Shrinkage-induced Protein Tyrosine Phosphorylation in Chinese Hamster Ovary Cells*

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To investigate the signal transduction of osmotic stress, we examined hypertonicity-induced tyrosine phosphorylations in Chinese hamster ovary cells. Hyperosmotic stress elicits characteristic phosphotyrosine accumulations in at least 3 proteins (~42, ~85, and ~120 kDa). The most prominent response occurred in the 85-kDa band (p85) whose phosphorylation was rapid, sustained, and apparent already at mild hypertonicity (350 mosM), proportional to the extracellular osmotic concentration, and reversible. Hyperosmotic environment could not induce tyrosine phosphorylation if cell shrinkage was prevented by nystatin and appropriately composed media. Conversely, isotonic shrinkage caused strong tyrosine phosphorylation. Thus, the initial signal is a decrease in cell volume and not an increase in the intra- or extracellular osmotic concentration, or a rise in cytosolic K+ and Cl− levels. Tyrosine phosphorylation of p85 was not due to the hypertonicity-induced protein kinase C-dependent stimulation of the extracellular signal-regulated protein kinase, nor to the activation of stress-activated protein kinases. Tonicity-responsive proteins interacted with Grb2-glutathione S-transferase fusion proteins: the 120-kDa protein complexed with the SH2 and both SH3 domains, whereas p85 associated with the SH2 and the N-terminal SH3 domains of the adapter. Tyrosine phosphorylation of p85 is a sensitive indicator of reduced intracellular hydration and might signify a hitherto unrecognized, early volume-dependent signaling event.

Osmotic changes in the extracellular environment can influence a variety of vitally important cell functions (reviewed in Ref. 1). Alterations in tonicity has been reported to regulate several ion transporters (including different isoforms of the Na+ /H+ exchanger (NHE-1–4)1 (2–5), the Na+/K+/Cl− cotransporter (6), K+ and Cl− channels (see Ref. 7)) to modify the activity of key metabolic enzymes (1) and to affect the transcription of certain genes (8, 9). Although many of these events are of homeostatic nature which serve the restoration of the near-normal cell volume (7, 10), ample evidence has been accumulating that moderate volume changes themselves constitute an important signal that can be induced by and may be necessary for the action of different metabolic hormones (1). While the role of cellular hydration state as both a regulated and a regulating factor has been well established, very little is known about the mechanisms through which it is detected and about the subsequent signaling steps that convey the information to the effectors. Not even the exact parameter sensed during aniso-osmotic challenge is clear: it may be a change in the extra- and/or intracellular total osmotic activity, or an alteration in the cell volume. Furthermore, the volume change itself may exert its effect via various mechanisms. Conceivably, it may act by eliciting changes in the intracellular ionic strength or in the concentration of specific ions or other regulatory constituents. It is noteworthy that cytosolic Cl− concentration was found to modulate the operation of the Na+/H+ exchanger (11, 12) and the Na+/K+/Cl− cotransporter (13–15) as well as the activity of different enzymes including glycogen synthase (16) and yet unidentified protein kinases (17, 18). Alternatively, the mechanical strain of various cytoskeletal components may also initiate some of the effector responses (see Ref. 7).

Whatever the initial events are, the further processing of the signal is believed to involve a set of protein phosphorylations. Seminal discoveries, first made on yeast (19–21) and later verified in mammalian cells, have indicated that hyperosmotic stress stimulates certain extracellular signal-regulated protein kinases (ERKs or mitogen-activated protein kinases) and a related group of enzymes, the stress-activated protein kinases (SAPKs or JNKs). Serine/threonine kinases which were shown to be stimulated by hyperosmolarity in mammalian cells now include ERK-1 and -2 (22–24), SAPK46 and 55 (23, 25–27), p38 (28, 29), and the recently described Big MAP kinase (also known as ERK-5) (30). Interestingly in certain cell types hypotonicity also was found to activate ERK-1 and -2 (31, 32). While the potential link between these serine/threonine kinases and the effector functions (e.g. membrane transporters) remained unresolved, pharmacological data indicate that protein tyrosine phosphorylations may also play a pivotal role in mediating some of the hypo- or hyperosmotic stress-induced functional responses. Tyrosine kinase inhibitors abrogated the hypotonicity-triggered ion efflux from epithelial cells (31) and prevented the hypertonicity-evoked inhibition of the apical Na+/H+ exchanger (NHE-3) in kidney cells (33). These drugs were reported to suppress ERK activation induced by hypotonicity (32) but did not abolish the hypertonicity-elicited stimulation of this...
MAP kinase (22). This finding suggests that their effects may not be ascribed to the inhibition of MEKs and SEKs, the only identified members of the above cascade exhibiting tyrosine kinase activity. Despite their likely significance, the osmotic stress-induced protein tyrosine phosphorylations have never been systematically studied. Very little information is available about tonicity-regulated tyrosine kinases and their substrates are mostly unknown.

The aim of the present work was to investigate whether hypertonicity provokes characteristic tyrosine phosphorylations in cellular proteins and to establish whether the primary signal for these reactions is a change in the intracellular osmotic concentration or in the cell volume. We also addressed whether the observed tyrosine phosphorylations might be downstream of the activity of ERKs and SAPKs or may signify upstream or independent events. These experiments were performed on Chinese hamster ovary (CHO) cells expressing known isoforms of the Na+/H+ exchanger in which the functional responses to hypertonicity (i.e. stimulation of NHE-1 and inhibition of NHE-3) have been well characterized (2, 34, 35).

Our results indicate that hypertonicity induces tyrosine phosphorylation in several proteins. The most prominent of these is an approximate 85-kDa band. Its phosphorylation is quick, proportional, and specific to the imposed osmotic stress, and tissue culture reagents were purchased from Sigma. Genistein was from Calbiochem, PD 98059 was a kind gift from Dr. Julian Downward. Anti-phosphotyrosine (clone 4G10) was obtained from Upstate Biotechnology Inc. (UBI). Anti-ERK-2 and anti-Pan-ERK were from Transduction Laboratories. Polyclonal anti-phosphoinositide 3-kinase used for Western blotting was from UBI. Since this antibody was generated against a GST fusion protein, the association of GST-Grb2 with PI 3-kinase was detected with another anti-PI-3-kinase, obtained from Sigma. Anti-phospho-p88 and anti-phospho-SAPK antibodies which specifically react with the phosphorylated forms of the respective kinases were from New England Biolabs. Protein-A Sepharose was obtained from Pierce. Peroxidase-conjugated anti-mouse IgG and the Enhanced chemiluminescence kit were purchased from Amersham. [14C]Succrose (540 mCi/mmol) was from Amersham and Bicarbonate-free medium RPMI 1640 was buffered with 25 mM NaHCO3, 10 mM NaCl, 1 mM MgCl2, and 1 mM glucose, pH 7.4. The osmolarity of the medium was adjusted to 290 mOsm/kg water and a NaCl solution of 400 mosM.

When required, media were made hypertonic to the indicated level by the addition of sucrose or KCl as detailed in the text. Osmolarity was determined on Chinese hamster ovary (CHO) cells expressing known isoforms of the Na+/H+ exchanger in which the functional responses to hypertonicity (i.e. stimulation of NHE-1 and inhibition of NHE-3) have been well characterized (2, 34, 35).

Detection of Tyrosine Phosphorylation—Aliquots of total cell lysates were diluted (1:1) into 2× Laemmli sample buffer, boiled for 3 min, and subjected to electrophoresis on 10% SDS-polyacrylamide gels. The separated proteins were transferred to nitrocellulose (Hoefer Pharmacia Biotech Inc.) using a Bio-Rad Mini Protein II apparatus. To check effectiveness of transfer and similarity of protein amount, lanes were visualized by staining with Ponceau S. Blots were then blocked in Tris-buffered saline containing 2% bovine serum albumin and incubated with the anti-phosphotyrosine antibody (dilution 1:5000). The binding of the antibody was visualized by peroxidase-coupled goat anti-mouse IgG, using the enhanced chemiluminescence method according to the manufacturer’s instructions.

Detection of Phosphorylation of SAPK and p88—This was carried out by using an anti-phospho-specific SAPK antibody (dilution 1:1000), and a phospho-specific p88 antibody (dilution 1:500).

Detection of In Vitro Interaction of Proteins with Grb2-GST—To precipitate proteins interacting with the full-size Grb2 or with certain domains of the adapter, cells were treated and lysed as described earlier then Triton X-100-insoluble material was sedimented out. The various Grb2-GST fusion proteins (5 μg/sample) coupled to agarose resin and coupled to agarose resin and subjected to electrophoresis on 10% SDS-polyacrylamide gels. The supernatants were incubated with anti-ERK-2 or anti-Pan-ERK antibodies (1:1000 dilution) and subjected to Western blotting with anti-phosphotyrosine. After stripping, the same blot was developed with anti-phosphoinositide 3-kinase followed by a 3-h incubation with immobilized protein A-agarose (20 μl/sample)

Detection of Tyrosine Phosphorylation of Phosphoinositide 3-Kinase—Lysates containing equivalent amounts of proteins obtained from iso- or hypertonicity-treated cells were clarified by centrifugation at 15,600 x g for 5 min. Extracts were incubated overnight with 8 μg of anti-phosphoinositide 3-kinase followed by a 3-h incubation with immobilized protein A at 4 °C. Then beads were washed and washed four times with lysis buffer containing 0.25% Triton X-100 and 1 mM Na3VO4. Precipitated proteins and aliquots of the first supernatant were electrophoresed and probed with anti-phosphotyrosine. After stripping, the same blot was used as a positive control and probed with another anti-PI-3 kinase antibody.
an aspecific binding of the primary or secondary antibody as evidenced by the following observations. 1) No labeling was found in this region when the blots were incubated with the primary antibody in the presence of 1 mM phosphotyrosine (not shown); 2) the band was not detected either when only the secondary antibody was applied (not shown); and 3) pretreatment of the cells with the protein tyrosine kinase inhibitor genistein (50 μM, 30 min) largely reduced (but did not completely abolish) the change provoked by hypertonicity (Fig. 1C). As the phosphorylation of the 85-kDa protein (referred below as p85) seemed to be far the most sensitive to increased tonicity, we decided to use this band as a semi-quantitative indicator for further characterization of the hyperosmotic response.

To establish the dependence of tyrosine phosphorylation on the extracellular osmotic concentration, we treated the cells with sodium medium supplemented with varying concentrations of sucrose. Increased tyrosine phosphorylation of p85 was occasionally observable upon addition of as low as 25 mM sucrose, whereas a clearly detectable rise consistently occurred after using 50 mM sugar (total osmolarity approximately 350 mosM). Other non-permeable osmolytes (NaCl and KCl) were equally effective in eliciting phosphorylation (see below). The signal became markedly stronger with increasing osmolarity, approaching a plateau above 600 mosM (Fig. 2, A and B). Fig. 2, C and D, reports the kinetic of the response. Using an osmotic concentration of 500 mosM, the signal was readily visible after 0.5–1 min (the earliest time points that could be reliably tested) and it increased further with time. Maximal effects were generally reached between 5 and 10 min. In most samples the signal showed a slow decay at times longer than 20–30 min. To test the reversibility of tyrosine phosphorylation, cells were first treated with a hypertonic solution for 10 min after which isonicotonicity was re-established for varying times before lysing the cells. As illustrated on Fig. 2E, upon readdition of the isonicotonic sodium medium a quick and large decrease occurred in the phosphotyrosine content of p85: after 1 min the labeling became 80% weaker and by 2 min it dropped to the background level. To decide whether downward departures from isonicotonicity could also elicit the phosphorylation of p85, we reduced the osmolality of the extracellular medium by 33%. This maneuver did not cause phosphorysine accumulation in this band. Taken together, the tyrosine phosphorylation (and dephosphorylation) of the 85-kDa protein is a rapid, reversible, time- and osmotic concentration-dependent process, the level of which reflects the course and magnitude of changes in extracellular osmotic activity in a wide range above the isonicotonic values.

Is Cell Shrinkage a Prerequisite for Inducing Tyrosine Phosphorylation by Hypertonicity?—When the extracellular tonicity is increased by non-permeable osmolytes, cells undergo a proportional shrinkage. It was of interest to establish whether the trigger for the observed phosphorylation events is a change in osmotic concentration itself, or rather the consequent change in the cell volume.

To dissect the imposed changes in intra- and extracellular tonicity (and ionic strength) on the one hand from the changes in the cell volume on the other, we used two approaches. 1) We applied hypertonicity while the shrinkage was prevented and 2) we induced cell shrinkage under isoosmotic conditions. These manipulations were brought about by using appropriately composed extracellular media and the non-selective, monovalent ionophore, nystatin as detailed below.

To mimic cytoplasmic ionic concentrations the following experiments were performed in an isonicotinic KCl medium. The volume of the cells bathed in this iso-potassium solution was stable and identical with the value measured in the iso-sodium medium (not shown). Keeping the cells in iso-KCl medium did
not induce any change in the level of tyrosine phosphorylation of the 85-kDa protein (Fig. 3A, lane 1) or in any other toxicity-sensitive band. Raising the extracellular osmolarity by addition of 100 mM KCl resulted in approximately 30% cell shrinkage and in a substantial increase in the tyrosine phosphorylation of the 85-kDa band (Fig. 3A, lanes 1 and 3, and columns 1 and 3). An entirely different pattern was obtained if this experiment was carried out on cells which had previously been permeabilized for monovalent ions with nystatin. The ionophore added to the cells induced a sizable (3.5-fold) swelling (Fig. 3A, column 2) in complete agreement with earlier observations made on HeLa cells (38). This volume increase is due to a colloid-osmotic water uptake because in these permeabilized cells the major intracellular ions are no more capable of counterbalancing the osmotic pressure exerted by the intracellular proteins (38). Under this condition of isotonic hyper-osmolality the basal level of phosphorylation was identical with that observed in control cells (compare lanes 1 and 2 on Fig. 3A). As expected, raising the extracellular osmolality by the addition of 100 mM KCl to the permeabilized cells did not induce any decrease in their volume (column 4). Importantly, this treatment failed to elicit tyrosine phosphorylation either (lane 4). It is noteworthy, that although the cell volume remained unaffected in nystatin-permeabilized cells, the addition of KCl increased both the intra- and extracellular osmotic activity, ionic strength, K⁺, and Cl⁻ concentrations. Despite these changes, however, no phosphorylation occurred. An alternative interpretation of this lack of the response to KCl in the ionophore-treated cells could be that nystatin unspecifically interfered with the normal operation of tyrosine kinases or other toxicity-sensitive elements. To test this assumption, control and nystatin-treated cells were exposed to a hypertonic medium in which osmolarity was elevated by sucrose (200 mM) instead of KCl. As sucrose cannot permeate through the nystatin-formed pores, in this case hypertonicity led to a pronounced shrinkage in both the control and in the drug-treated cells (Fig. 3A, columns 5 and 6). In fact the sucrose-induced shrinkage was even bigger in the presence of nystatin. In keeping with this observation, sucrose induced substantial tyrosine phosphorylation both in the absence and presence of nystatin. The intensity of this reaction was proportional to the corresponding volume changes (Fig. 3A, lanes 5 and 6).

To further substantiate that neither nystatin itself nor the transient swollen state of the cells caused by the drug were responsible for the prevention of tyrosine phosphorylation upon addition of KCl, the same experiments were repeated using an isotonic medium in which 30 mM KCl was replaced by 60 mM sucrose. In this solution the colloid-osmotic pressure of cellular proteins was mostly counterbalanced by the impermeable sugar. This is reflected by the fact that under these conditions nystatin caused only a marginal swelling (<15%). In all other respects, however, the results were identical with those reported on Fig. 3A: nystatin prevented the KCl- but not the sucrose-induced tyrosine phosphorylation (not shown).

Thus we have established that hypertonic environment without cell shrinkage is not capable of facilitating tyrosine phosphorylation of p85. Next we asked whether a decrease in cell volume in the absence of hyperosmosis is sufficient to induce the phenomenon. To test this, cells were exposed to an isotonic medium (NMG-glucuronate) devoid of the major intracellular ionic constituents. This composition was chosen to generate extracellularly directed gradients which can serve as a driving force for the spontaneous efflux of ions (mostly K⁺ and Cl⁻) and the accompanying water leading to cell shrinkage. Incubation of the cells for 15 min in this environment resulted in a huge loss in their water content and a strong tyrosine phosphorylation of the 85-kDa band (Fig. 3B). In this case nystatin (i.e. rising the ion permeability of the membrane) did not inhibit but rather facilitated tyrosine phosphorylation (not shown). Thus the same ionophore could prevent or promote tyrosine phosphorylation of p85 in perfect concordance with its effect on cell volume under various conditions.

Taken together, we conclude that a decrease in cell volume is certainly sufficient to trigger tyrosine phosphorylation of p85. On the other hand an increase in the intra- or extracellular osmotic concentration, ionic strength, or KCl level do not seem to be necessary or required for this reaction.

Investigation of the Possible Relationship of p85 Tyrosine Phosphorylation to the Osmo-sensitive Kinase Cascades—In Madin-Darby canine kidney cells hypertonicity was reported to turn on a kinase cascade involving the sequential activation of protein kinase C, Raf1 kinase, MAP kinase kinase (MEK), and ERK (22, 24). The aim of the following experiments was to establish whether a similar signaling route operates also in CHO cells and to clarify whether the tyrosine phosphorylation of p85 might be a consequence of the activation of one or more members of this cascade. We applied different treatments known to influence the activity of PKC and the activation of the
kinase also in CHO cells. However, neither PKC nor MEK or sequential activation of protein kinase C and MAP kinase pertonicity-induced stimulation of ERK-2 is mediated by the inhibitor of MEK (41) (Fig. 4).

Tyrosine phosphorylation was completely abolished by PD 098095, a specific inhibitor of MEK (41), verifying that the down-regulation of PKC was effective. Importantly, in PKC-depleted cells also, hypertonicity lost its potential to activate ERK-2 but it preserved its effect on p85 phosphorylation (lane 5, upper panel). Moreover in non-depleted cells both the PMA- and the osmotic shock-promoted ERK-2 activation but not p85 tyrosine phosphorylation was completely abolished by PD 098095, a specific inhibitor of MEK (41) (Fig. 4B).

Together these results unambiguously indicate that the hypertonicity-induced stimulation of ERK-2 is mediated by the sequential activation of protein kinase C and MAP kinase kinase also in CHO cells. However, neither PKC nor MEK or classical MAP kinase pathway, and compared the effect of these manipulations on ERK stimulation and p85 phosphorylation. The most abundant MAP kinase variant in our CHO cells was ERK-2, since using a PAN-ERK antibody (raised against an epitope that is common in several MAP kinases) resulted in the predominant labeling of a 42-kDa band which was also visualized by a specific anti-ERK-2 antibody. Stimulation of ERK-2 was detected as a reduction in the electrophoretic mobility of the enzyme indicative of its phosphorylation and consequent activation (32). Protein kinase C-activating phorbol esters were shown to stimulate ERK-2 in several cell types (26, 39, 40). Indeed PMA induced a prominent shift in the mobility of a substantial portion of ERK-2 also in CHO cells as evidenced by the appearance of two distinct anti-ERK-2 immunoreactive bands (Fig. 4A, compare lanes 1 and 2 on the lower panel). In sharp contrast, PMA failed to elicit tyrosine phosphorylation of the 85-kDa band (compare lanes 1 and 2 on the upper panel). Application of strong hypertonicity (900 mosM) also caused a well detectable ERK-2 activation (lane 4, lower panel) and, as expected, provoked the tyrosine phosphorylation of p85 (lane 4, upper panel). To determine the potential role of PKC in the hypertonicity-related phosphorylations, cells were depleted of PKC by overnight incubation with PMA, and subsequently they were challenged either by the phorbol ester again or by hypertonicity. Under these conditions PMA was no more capable of inducing ERK-2 activation (lane 3, lower panel) verifying that the down-regulation of PKC was effective. Importantly, in PKC-depleted cells also, hypertonicity lost its potential to activate ERK-2 but it preserved its effect on p85 phosphorylation (lane 5, upper panel). Moreover in non-depleted cells both the PMA- and the osmotic shock-promoted ERK-2 activation but not p85 tyrosine phosphorylation was completely abolished by PD 098095, a specific inhibitor of MEK (41) (Fig. 4B).

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**Fig. 3.** Tyrosine phosphorylation is induced by cells shrinkage and not by an increase in intra- or extracellular osmolarity. Changes in cell volume (columns, upper panels) and tyrosine phosphorylation of p85 (blots, lower panels) were followed in parallel. A, cells were kept in iso-potassium medium and treated as indicated in the table under the blots. Nystatin (400 units/ml), when present, was added to the cells 5 min prior to any further treatment. Hypertonic exposure (10 min) was achieved by the addition of either 100 mM KCl or 200 mM sucrose. For volume determinations, cells were used in suspension (10^6/ml) and the intracellular water space was measured by [14C]sucrose and 3H2O as detailed under "Experimental Procedures." Data were normalized to the average volume of untreated cells obtained in iso-potassium medium (n = 3). Tyrosine phosphorylation was detected as described in the legend to Fig. 1. B, cell volume and tyrosine phosphorylation were determined after a 10-min incubation either in iso-sodium or iso-NMG medium as indicated. Neither of the above treatments caused cell detachment or lysis as verified by microscopic inspection and protein determinations.

**Fig. 4.** The hypertonicity-induced tyrosine phosphorylation of p85 is not dependent on the osmotic stress-promoted activation of PKC and ERK-2. The activation of ERK-2 (as detected by the mobility shift assay) and tyrosine phosphorylation of the 85-kDa band were followed in parallel. Aliquots of the sample were electrophoresed either on big-sized (16 x 18 cm) 15% gels or 10% mini gels, blotted onto nitrocellulose, and probed with anti-ERK-2 (lower panels, marked as ERK-2) or anti-phosphotyrosine (upper panels marked as PY) antibodies, respectively. A, cells either untreated or depleted of PKC (Depl) were incubated in iso-sodium medium for 10 min and then challenged with the following additions: none (cont); 50 nM PMA for 5 min (PMA); 600 mM sucrose (~900 mosM) for 10 min (900). Details of the PKC depletion are described under "Experimental Procedures." B, cells were preincubated for 20 min in iso-sodium medium without (lanes 1 and 3) or with (lanes 2 and 4) the MEK inhibitor, PD098059, and then exposed to PMA (lanes 1 and 2) or hyperosmolarity (lanes 3 and 4) for 10 min. Lysates were made and processed as detailed above.

ERK-2 are involved in the hyperosmolarity-related tyrosine phosphorylation of the 85-kDa protein. The SAPKs (also referred to as c-Jun N-terminal kinases or JNKs) constitute another branch of the MAP kinase superfamily that was shown to be activated by various cellular stresses including hyperosmolarity (42, 43). The question whether these enzymes could be upstream of the phosphorylation of p85 was approached by pharmacological means and by applying different stress conditions. The protein synthesis inhibitor anisomycin was demonstrated to be the most potent activator of SAPKs in various cell types including CHO cells (26, 44, 45). This drug,
Aniso 42°C H$_2$O$_2$

![Diagram](image)

**Fig. 5.** Tyrosine phosphorylation of p85 is induced by osmotic shock but not by other cellular stresses. Cells were preincubated in iso-sodium for 10 min followed by incubation in iso-sodium (I) or hyper-sodium (H) supplemented with 10 μg/ml anisomycin (Aniso) or 200 μM H$_2$O$_2$ (H$_2$O$_2$) for 10 min. Heat shock (lanes marked as 42°C) was induced by incubation of cells at 42°C in iso-sodium medium (I) for 30 min or in iso-sodium for the first 20 min and hyper-sodium for an additional 10 min (H). None of these treatments resulted in detachment of cells from the plate as controlled by microscopic inspection at the end of the incubation period. Tyrosine phosphorylation of p85 was detected as in Fig. 1. The p85 phosphorylation in iso- or hypertonicity-treated control samples which were not challenged by any pretreatment was not different from the pattern observed in the presence of anisomycin, heat shock, and H$_2$O$_2$ (not shown).

However, failed to induce tyrosine phosphorylation of p85 and did not interfere with the appearance of the reaction upon exposing the cells to a hypertonic environment (Fig. 5, lanes marked as aniso). Similarly, heat shock (warming up the cells to 42°C for 30 min), another well known inducer of SAPKs (26, 46) did not elicit p85 phosphorylation and did not affect the hypertonic response (lanes marked as 42°C). Finally the potential action of oxidative stress was tested. Incubating the cells for 5 or 10 min with 200 or 400 μM H$_2$O$_2$ had either no effect or resulted in a slight (hardly detectable) increase in the level of phosphotyrosine accumulation into p85. Osmotic stimulation could always markedly increase the level of phosphorylation of p85 also in H$_2$O$_2$-treated cells (lanes marked as H$_2$O$_2$). Similar results were obtained when the xanthine/xanthine oxidase system was applied to generate superoxide anions initiating the formation of other aggressive oxygen radicals (not shown).

These findings lend credence to the conclusion that tyrosine phosphorylation of the 85-kDa protein is not downstream to the activation of SAPKs. Moreover hyperosmolarity seems to be a rather specific stimulus for p85 phosphorylation since other cellular stresses are not (or only very weak) inducers of this phenomenon.

To test the possibility that the tyrosine phosphorylation of p85 may be upstream of the phosphorylation of osmo-sensitive MAP kinases, we examined the pattern of phosphorylation of ERK-2, SAPKs (p54, p46), and p38 under conditions when osmolality and cell volume were varied independently, exactly as described for p85 (compare Fig. 3 and Fig. 6). The phosphorylation of SAPKs and p38 was followed by phosphorylation-specific antibodies. The stimulation of ERK-2 correlated with cell shrinkage, however, the effect was clearly detectable only at strong volume reduction (Fig. 6, ERK-2, lane 6). The phosphorylation of SAPK p54 was very similar to that of p85: the trigger was the decreased volume and not the increased osmolality (Fig. 6, SAPK, compare lanes 3 and 4) and the response was proportional to the reduction in cell volume (lanes 3, 5, and 6). SAPK p46 reacted in a similar manner but apparently it required a stronger shrinkage. In contrast, the behavior of p38 was entirely different: its phosphorylation was strongly stimulated both under iso- or hypertonic swelling (p38, lanes 2 and 4) and under hypotonic shrinkage (lanes 3, 5, and 6). In conclusion, p85 tyrosine phosphorylation might be either upstream of SAPK (and perhaps ERK) activation, or it may be a consequence of an early event necessary for the activation of these MAP kinases as well. The activation of p38 does not correlate with p85 phosphorylation.

**Interaction of p85 and Other Osmo-sensitive Proteins with SH2 and SH3 Domains of Grb2—Interactions between tyrosine-phosphorylated proteins and adapter molecules (e.g. Grb2) constitute an important step in numerous signal-transducing pathways (47). Besides providing information about the signaling route itself, investigation of these interactions may help to find characteristic structural domains on the osmo-sensitive proteins, and can be useful for the future purification attempts. Therefore we tested whether hyperosmotic stress could induce a detectable change in the pattern of tyrosine-phosphorylated proteins that complex with Grb2, or certain domains of this adapter molecule. For these experiments fusion proteins were used which contained glutathione S-transferase (GST) linked either to the full-length Grb2 sequence (consisting of one SH2 domain and two SH3 domains) or to certain parts of it. Three mutant Grb2 proteins were applied: the first possessed only a functional SH2 domain (double SH3 mutant), the second had only the N-terminal SH3, whereas the third had only the C-terminal SH3 domain. Lysates of iso- or hypertonicity-treated cells were incubated with the appropriate fusion protein immobilized on glutathione-agarose beads. The material precipitated by the beads was subjected to electrophoresis and blotted with anti-phosphotyrosine antibodies. As shown on Fig. 7, the whole Grb2 molecule could precipitate multiple phosphotyrosine-containing proteins (lanes Grb2). The association of these to the beads was due to the presence of Grb2 since no proteins were isolated with beads covered only with GST (not shown). Comparing the pattern obtained from iso- or hypertonicity-treated samples showed that hyperosmosis induced an approximately 4-fold increase in the content of a Grb2-precipitable, tyrosine-phosphorylated protein of 85-kDa. The location of this band corresponded perfectly to the osmo-sensitive band observed in whole cell lysates. Using Grb2 possessing only a functional SH2 domain gave similar results except that the difference at the level of p85 was even more pronounced and other hypertonicity-related bands also became apparent with molecular masses of ~100 and ~120 kDa (see lanes SH2). It is noteworthy that an increase in the signal in these regions (especially at 120 kDa) was often observed also in whole cell lysates (see e.g. Fig. 1A) but these proteins, being just below a huge, phosphotyrosine-containing, and osmotically not respon-
interaction of proteins tyrosine phosphorylated in response to osmotic shock with Grb2-GST fusion proteins. Lysates from cells which have been exposed for 10 min to iso- or hypertonic (600 mosm) media (marked as I and H, respectively) were centrifuged to remove Triton X-100-insoluble material and the supernatant was incubated for 1 h with agarose resin-coupled Grb2-GST proteins containing full-length Grb2 (left panel), or different mutants possessing only one functional domain of the adapter (labeled as SH2, N-SH3, and C-SH3). Beads were sedimented, washed several times, and the precipitated proteins were subjected to Western blotting with anti-phosphotyrosine antibody.

Figure 7. Interaction of proteins tyrosine phosphorylated in response to osmotic shock with Grb2-GST fusion proteins. Lysates from iso- or hypertonic (600 mosm) media (marked as I and H, respectively) were centrifuged to remove Triton X-100-insoluble material and the supernatant was incubated for 1 h with agarose resin-coupled Grb2-GST proteins containing full-length Grb2 (left panel), or different mutants possessing only one functional domain of the adapter (labeled as SH2, N-SH3, and C-SH3). Beads were sedimented, washed several times, and the precipitated proteins were subjected to Western blotting with anti-phosphotyrosine antibody.

Figure 8. Distinctive properties of the regulatory subunit of PI 3-kinase and the osmo-sensitive p85 protein. A, left panel: PI 3-kinase was immunoprecipitated from lysates of iso- or hypertonicity-treated samples and the precipitates were probed with anti-phosphotyrosine (upper lanes), and after stripping, with anti-PI 3-kinase (lower lanes). Right panel: Western blots of total cell lysates were probed with anti-phosphotyrosine or anti-PI 3-kinase. The amount of cell lysates were selected to give a comparable PI 3-kinase signal as observed in the immunoprecipitates. Samples from the precipitates and the lysates were probed with the same antibodies and developed in parallel, for the same time, on the same film. B, lysates from iso- (I) or hypertonicity (H)-challenged cells were incubated with beads coupled to various domains of Grb2 and processed as described under Fig. 7. Precipitated proteins were subjected to Western blotting and probed with anti-PI 3-kinase.

These results show that, at least in vitro, three osmotically responsive proteins can bind to Grb2. Two of these proteins can probably form a complex with Grb2 by two distinct mechanisms involving both the SH2 and SH3 domains of the adapter. Currently it cannot be decided whether this complex formation occurs by direct binding of the respective osmo-sensitive phosphoproteins to the various domains of Grb2 or through participation of other connecting proteins. Nevertheless a possible interpretation of these results is that p85 and p120 might contain proline-rich motifs through which they can associate with SH3 domains. A certain conclusion is that binding of p85 and p120 to Grb2 occurs independently: while the binding of p85 is lost if only the C-SH3 domain is intact, this alteration does not affect the association of p120.

The regulatory subunit of phosphoinositide 3-kinase (PI 3-kinase) is an 85-kDa protein which can be tyrosine phosphorylated and was shown to interact with Grb2 (48). To establish whether the osmo-sensitive p85 could be identical with PI 3-kinase, we immunoprecipitated the regulatory subunit from the lysates of iso- and hypertonicity-treated cells and probed these precipitates with anti-phosphotyrosine antibodies. Absolutely no labeling occurred in the precipitates obtained from control or osmotically challenged cells. The presence of PI 3-kinase in these samples was verified by probing the precipitates with anti-PI 3-kinase (Fig. 8A, left panels). Importantly, in aliquots of total cell lysates, which contained comparable amounts of PI 3-kinase as the precipitates, the hypertonicity-induced tyrosine phosphorylation was readily detectable (Fig. 8A, right panels). This finding indicates that the absence of the phosphotyrosine signal in the precipitates could not be accounted for by the insufficient immunoprecipitation of PI 3-kinase. To further investigate whether PI 3-kinase and the osmo-sensitive p85 are in fact distinct, we compared the binding pattern of these proteins to the various domains of Grb-2 (compare Fig. 8B and Fig. 7). PI 3-kinase could bind to both the N- and the C-terminal SH3 domains of the adapter (in agreement with earlier reports (48)), while, as described above, p85 did not complex with the C-SH3 domain. Furthermore, the SH2 domain-containing beads, which bound p85 in lysates of hypertonicity-treated cells, did not precipitate out PI 3-kinase from these samples. Taken together, these findings argue against the possibility that the osmo-sensitive p85 is the regulatory subunit of PI 3-kinase.

DISCUSSION

The present study demonstrates that an increase in extracellular osmotic activity induces characteristic tyrosine phosphorylations in at least three proteins (with apparent molecular masses of approximately 42, 85, and 120 kDa) of CHO cells (Fig. 1). This effect is most pronounced in an 85-kDa protein whose response is quick (detectable within 1 min), reversible, proportional to the applied osmotic concentration and occurs already at mild hypertonicity (plus 50 mosm) (Fig. 2). All these features render the tyrosine phosphorylation of p85 a sensitive and accurate indicator of the decrease in cellular hydration. We have provided evidence that the triggering factor is not an increase in extra- or intracellular osmotic concentration per se, but rather a decrease in cell volume. Cell shrinkage seems to be
a prerequisite for the process, and it is equally effective, irrespec-
tively whether it occurs under iso- or hyperosmotic conditions. Changes in Cl\(^-\) and K\(^+\) concentration are thought to regulate kinases and were reported to affect the phosphoryla-
tion of ion channels (18) and the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransporter (14, 15). In contrast, the shrinkage-induced rise in the cytosolic concentration of these ions is certainly not responsible for the observed tyrosine phosphorylations (Fig. 3). Pharmacological interference with tubulin assembly or with actin polymeriza-
tion did not inhibit the shrinkage-dependent tyrosine phospho-
rylation (data not shown), suggesting that the integrity of these systems is not an absolute requirement for this response. It awaits elucidation whether the initializing volume-dependent event is the increase of a yet unidentified cytoplasmic regulator and/or the occurrence of specific protein-protein interactions due to macromolecular crowding (49).

Hyperosmolar environment was found to stimulate several subgroups of the MAP kinase superfamily including ERKs, SAPKs, and p38 (43). The tyrosine-phosphorylated 42-kDa protein is very likely to be ERK-2 since this band co-migrated with the anti-ERK-2 reactive protein and ERK-2 became indeed phosphorylated under our conditions (Figs. 4 and 6).

The molecular identity of p85, the most osmo-responsive protein, remains unresolved. Nevertheless our functional data obtained by using activators and inhibitors of various signaling pathways (Figs. 4 and 5) together with the structural information regarding the interaction of this band with different do-
 mains of Grb2 (Figs. 7 and 8) provide a basis to relate p85 to identified members of tonicity-responsive kinase cascades and to narrow down the number of potential candidates. The phosphor-
ylation of p85 is not downstream to the activation of PKC or ERK-2, since the prevention of the hyperosmosis-induced ERK activation by PKC depletion or by inhibition of MEK leaves the tyrosine phosphorylation of p85 intact. Similarly, p85 phosphorylation is not a consequence of SAPK stimulation, because activators of these kinases (e.g. anisomycin, heat shock, or oxidative stress) fail to provoke the effect. ERK-5, another osmo-sensitive MAP kinase contains unique proline-
rich regions (50, 51) which might be able to associate with SH3 domains. On the other hand several findings argue against ERK-5 being the 85-kDa tyrosine-phosphorylated protein. First, the apparent molecular mass of ERK-5 was found to be 110 kDa (30). Second, an anti-PAN-ERK antibody raised against a region of ERK-2 which has 60% identity with the corresponding portion of ERK-5, and which was shown to react with a high molecular weight MAP kinase, failed to react with any band >60 kDa in our cell lysates. Third, ERK-5 was de-
scribed as a redox-sensitive kinase, whereas oxidative stress was ineffective in eliciting p85 tyrosine phosphorylation. MAP kinase kinases (i.e. MEKS, SEK1, and MKK3) which constitute the next level of these pathways and might autophosphorylate on tyrosines can also be excluded simply on the basis of their size. Finally, we have shown that p85 is probably not identical with another important signaling enzyme of similar molecular mass, the regulatory subunit of PI 3-kinase (Fig. 8).

In the light of all these considerations we suggest that the tyrosine phosphorylation of p85 likely represents a hitherto unrecognized, cell shrinkage-dependent, early event. This re-
action may be one of the upstream mechanisms through which extracellular physical parameters are converted to intracellu-
lar biochemical signals.

While in the plasma membrane of yeast cells a two-compo-
nent osmosensor system that regulates MAP kinases have been identified (20, 21), the initial steps of the hypertonicity-related signal transduction in mammalian cells are largely unknown. Emerging evidence, however, suggests that tyrosine phospho-
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