucleolin (Fig. 1). When 200 mM sorbitol was present, we found that in many cells there was a near-complete expulsion of nucleolin from the innermost fibrillar core of the nucleolus, although there remained a persistent annular signal (Fig. 1) within the outermost, "granular" zone. The absence of nucleolin from the fibrillar core, where it normally facilitates transcription, replication, and recombination of rDNA (28), suggests that these early stages of ribosome biogenesis are halted following hyperosmotic stress.

The addition of 200 mM sorbitol had an additional effect upon nucleolin. A significant quantity of nucleolin was released into the nucleoplasm (Fig. 1). We estimated (see "Experimental Procedures") that there was up to a 10-fold increase in the average signal intensity for nucleoplasmic nucleolin, following 30-min treatment with 200 mM sorbitol (Fig. 1, *A* and *B*). Importantly, nucleolin was not distributed homogeneously throughout the nucleoplasm. Instead, a granular pattern was observed with locally enriched, nucleolin foci (some of which are highlighted by *yellow arrows* in Fig. 1). These foci tended to be most prominent in discrete zones that were stained poorly by DAPI, that is, areas that are enriched in transcriptionally active euchromatin. A further increase in the sorbitol concentration to 300 mM did not significantly change the characteristics of nucleolin mobilization (Fig. 1, *A* and *B*). This is the greatest degree of hyperosmotic stress that we have used, because it is below the level that is generally considered to be toxic (2). Our data were therefore obtained in a physiologically relevant context. Using 200 mM sorbitol, we studied the time course of these effects. There was a statistically significant effect within 15 min, and nucleolin mobilization was completed by 30 min (Fig. 1, *C* and *D*).

Hyperosmotic stress also influenced the spatial dynamics of nucleolin in HeLa cells (Fig. 2) and H1299 cells (data not shown). In both cell types, nucleolin was purged from the fibrillar core of the nucleolus (Fig. 2 and data not shown). Compared with U2-OS cells, HeLa cells were more sensitive to osmotic

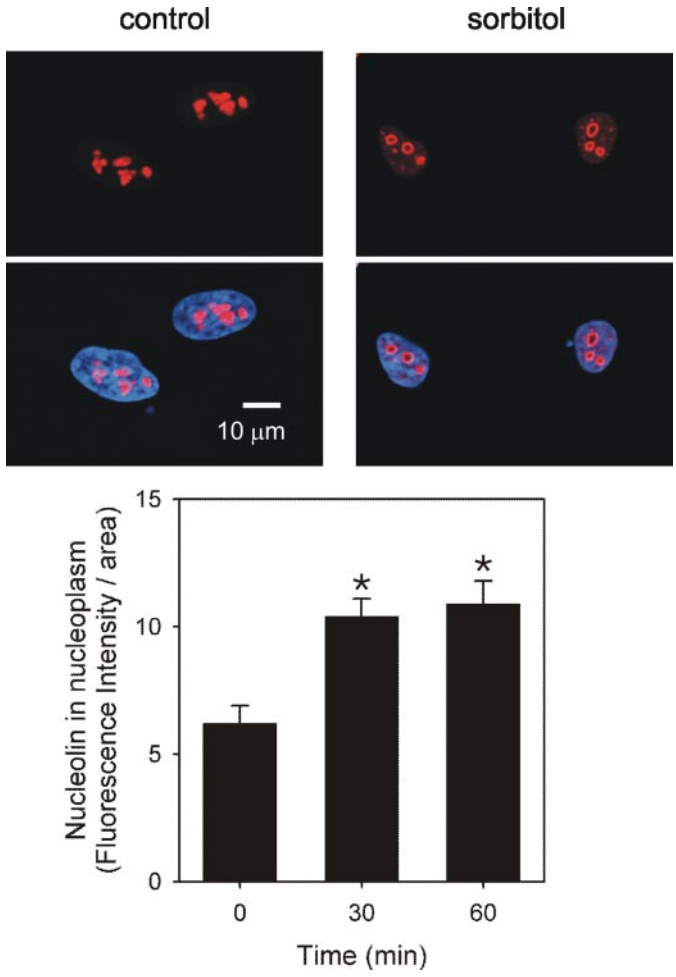

FIGURE 2. **Hyperosmotic stress causes nucleolin to be released from the nucleolus of HeLa cells.** HeLa cells were fixed and stained as described under "Experimental Procedures." Nucleolin staining is shown in *red*, whereas DAPI staining is shown in *blue*. Cells were treated for either 30 or 60 min with 100 mM sorbitol. The *upper panel* compares cells treated with vehicle or sorbitol for 30 min. The *lower panel* shows the average intensity of nucleolin staining throughout the nucleoplasm (see "Experimental Procedures" for details), and data represent mean ± S.E. of 15–19 cells. *, $p < 0.005$.

stress; as little as 100 mM sorbitol was sufficient to mobilize nucleolin (Fig. 2). Hyperosmotic stress also led to an increase in the nucleoplasmic staining of nucleolin (Fig. 2). Interestingly, this increase in the concentration of nucleoplasmic nucleolin may in part reflect some nuclear shrinkage following hyperosmotic stress (Fig. 2). This particular effect was not observed in U2-OS cells (Fig. 1).

All of the experiments described above used a commercial human anti-nucleolin antibody (see "Experimental Procedures"), but similar data (not shown) were also obtained using a different human anti-nucleolin monoclonal antibody (see "Experimental Procedures" and Ref. 34).

The Effects of Hyperosmotic Stress upon Levels of Higher Inositol Polyphosphates in U2-OS Cells—In vitro, inositol pyrophosphates have been shown to phosphorylate nucleolin in a concentration-dependent manner (21, 22).[4] Could this be a mechanism by which physiologically relevant changes in the cellular levels of inositol pyrophosphates alter nucleolin function? To pursue this question, we have used HPLC analysis of [^{3}H]inositol-labeled U2-OS cells to measure the cellular levels

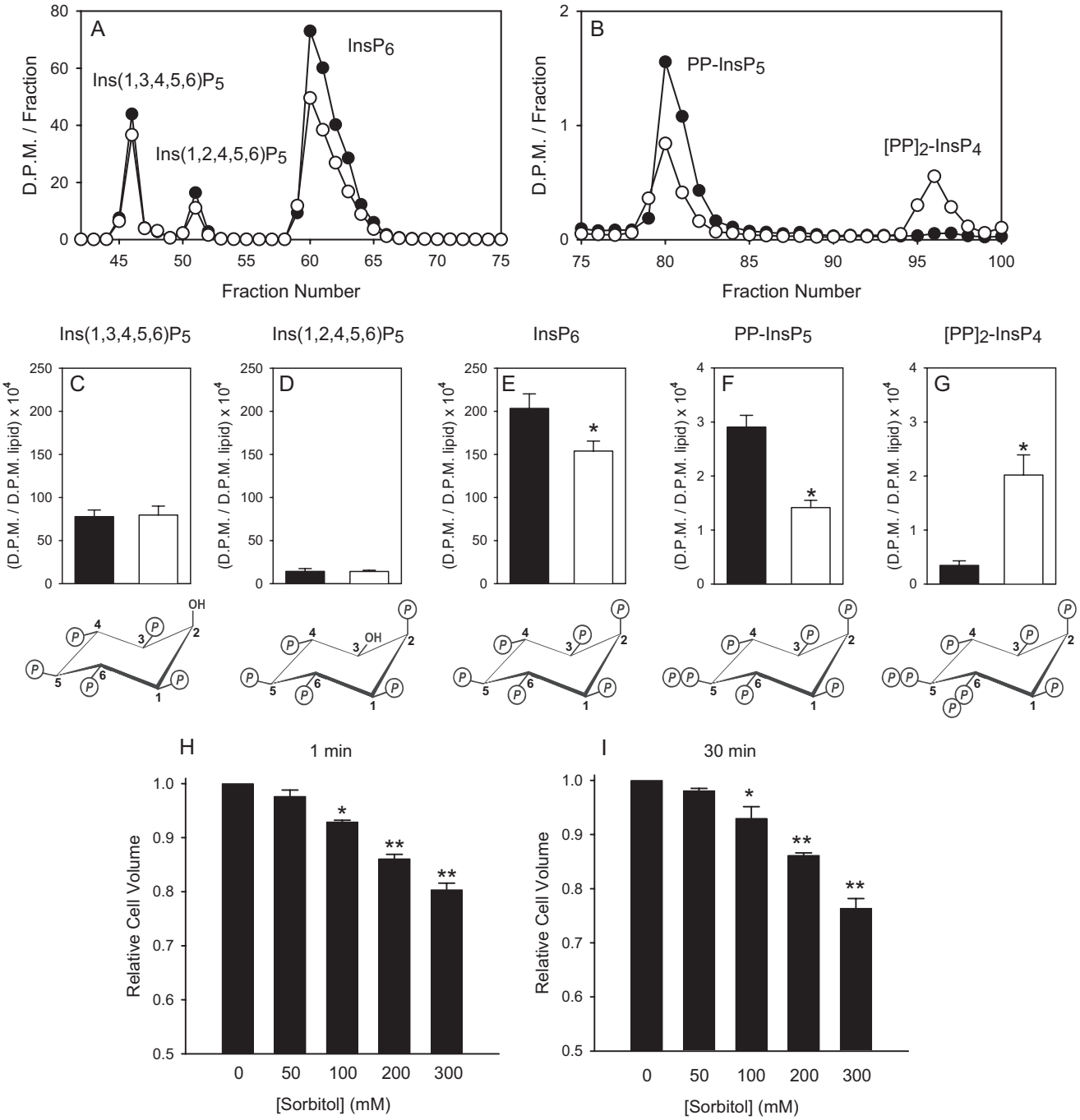

FIGURE 3. **The effects of hyperosmotic stress upon cell volume and cellular levels of inositol polyphosphates in U2-OS cells.** U2-OS cells were labeled with [^{3}H]inositol, and then cells were quenched and extracted, and individual inositol phosphates were separated by HPLC. The identity of each inositol phosphate was established by their co-elution with standards (53). *A* and *B*, representative HPLC traces from cells treated for 30 min with either vehicle (*closed symbols*) or 200 mM sorbitol (*open symbols*). The chromatograms are split into two panels, so as to highlight differences in the scales of the *y*-axes. *C–G*, levels of individual inositol phosphates from six experiments (means ± S.E.; *filled bars* = control, *open bars* = sorbitol; *, $p < 0.05$), beneath which the structures of the compounds are depicted. Note that the placement of the 6-diphosphate group in [PP]$_2$-InsP$_4$ is only tentative, based on an analysis of this material in *Dictyostelium* (54); the structure of [PP]$_2$-InsP$_4$ has yet to be defined in mammalian cells. *H* and *I*, relative cell volume after 1- and 30-min treatment with either 0, 50, 100, 200, or 300 mM sorbitol. Cell volume was determined by electronic cell sizing (see "Experimental Procedures").

of these polyphosphates (Fig. 3, *A* and *B*). As is the case with other mammalian cell types (*e.g.* Refs. 35 and 36), the levels of PP-InsP$_5$ and [PP]$_2$-InsP$_4$ in control cells (*closed symbols*, Fig. 3*B*) were, respectively, ~2% and 0.2% of the level of InsP$_6$. We also noted the presence of two InsP$_5$ isomers, Ins(1,3,4,5,6)P$_5$ and Ins(1,2,4,5,6)P$_5$, with the former predominating (Fig. 3, *C* and *D*); again, this is a typical result (35, 36).

InsP$_6$ is an important metabolite to monitor, because it is a precursor for inositol pyrophosphate synthesis and also an inhibitor of protein phosphorylation by these pyrophosphates (22). Following 30-min exposure to 200 mM sorbitol, there was a 20–25% decrease in InsP$_6$ levels (Fig. 3*E*). It should be noted that no other biological stimulus has previously been shown to elicit such a large and rapid change in cellular InsP$_6$ turnover,

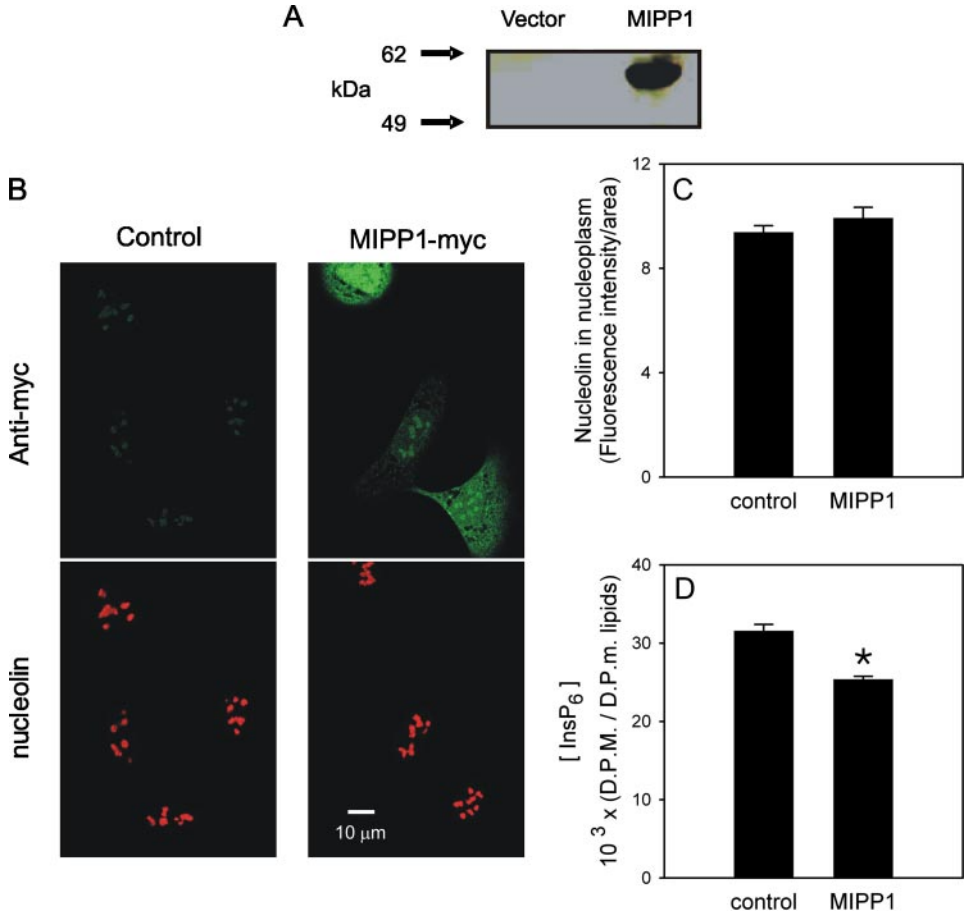

FIGURE 4. **The effect of MINPP expression upon cellular $InsP_6$ levels and intracellular nucleolin distribution.** U2-OS cells were transfected with either MINPP-*myc* or vector alone as described under "Experimental Procedures." *A*, Western analysis (using anti-*myc* antibody) of extracts of U2-OS cells. *B*, lack of effect of transfection of MINPP upon intracellular nucleolin distribution. *C*, provides the mean (±S.E.) nucleolin intensity in the nucleoplasm from 7–13 cells. *D*, compares the $InsP_6$ levels in vector- and MINPP-transfected cells, determined by HPLC analysis. *, $p < 0.05$.

when it is driven by endogenous enzymes. Because the total ^3H dpm in the inositol pyrophosphate region of the chromatograph (*i.e.* PP-[^{3}H]$InsP_5$ plus $[PP]_2$-[^{3}H]$InsP_4$) did not change following hyperosmotic stress (see below), we conclude that the $InsP_6$ that was metabolized must be dephosphorylated rather than phosphorylated. However, this was not associated with any corresponding elevation in $InsP_5$ levels (Fig. 3). There were increases in the less phosphorylated inositol phosphates (data not shown), but we cannot tell if this accounts for the $InsP_6$ that was dephosphorylated, because lower inositol phosphates accumulate during hyperosmotic stress by an independent mechanism involving activation of PLC (37).

An immediate cell shrinkage is expected to be induced by hyperosmotic stress. Indeed, we found that, following the addition of sorbitol to the culture medium, U2-OS cells showed a significant and rapid (within 1 min) decrease in cell volume, by a value of 9% when 100 mM sorbitol was added ($p < 0.01$), increasing to 25% when 300 mM was present ($p < 0.001$) (Fig. 3*H*). Osmotically induced cell shrinkage is typically followed by a regulatory volume increase, although the time frame of this compensatory cell swelling can vary from minutes to hours (38). Our U2-OS cells remained shrunken by hyperosmotic stress for at least 30 min (Fig. 3*I*). It is intriguing that the magnitude of the decrease in cellular $InsP_6$ levels in response to the addition of 200 mM sorbitol (20–25% Fig. 3*E*) is very similar to the size of the accompanying decrease in cell volume (20%, Fig. 3, *H* and *I*). Thus, we conclude that the concentration of $InsP_6$ is stabilized during cell shrinkage. It has frequently been noted that cellular concentration of $InsP_6$ is remarkably constant (39, 40). This has generally been interpreted as evidence that there is relatively slow metabolic flux through $InsP_6$. On the contrary, our new data indicate that $InsP_6$ turnover can be quite high, thereby enabling rapidly acting homeostatic mechanisms that minimize fluctuations in intracellular concentration of $InsP_6$, when cell volume changes.

We also observed that the total cellular levels of $[PP]_2$-$InsP_4$ increased nearly 6-fold in response to hyperosmotic stress (Fig. 3*G*), reflecting a 7-fold concentration change when the accompanying cell shrinkage is taken into account. This was near quantitatively balanced by a corresponding decrease in PP-$InsP_5$ levels (Fig. 3*F*), consistent with an earlier demonstration that the PP-$InsP_5$ kinase is activated following a hypertonic challenge (15).

The Effect of MINPP upon $InsP_6$ Levels and Nucleolin Mobilization—Because $InsP_6$ is an inhibitor of nucleolin phosphorylation by inositol pyrophosphates (22), we wanted to investigate whether a decrease in $InsP_6$ levels *per se* could drive nucleolin mobilization. We did not use RNA interference to reduce expression of the $InsP_5$ kinase that synthesizes $InsP_6$. This decision was based on previous experiments showing that "knockdown" of mammalian $InsP_5$ 2-kinase expression induces gross changes in nuclear morphology (41), is pro-apoptotic (42), and generally toxic (43, 44). Instead, we overexpressed MINPP, an $InsP_6$ phosphatase. Overexpression of full-length MINPP in its natural habitat, the lumen of the endoplasmic reticulum, often has little impact upon the cytosolic pool of $InsP_6$ (45). Therefore, we overexpressed a truncated version of MINPP, which is expressed in the cytoplasm, because it was missing its N-terminal signal peptide that normally directs the enzyme into the endoplasmic reticulum (46). Western analysis confirmed that the protein was expressed: the apparent size was virtually identical to the expected size of 57 kDa (Fig. 4*A*). The degree of transfection in these experiments was ~80%. MINPP overexpression reduced levels of $InsP_6$ in the cell population by ~20% (Fig. 4*D*), which equates to an average 25% reduction in the cells that were successfully transfected. Bearing in mind

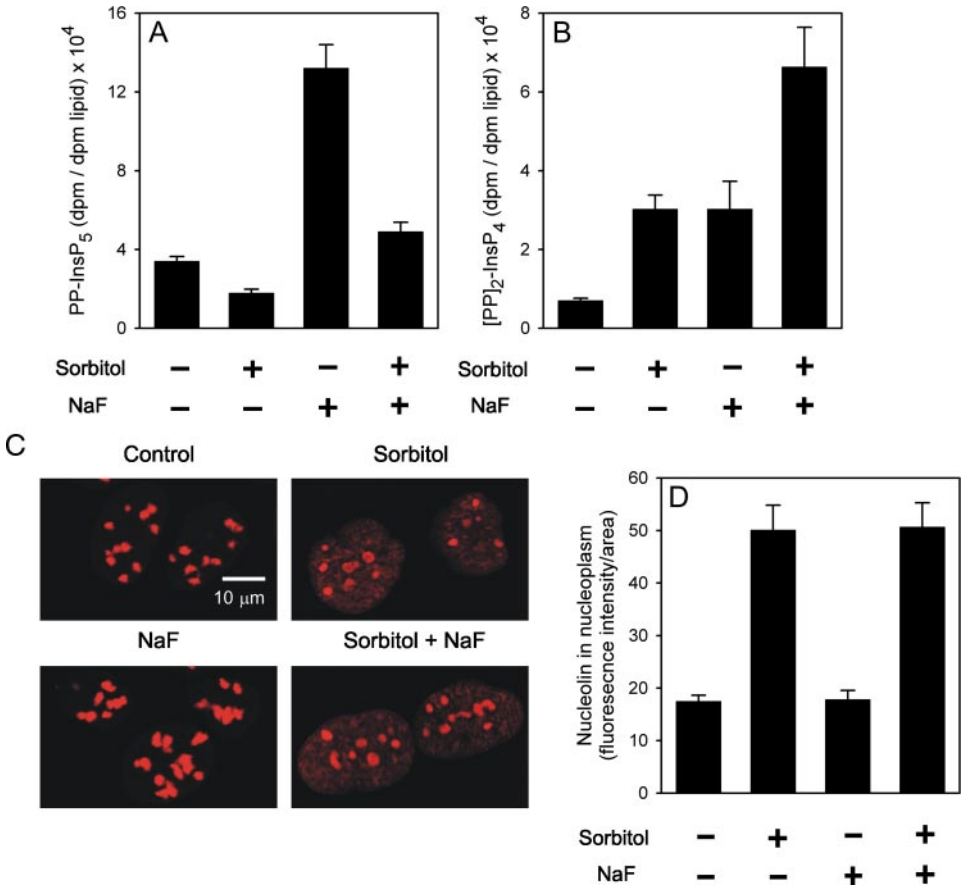

FIGURE 5. **The effect of NaF and hyperosmotic stress upon nucleolin mobilization and cellular levels of PP-InsP$_5$ and [PP]$_2$-InsP$_4$.** The levels of PP-InsP$_5$ (*A*) and [PP]$_2$-InsP$_4$ (*B*) in [^{3}H]inositol-labeled U2-OS cells were determined by HPLC. Cells were treated with either vehicle or NaF (1 mM for 30 min) followed 30 min later by either vehicle or sorbitol (200 mM for 30 min). In parallel, non-radiolabeled cells were fixed and stained for nucleolin (*red signal*) as described under "Experimental Procedures." Nucleolin distribution was determined by confocal microscopy. *C*, representative images; *D*, mean (±S.E.) values for nucleolin intensity in the nucleoplasm from 18–24 cells for each condition.

how aggressively the cell normally defends a stable InsP$_6$ concentration (see above), this particular effect of MINPP is arguably quite dramatic. Nevertheless, overexpression of MINPP did not promote significant release any nucleolin from the nucleolus (Fig. 4, *B* and *C*).

Do Changes in Inositol Pyrophosphate Levels Drive Nucleolin Mobilization?—In vitro, at least, inositol pyrophosphates phosphorylate nucleolin in a concentration-dependent manner (21, 22). The challenge for this field is now to establish a paradigm for investigating if, *in vivo*, physiologically relevant changes in the cellular levels of inositol pyrophosphates can be directly linked to functional changes in any of the proteins that these molecules can phosphorylate. Clearly, cellular levels of inositol pyrophosphates respond to osmotic stress; does this affect nucleolin function? To address this question, we developed a pharmacological strategy for examining if stress-dependent changes in inositol pyrophosphate turnover are directly responsible for alterations in nucleolin behavior.

Fluoride inhibits the phosphatases that rapidly hydrolyze PP-InsP$_5$ and [PP]$_2$-InsP$_4$ (47). This inhibition exposes the ongoing activities of the InsP$_6$ and PP-InsP$_5$ kinases (47). In the current study, the addition of 1 mM NaF for 60 min caused the cellular levels of PP-InsP$_5$ and [PP]$_2$-InsP$_4$ to each increase 4-fold (Fig. 5, *A* and *B*). This represents a considerable increase in total mass levels of inositol pyrophosphates, but it did not have any effects upon nucleolin's spatial dynamics (Fig. 5, *C* and *D*).

We further found that the separate effects of fluoride and sorbitol upon [PP]$_2$-InsP$_4$ levels were approximately additive (Fig. 5*B*), but fluoride did not affect the degree of nucleolin mobilization caused by sorbitol addition (Fig. 5, *C* and *D*). We also found that fluoride prevented hyperosmotic stress from decreasing total cellular levels of PP-InsP$_5$ (Fig. 5*A*). However, this was also without any effect upon nucleolin mobilization.

The possible significance of the stress-dependent increases in [PP]$_2$-InsP$_4$ levels was also studied using the MEK inhibitor, PD98059. We have previously shown that this inhibitor antagonizes the increases in cellular [PP]$_2$-InsP$_4$ levels brought about by hyperosmotic stress (14). A similar effect was observed in the current study (Fig. 6). PD98059 did not significantly alter the cellular levels of other inositol phosphates (Fig. 6 and data not shown). Despite its effects upon [PP]$_2$-InsP$_4$ synthesis, PD98059 did not affect the intranuclear mobilization of nucleolin, either in control or in sorbitol-stressed cells (Fig. 6). These data consolidate the conclusion (see above) that inositol pyrophosphates do not mediate the effects of hyperosmotic stress upon the intranuclear distribution of nucleolin. An additional conclusion to emerge from these particular experiments (Fig. 6), although this is not directly relevant to the goals of the current study, is that the MEK/ERK pathway does not drive nucleolin mobilization.

Concluding Comments—In U2-OS cells and HeLa cells incubated under isotonic conditions, nucleolin is entirely restricted to the nucleolus (Figs. 1 and 2). This presumably facilitates a key function for nucleolin, namely, its participation in all aspects of ribosome biogenesis (28). Here, we demonstrated that mild hypertonic stress caused nucleolin to be expelled from the innermost fibrillar core of the nucleolus. This likely prevents nucleolin from facilitating early stages of ribosomal biogenesis, namely, transcription, replication, and recombination of rDNA (28). Instead, nucleolin migrated into the nucleoplasm, where it preferentially localized in zones containing euchromatic (transcriptionally active) DNA (Fig. 1). This is where DNA replication normally begins, prior to replication of heterochromatic DNA (48). Studies from other laboratories (30, 49) indicate that a major assignment for nucleoplasmic nucleolin is to constrain

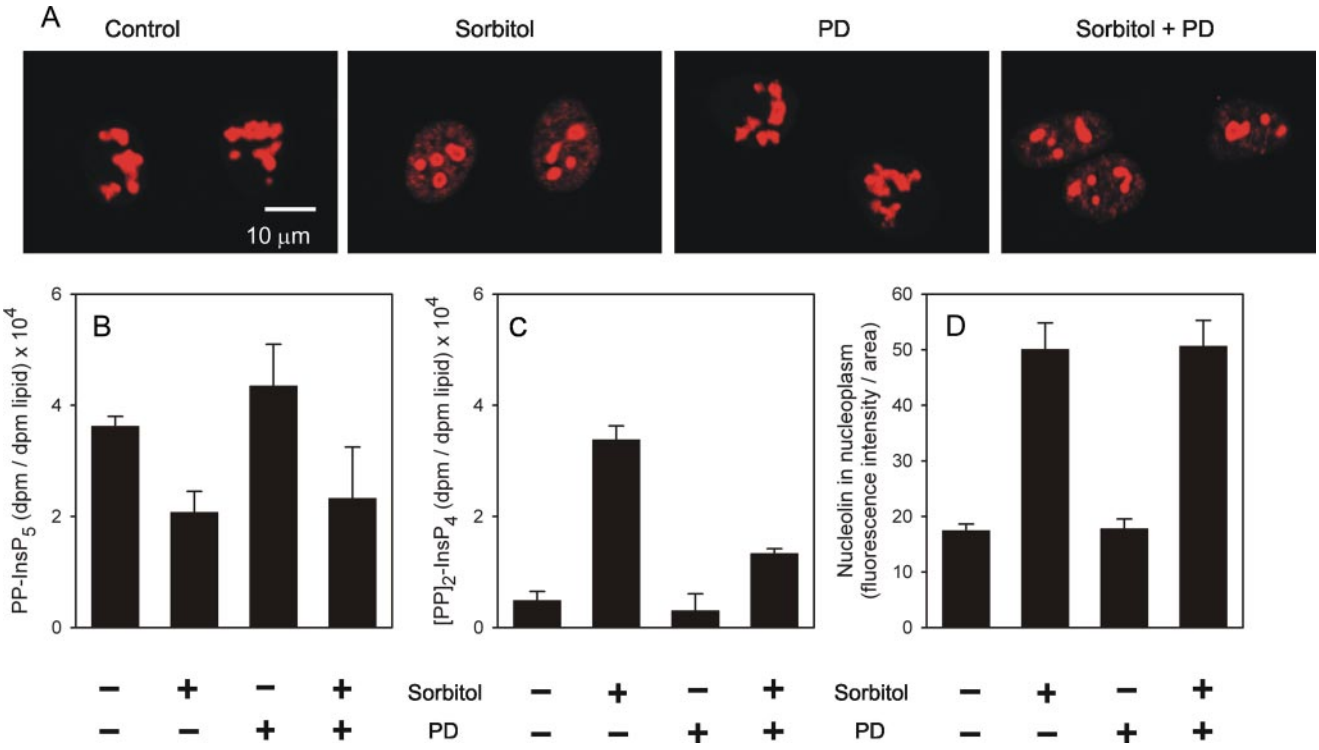

FIGURE 6. **The effect of PD98059 and hyperosmotic stress upon nucleolin mobilization and cellular levels of PP-InsP$_5$ and [PP]$_2$-InsP$_4$.** U2-OS cells were treated with either vehicle or PD98059 (50 μM for 30 min) followed 30 min later by either vehicle or sorbitol (200 mM for 30 min). Then, cells were fixed and stained for nucleolin (*red signal*) as described under "Experimental Procedures." Nucleolin distribution was determined by confocal microscopy. *A*, representative images. In parallel, the levels of PP-InsP$_5$ and [PP]$_2$-InsP$_4$ in [^{3}H]inositol-labeled cells was determined by HPLC (*B* and *C*). *D*, mean (±S.E.) values for nucleolin intensity in the nucleoplasm from 11–16 cells for each condition.

DNA replication. We therefore conclude that nucleolin switches its functions following hyperosmotic stress. Thus, the gatekeeping activity of the nucleolus is the target of a fast-acting signaling mechanism that modulates the biological activities of nucleolin by affecting its subcellular localization.

An increase in nucleoplasmic nucleolin levels has previously been observed in response to some pathological stimuli: a chronic thermal challenge (up to 90-min exposure to 44 °C), or after chemically induced genotoxicity (30, 49, 50). However, another environmental insult, UV irradiation, does not elicit these effects (49). The latter observations illustrate how the cell's response to one form of stress does not predict how it will react to another. A cell tailors its individual molecular responses to meet the particular challenges from the type of stress to which it is exposed (7, 51), just as there are distinct cellular responses to different hormones. There are also important distinctions that can be drawn between different stresses that are either pathological or physiological in nature. The extent of the hyperosmotic stress to which our U2-OS cells were routinely exposed, 200 mosM, is within the range that bone cells normally experience (9). Because the degree of hyperosmotic stress that we have used is sublethal (2), no apoptosis was observed (data not shown).

Nucleolin was a particular focus of the current study, because it is one of a select group of proteins that can be phosphorylated by inositol pyrophosphates, *in vitro* at least, through a kinase-independent reaction (21, 22). It is now being predicted in some publications that this phenomenon provides a molecular foundation for a new signaling mechanism, permitting stimulus-dependent fluctuations in the cellular levels of inositol pyrophosphates to alter the functions of target proteins (25, 26). However, there are others who advocate a more cautious interpretation of these data, until this provocative new mode of covalent modification can be either validated or disproved to occur *in vivo* (23, 52). The current study is the first attempt to deploy an *in vivo* model to investigate the potential signaling significance of protein phosphorylation by inositol pyrophosphates. In choosing nucleolin for these experiments, we selected one of best characterized *in vitro* substrates of the inositol pyrophosphates. All of our evidence points to inositol pyrophosphates not supervising the responses of nucleolin to osmotic stress *in vivo*. The protocols that we have deployed in our study can be more widely applied to a more general search for other molecular targets of the inositol pyrophosphates, irrespective of their molecular mechanism of action.

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