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 fibrous 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, diposphoinositol 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/ddepolymerization 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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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 phosphorylation of proteins 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 and yeast versions of this protein are 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.

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(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% CO2 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

Intracellular 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. 1A), 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 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). 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 [3H]inositol-labeled U2-OS cells to measure the cellular levels

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**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.
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-InsP₅ and [PP]₂-InsP₄ in control cells (closed symbols, Fig. 3B) were, respectively, ~2% and 0.2% of the level of InsP₆. We also noted the presence of two InsP₅ isomers, Ins(1,3,4,5,6)P₅ and Ins(1,2,4,5,6)P₅, with the former predominating (Fig. 3, C and D); again, this is a typical result (35, 36).

InsP₆ 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 InsP₆ levels (Fig. 3E). It should be noted that no other biological stimulus has previously been shown to elicit such a large and rapid change in cellular InsP₆ turnover,

**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 [³H]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-InsP₄ is only tentative, based on an analysis of this material in Dictyostelium (54); the structure of [PP]₂-InsP₄ 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”).
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**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 $^3$H 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. 3H). 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. 3I). 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. 3E) 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. 3G), 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. 3F), 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 “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. 4A). The degree of transfection in these experiments was ~80%. MINPP overexpression reduced levels of InsP$_6$ in the cell population by ~20% (Fig. 4D), which equates to an average 25% reduction in the cells that were successfully transfected. Bearing in mind
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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 [mH]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 increases in [PP]$_2$-InsP$_4$ levels, nucleolin’s spatial dynamics (Fig. 5A). 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, A and B). We also found that fluoride prevented hyperosmotic stress from decreasing total cellular levels of PP-InsP$_5$ (Fig. 5A). However, this was also without any effect upon nucleolin mobilization.

We further found that the separate effects of fluoride and sorbitol upon [PP]$_2$-InsP$_4$ levels were approximately additive (Fig. 5B), 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. 5A). However, this was also without any effect upon nucleolin mobilization.

The possible significance of the stress-dependent decreases 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. 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
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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