Hyposmotic Stress Induces Cell Growth Arrest via Proteasome Activation and Cyclin/Cyclin-dependent Kinase Degradation*

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Ordered cell cycle progression requires the expression and activation of several cyclins and cyclin-dependent kinases (Cdks). Hyposmotic stress causes growth arrest possibly via proteasome-mediated degradation of cyclin D1. We studied the effect of hyposmotic conditions on three colon (Caco2, HRT18, HT29) and two pancreatic (AsPC-1 and PaCa-2) cell lines. Hyposmosis caused reversible cell growth arrest of the five cell lines in a cell cycle-independent fashion, although some cell lines accumulated at the G1/S interface. Growth arrest was followed by apoptosis or by formation of multinucleated giant cells, which is consistent with cell cycle catastrophe. Hyposmosis dramatically decreased Cdc2, Cdk2, Cdk4, cyclin B1, and cyclin D3 expression in a time-dependent fashion, in association with an overall decrease in cellular protein synthesis. However, some protein levels remained unaltered, including cyclin E and keratin 8. Selective proteasome inhibition prevented Cdk and cyclin degradation and reversed hyposmotic stress-induced growth arrest, whereas calpain and lysosome enzyme inhibitors had no measurable effect on cell cycle protein degradation. Therefore, hyposmotic stress inhibits cell growth and, depending on the cell type, causes cell cycle catastrophe with or without apoptosis. The growth arrest is due to decreased protein synthesis and proteasome activation, with subsequent degradation of several cyclins and Cdks.

The cell cycle involves a meticulously ordered series of events that control defined cell cycle stage checkpoints and ultimately cell division. These events are tightly regulated by the expression and degradation, activation and inactivation, and subcellular localization of cyclins and cyclin-dependent kinases (Cdks) (1–3). Cyclins associate with, and activate, Cdks and are periodically synthesized then degraded during cell cycle progression, whereas cellular Cdk levels tend to remain in a cell cycle-independent fashion, although some cell lines accumulated at the G1/S interface. Growth arrest was followed by apoptosis or by formation of multinucleated giant cells, which is consistent with cell cycle catastrophe. Hyposmosis dramatically decreased Cdc2, Cdk2, Cdk4, cyclin B1, and cyclin D3 expression in a time-dependent fashion, in association with an overall decrease in cellular protein synthesis. However, some protein levels remained unaltered, including cyclin E and keratin 8. Selective proteasome inhibition prevented Cdk and cyclin degradation and reversed hyposmotic stress-induced growth arrest, whereas calpain and lysosome enzyme inhibitors had no measurable effect on cell cycle protein degradation. Therefore, hyposmotic stress inhibits cell growth and, depending on the cell type, causes cell cycle catastrophe with or without apoptosis. The growth arrest is due to decreased protein synthesis and proteasome activation, with subsequent degradation of several cyclins and Cdks.

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§ The abbreviations used are: Cdks, cyclin-dependent kinases; ALLN, N-acetyl-L-leucyl-L-leucinal-Norleucinal; K, keratin; PBS, phosphate-buffered saline; mAb, monoclonal antibody; GADD, growth arrest and DNA damage-inducible protein.

EXPERIMENTAL PROCEDURES

Cell Culture and Hyposmotic Treatment—Three human colon (Caco2, HRT18, HT29) and two pancreas (AsPC-1, PaCa-2) carcinoma cell lines were used (American Type Culture Collection, Manassas, VA). All the cell lines were cultured at 37 °C, in 5% CO2 humidified incubators. Hyposmotic treatment was done by dilution of the culture medium with distilled water (1:1) to a final osmolarity of 150 mosM. As a control, cells were cultured in iso-osmotic medium by diluting the medium with an.
based on three independent experiments.

Numbers (shown on the adherent) were counted followed by calculating the -fold change in cell number. At the indicated time points, the total cells (floater and adherent) were counted followed by calculating the -fold change in cell numbers (shown on the y-axis). The histograms show mean ± S.D. based on three independent experiments.

**Equal volume of distilled water containing 0.15 M sodium chloride (24, 26).** Floater cells were collected after centrifugation of the culture medium, and adherent cells were collected after trypsinization. Cell death was assessed by trypan blue staining. All data are presented as the mean ± S.D. Student’s paired t tests were used for statistical analysis.

**Detection of Apoptosis and Cell Cycle Analysis—**Monoclonal antibody (mAb) M30 “CytoDEATH antibody” (Roche Molecular Biochemicals, Indianapolis, IN), which recognizes a keratin 18 (K18) fragment generated after caspase digestion, was used for immunoblotting of cell lysates. M30 reactivity is indicative of cells undergoing apoptosis (34, 35). A rabbit polyclonal anti-keratin polypeptide 18 (K18), Ab 4668, was generated against an N-terminal K18 peptide (RPVSSAASVYAGA38) as described previously (36). This Ab was used to detect the intact and caspase-generated N-terminal K18 fragment (35). Formation of DNA fragments was assessed after genomic DNA isolation (using a Qiagen kit) then separation using 1.5% agarose gels. For cell cycle analysis, the five cell lines analyzed in Fig. 1 were cultured in hypotonic (H) or isotonic (I) control conditions for 24 h. The numbers of floater (dark bars) and adherent (light bars) cells were counted. Note that most of the cells remain adherent after exposure to hypotonic conditions. In a separate but similar experiment to that shown in panel A, the percent cell death in the proportionately small floater cell populations was determined. C, genomic DNA was purified from the floater cells, and an equal amount of DNA from each cell line was resolved using a 1.5% agarose gel, stained with ethidium bromide followed by visualization using a UV trans-illuminator (subpanel a). The lower subpanel b shows a lighter exposure of the high molecular weight dense DNA bands (as compared with subpanel a) to demonstrate that the ladder formation noted in HRT18 cells is not related to sample loading. H, hypotonic; I, isotonic.

**Gel and Protein Analysis—**Cells were solubilized in hot (90 °C) SDS-PAGE sample buffer. The protein samples were resolved by SDS-PAGE (38) then transferred to polyvinylidene difluoride membranes followed by Western blot analysis (39). The primary antibodies, directed toward cell cycle related and other proteins, included antibodies to Cdc2, cyclin B1, Cdk2, cyclin E, Cdk4, and cyclin D3 (CLONTECH, Palo Alto, CA), keratin polypeptide 8 (K8) mAb M20 (NeoMarkers, Fremont, CA), B1, Cdk2, cyclin E, Cdk4, and cyclin D3 (CLONTECH, Palo Alto, CA), keratin polypeptide 8 (K8) mAb M20 (NeoMarkers, Fremont, CA), B1, Cdk2, cyclin E, Cdk4, and cyclin D3 (CLONTECH, Palo Alto, CA), keratin polypeptide 8 (K8) mAb M20 (NeoMarkers, Fremont, CA), B1, Cdk2, cyclin E, Cdk4, and cyclin D3 (CLONTECH, Palo Alto, CA), keratin polypeptide 8 (K8) mAb M20 (NeoMarkers, Fremont, CA).

**Metabolic Labeling of Cells—**Cells were cultured for 5 h in iso- or hypo-osmotic medium. Cell culture media was then adjusted to hypo-(H11005) or iso-osmotic methionine-free labeling media after rinsing twice with the same labeling media. [35S]Methionine (100 μCi/ml) was added, and the cells were further cultured for one additional hour (total culture time = 6 h) in the presence of iso- or hypo-osmotic media. Labeled cells were washed with cold PBS and solubilized with 1% Nonidet-P40 in PBS containing 5 mM EDTA. After measurement of protein concentration using the BCA method (Pierce, Rockford, IL), protein synthesis was estimated by measuring the incorporated radioactivity per microgram of protein.

**Assessment of Protein Degradation—**Cells were preincubated for 2 h with the proteasome inhibitors lactacystin (20 μM) or N-acetyl-I-leucyl-I-leucyl-I-norleucinal (ALLN) (270 μM), the calpain inhibitor PD150606 (100 μM), the lysosomal protease inhibitors chloroquine (100 μM), or a mixture of pepstatin-A (100 μM) and leupeptin (50 μM). Cells...
were then cultured for 6 h in hyposmotic media containing the same inhibitors used during the preincubation. Proteins were isolated after 6 h followed by immunoblotting with antibodies directed to cell cycle-related proteins.

Electron Microscopy—Cell lines were cultured under hyposmotic conditions for 48 h followed by collection of the floater cells, washing with PBS (pH 7.4) then fixing with 0.1 M sodium phosphate buffer (pH 7.2) containing 2% glutaraldehyde (60 min, 4 °C). Alternatively, asynchronously growing cells were collected by trypsinization followed by a similar glutaraldehyde fixation. Further processing of the fixed cells was done as described previously (40). Cells were examined and photographed using a Philips CM-12 transmission electron microscope.

**RESULTS**

**Cell Growth Arrest upon Exposure to an Hyposmotic Stress**—To assess the effect of hyposmotic culture conditions on cell proliferation, three colonic (Caco2, HRT18, HT29) and two pancreatic (AsPC-1 and PaCa-2) cell lines were exposed to hypotonic medium for different times as indicated (Fig. 1). As compared with isotonic control medium, the hyposmotic culture conditions were associated with a reduced total cell number (floater and adherent) in all the cell lines beginning at 6 h (Fig. 1). The reduced cell numbers upon culturing in hyposmotic conditions reached statistical significance (Student’s t test, p < 0.05) at different time points depending on the tested cell lines (HRT18 and AsPC-1 cells at 6 and 12 h, respectively; HT29, Caco2, and PaCa-2 cells at 24 h). Notably, the growth arrest was reversible, after switching to isotonic control medium, in the five cell lines up to 24 h after exposure to the hyposmotic treatment (not shown). In addition and as may be expected, the isotonic control medium has a 50% dilution of serum and other normal culture medium factors, which can result in up to a 30% decrease in growth rate (depending on the culture duration and the cell line, not shown). However, for all the experiments shown we used the isotonic control medium for appropriate comparison with the hyposmotic conditions.

**Hyposmosis-induced Cell Growth Arrest Leads to Apoptosis in HRT18 Cells**—We examined the effect of the hyposmotic-induced cell-growth arrest by determining if this arrest was accompanied by cell death. Most of the cells (>90%) remain attached to the tissue culture dish even after 24 h of exposure to hyposmotic conditions (Fig. 2A). The viability of the adherent cells, as determined by trypan blue exclusion, was >95% (not shown) whereas the viability of the floater cells was >60% in all the cell lines tested except for HRT18 where >80% of the floater cells were dead (Fig. 2B). The percent death of floating cells increased significantly upon hypotonic exposure (p < 0.05) in HRT18 (28% to 86%) and Caco2 (17% to 36%) cells but not in other cell lines. Genomic DNA analysis of the floating cells showed ladder formation only in HRT18 cells (Fig. 2C), which is consistent with apoptosis, whereas none of the adherent cells in the five cell lines manifested any DNA ladder formation (not shown). The DNA ladder formation occurs only in the floater cells isolated from HRT18 cells grown under hyposmotic conditions but not cells grown under isotonic conditions (Fig. 2C, compare lanes 2 and 6). The apoptotic cell death of a fraction of HRT18 cells that have been exposed to hyposmotic conditions was confirmed by the caspase-mediated formation of the two K18 fragments, p29 and p43 (Fig. 3). The two keratin-degradation fragments were detected only in HRT18 cells, and the stoichiometry of the p29 fragment (which is generated after formation of p43 (35)), as compared with uncleaved K18, increases with increasing exposure time to hyposmotic conditions (compare Fig. 3B with 3A). Therefore, the results of Figs. 1–3 indicate that hyposmotic stress causes minimal cell death and suggest that such stress leads primarily to growth arrest with some cell death occurring primarily in HRT18 cells via apoptosis.

**Hyposmotic Stress Results in a Stage-independent Cell Cycle Growth Arrest**—The rapid effect of hyposmotic stress (e.g. within 6 h) on cell growth suggested that this stress could act via a generalized and/or a cell cycle stage-specific arrest. We tested these possibilities by cell cycle analysis after culturing cells in the presence or absence of hyposmotic conditions. As shown in Fig. 4, the colonic cell lines (Caco2, HRT18, and HT29) manifested a preferential accumulation of cells at the G1/S interface after 6 h of culture, whereas the two pancreatic cell lines (AsPC-1 and PaCa-2) had a more generalized arrest. Cell cycle analysis after 12, 24, or 48 h of culture in hyposmotic conditions manifested a similar trend for all the five cell lines (not shown) thereby indicating that the growth arrest was not necessarily related to a single cell cycle stage per se, and that...
FIG. 4. Cell cycle analysis of cultured colonic and pancreatic cell lines in the presence or absence of hypotonic stress. The five cell lines used in Figs. 1–3 were cultured in hypo- or iso-osmotic conditions for 6 h. Floater cells were collected and pooled with the adherent cells (isolated by trypsinization). After washing with PBS, cells were fixed then processed for cell cycle analysis as described under “Experimental Procedures.” In some cases, the total percentage of cells (i.e. G$_0$/G$_1$ + S + G$_2$/M) was slightly less than 100 (e.g. Caco2 cells) due to the presence of cell fragments or cells with DNA content of <2n or >4n, respectively.
Hyposmotic Stress and Cell Growth Arrest

FIG. 5. Ultrastructural analysis of cultured colonic and pancreatic cells after hyposmotic exposure. Cells were cultured for 48 h under hyposmotic conditions. Floater cells were collected and processed for transmission electron microscopy. Note the formation of: vacuoles in all the cell lines (a–f), nuclear condensation (b–f), multiple nuclei (c, d), giant cells (a, d, e), and nuclear fragments (b). Two images of HRT18 cells are shown to highlight the heterogeneity of the nuclear morphology of these cells. Cell sizes, based on measuring the largest diameter of the cells shown, are: 26 μm (Caco2 cell, panel a), 9 μm (apoptotic HRT18 cell, panel b), 13 μm (HRT18 cell, panel c), 26 μm (HT29 cell, panel d), 18 μm (AsPC-1 cell, panel e), and 12 μm (PaCa-2 cell, panel f). The basal size of cells growing under normal conditions is 10–14 μm (not shown), as exemplified by the control HT29 cell shown in the inset of panel d (which measured 13 μm). The bar pertains only to panel a and corresponds to 1.7 μm.

FIG. 6. Effect of hyposmotic stress on cell cycle-related protein levels. Cells were cultured in iso- (I) or hypo- (H) osmotic medium for 6 (A) or 24 h (B), followed by isolation of a total cellular protein homogenate by solubilization of the isolated cells (floater and adherent) in reducing sample buffer. Equal amounts of protein from each sample were separated on 10% acrylamide gels followed by blotting using antibodies to Cdc2, cyclin B1, Cdk2, cyclin E, Cdk4, cyclin D3, and K8. Note the dramatic and progressive decrease in the steady-state levels in most but not all of the cell cycle-related proteins. Equal protein loading was confirmed by the similar observed levels of K8.

more than one stage can be impacted depending on the cell line examined.

We also examined the ultrastructural features of the five cell lines after hyposmotic stress-induced growth arrest. For this, floater cells were collected from each cell line after 48 h of hyposmotic treatment then examined by transmission electron microscopy. As shown in Fig. 5, the ultrastructural features included formation of: variable-sized vacuoles in all the cell lines (panels a–f), nuclear condensation (panels b–f), multiple nuclei (panels c, d), and giant cells (panels a, d, e), and nuclear fragments (panel b). These features were found primarily in the floater cells (Fig. 2A and not shown) and were present in all of the cell types, although nuclear fragmentation was noted only in some of the HRT18 cells, which is consistent with some HRT18 cells undergoing apoptosis as noted in Figs. 2C and 3. Overall, these morphological changes and the cell cycle arrest and cell cycle analysis data suggest that hyposmosis causes a global cell cycle catastrophe.

Reduction of Cell Cycle Protein Levels after Hyposmotic Treatment—Because the cell cycle is controlled by expression and activation of several cyclins and Cdks, we asked whether their expression levels changed after cell exposure to a hyposmotic environment. As shown in Fig. 6, the expression level of the cell cycle-related proteins Cdc2, Cdk2, Cdk4, cyclin B1, and cyclin D3 were dramatically reduced in all the cell lines after hyposmotic treatment in a time-dependent fashion. However, this effect was not generalized in that some protein levels such as cyclin E and keratin 8 remained unaltered. Some cyclins and Cdks showed a profound rapid degradation/turover (e.g., Cdc2 and cyclin B1 in HRT18 cells) whereas others showed more gradual degradation (Cdc2 in Caco2 cells) (Fig. 6). Although the expression levels of individual cyclins/Cdks varied during iso-osmotic conditions among the cell lines, the overall effect for most but not all of the cell cycle-related proteins was either rapid or a gradual degradation/turover. Therefore, the observed cell cycle arrest upon exposure to hyposmotic conditions is likely due to decreased levels of several cell cycle-related proteins.

Activation of the Proteasome System Is Required for Hyposmosis-induced Degradation of Cell Cycle-related Proteins and for Cell Growth Arrest—The decreased levels of several cell cycle-related proteins in response to hyposmotic conditions implies that these altered protein levels result from a decreased biosynthetic rate and/or from increased degradation. Assess-
ment of the protein synthetic rate after hyposmotic stress showed that the overall protein synthetic rate decreased to 28–53% of the basal isotonic-condition rates (Fig. 7). This decreased synthetic rate does not, however, account for the dramatic decrease in some of the cell cycle-related proteins that became essentially undetectable within 6 h of exposure to hyposmotic conditions (e.g., Fig. 6A, see cyclin B1 and D3 and Cdk4 in HRT18 cells). Hence, protein degradation is also a likely major factor in the hyposmosis-induced increased turnover of the cell cycle-related proteins. To test this hypothesis, we examined the effect of proteasome and other protease inhibitors on the expression level of several cell cycle-related proteins in the presence or absence of 6-h exposure to a hyposmotic treatment. As shown in Fig. 8, lactacystin (a specific proteasome inhibitor) and ALLN (a nonspecific proteasome inhibitor) strongly inhibited the hyposmosis-induced reduction of cell cycle-related protein levels. In contrast, PD150606 (calpain inhibitor), chloroquine (lysosomal acidification inhibitor), and the pepstatin A/leupeptin (lysosome protease inhibitors) mixture did not have a significant effect on cyclin and Cdk expression levels.

Given the above findings, we examined whether proteasome

![Graph showing hyposmotic conditions partially inhibit overall protein biosynthesis](image1)

**Fig. 7.** Hyposmotic conditions partially inhibit overall protein biosynthesis. The five colonic and pancreatic cell lines were cultured in iso- or hypo-osmotic medium for 5 h then labeled for 1 h using [35S]methionine as described under “Experimental Procedures.” After washing off the labeling medium, cells were solubilized with 1% Nonidet P-40 in PBS containing 5 mM EDTA. Aliquots of the detergent lysate were counted, and the counts were normalized to the amount of solubilized protein.

![Graph showing effect of proteasome and other protease inhibitors on cell cycle-related protein degradation](image2)

**Fig. 8.** Effect of proteasome and other protease inhibitors on cell cycle-related protein degradation. HRT18 and AsPC-1 cells were preincubated with 0.1% MeSO (DMSO, as vehicle control), 20 μM lactacystin, 270 μM ALLN, 100 μM PD150606, 100 μM chloroquine, or a mix of 100 μM pepstatin A and 50 μM leupeptin. After 2 h, the medium was changed to hypo- or iso-osmotic medium (± lactacystin), and the cells were cultured at 37 °C for 20 h then counted. The histograms show mean ± S.D. based on three independent experiments. Asterisks highlight p values < 0.05 when comparing the hyposmotic treatments with lactacystin to those without.

![Graph showing effect of proteasome inhibition on hyposmosis-induced cell growth arrest](image3)

**Fig. 9.** Effect of proteasome inhibition on hyposmosis-induced cell growth arrest. Equal numbers of HT29 (A) or AsPC-1 cells (B) were seeded into cell culture dishes and incubated overnight in normal medium at 37 °C. Cells were then incubated in normal medium containing lactacystin (5 or 10 μM) or 0.1% MeSO (DMSO, as a vehicle control). After 2 h, the medium was changed to hypo- or iso-osmotic medium (± lactacystin), and the cells were cultured at 37 °C for 20 h then counted. The histograms show mean ± S.D. based on three independent experiments. Asterisks highlight p values < 0.05 when comparing the hyposmotic treatments with lactacystin to those without.
inhibition influenced hyposmosis-induced cell growth arrest. To do so, we compared the cell number in the presence or absence of lactacystin, under isotonic or hypotonic conditions. As shown in Fig. 9, lactacystin resulted in a slight decrease in cell number when cells were grown in isotonic control conditions, which may be related to the known effect of proteasome inhibitors as apoptosis-inducing agents in a variety of cell lines (e.g. Refs. 41, 42). In contrast, lactacystin reversed the hyposmosis-induced growth inhibition when tested in HT29 and AsPC-1 cells, reaching statistically significant reversal at doses of 10 \( \mu M \) for HT29 cells and 5 and 10 \( \mu M \) for AsPC-1 cells (Fig. 9). Doses of \( >10 \mu M \) were ineffective (not shown), likely due to overridding of the growth inhibitory effect of such compounds at the higher doses. These findings lend further strong support to the conclusion that hyposmosis-induced growth arrest is due in large part to proteasome activation and subsequent degradation of several key cell cycle-related proteins.

**DISCUSSION**

**Effect of Changes in Extracellular Osmosis on Cell Growth**—Due to the high water permeability of the mammalian plasma membrane, an imbalance between the osmolarity of the cytoplasm and the external environment will immediately lead to a redistribution of water and to a subsequent change in intracellular osmolarity and cell volume (9). Most cell types respond immediately to osmotic changes by activating plasma membrane transport pathways leading to net accumulation (upon hyperosmotic stress) or loss (upon hyposmotic stress) of osmotically active intracellular solutes (11, 43). The mechanism(s) whereby altered intracellular osmolarity interferes with physiological mammalian cell functions such as proliferation and differentiation is(are) poorly understood. Osmotically induced growth arrest has been observed in several organisms (44), including bacteria (45–48) and yeast (49, 50), and appears to be necessary for recovery from osmotic stress. Here we demonstrate for the first time that hyposmotic conditions induce reversible cell growth arrest within 6–24 h in all five human cell lines that we tested (Fig. 1).

Similar results of cell growth arrest were demonstrated in a murine kidney cell line after exposure to hyperosmotic conditions (22). The mechanism of this growth arrest is not known, but it correlates with induced expression of the so-called growth arrest and DNA damage-inducible (GADD) proteins GADD45 and GADD153 (22). It is speculated that the biological significance of cell growth arrest relates to allowing the arrested cells sufficient time for adaptive responses to be activated, and to switch mitotic energetic resources to cell protective systems to cope with the osmotic stress (10).

**Growth Arrest Leads Cells to Undergo Apoptosis or Cell Cycle Catastrophe**—Several studies point to the intimate association between apoptosis and cell cycle control (51, 52). In the present study, the hyposmosis-induced apoptosis was verified by formation of genomic DNA fragments (Fig. 2C), and by the caspase-mediated degradation of K18 (Fig. 3). Among the five cell lines we tested by exposing to hypotonic conditions, apoptotic death was observed only in HRT18 cells. The hyposmosis-associated partial G1/S arrest of HRT18 cells, and the subsequent apoptosis of a small subpopulation of these cells are consistent with previous studies showing that growth arrest (e.g. at G1 phase) can lead cells to undergo apoptosis (e.g. Refs. 53, 54). Caco2 and HT29 cells were also partially arrested at the G1 phase of the cell cycle by hyposmotic treatment (Fig. 4), but no apoptotic changes were noted in these cells. The reason for the lack of apoptosis in the remaining cell lines may be related to differences in susceptibility to apoptosis. Interestingly, inhibition of phosphatidylinositol 3-kinase by the compound LY294002 protected from cytosine arabinoside-induced apoptosis (55), so hyposmosis-induced degradation of related kinases may provide similar protection. In addition, the severity of the hyposmotic stress may also play a role in the induction of apoptosis. For example, apoptosis but not cell growth was studied and noted in several cell lines, including HT29 and AsPC-1 cells, under severe hypotonic conditions (84 mosM) (56) that we did not test in our cell systems.

Our results show that hyposmotic conditions result in growth arrest and cell cycle catastrophe (also called mitotic catastrophe) that may in some cell types lead to apoptosis as well. The features of generalized cell cycle arrest (Figs. 1 and 4), formation of giant and polyloid cells (Fig. 5) in the absence of significant cell death, are consistent with cell cycle catastrophe (57). Cell cycle/mitotic catastrophe can occur via multiple mechanisms, including modulation of checkpoint kinases (58) or adapter proteins (59), and has also been noted upon administration of anti-tumor drugs such as 5-fluorouracil (60). However, the growth arrest that we observe is independent of the formation of the giant and polyoid cells *per se*, because these cells are found in small numbers (primarily within the small...
subgroup of floater cells) and because cell cycle protein degradation involved the adherent cells.

**Hyposmosis-mediated Activation of the Proteasome as a Mechanism of Growth Arrest**—The findings of Figs. 8 and 9 indicate that activation of the proteasome pathway, and subsequent degradation of key cell cycle-related proteins, are largely responsible for the hyposmosis-induced cell growth arrest. Our studies add hyposmosis stress as a novel new mechanism for proteasome activation and also strongly implicate this degradation pathway in causing cell cycle arrest upon exposure of cells to hypotonic conditions (Fig. 10). The proteasome clearly plays an essential role in modulating the steady-state levels of a variety of proteins (61). To that end, degradation of cyclins, Cdk inhibitors, tumor suppressor proteins, and proto-oncogene products by the proteasome pathway highlights the important role of the proteasome in regulating cell growth (61). In addition, proteasome-mediated degradation of several individual proteins upon exposure to hypertonic stress has been reported for cyclin D1 (30), the insulin-regulated IRS-2 protein (62), and aquaporin-1 (63). In the case of hypotonic stress, it appears that several proteins become degraded upon hyposmosis exposure. Our hypothesis is that hyposmosis stress results in a generalized activation of the proteasome, with subsequent degradation of individual proteins that may be modulated by their: (i) steady-state half-life at the time of exposure to the stress, (ii) cellular localization, or (iii) post-translational modification state or whether they are bound to an associated protein that could affect their turnover.

The degradation of several cyclins and Cdk’s, among other likely proteasome substrates, provides a molecular explanation for the observed growth arrest upon hyposmosis exposure. For example, Cdk4/cyclin D is responsible for progression through the G1 cell cycle phase, so their decreased protein levels after exposure of cells to hyposmosis (Fig. 10) results in a generalized activation of the proteasome, with subsequent degradation of individual proteins that may be modulated by their: (i) steady-state half-life at the time of exposure to the stress, (ii) cellular localization, or (iii) post-translational modification state or whether they are bound to an associated protein that could affect their turnover.

Our working hypothesis (Fig. 10) is that hyposmosis stress results in growth arrest via proteasome activation and subsequent degradation of several cell cycle-regulating proteins. Growth arrest may also be related to the observed decrease in protein synthesis, which, in turn, affects the steady-state level of important cell cycle-related proteins. In addition, protein steady-state levels may be affected by mRNA stability, which can be rapidly degraded through AU-rich elements in the 3′-untranslated region upon proteasome activation (66).
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