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review

Dealing with osmostress through MAP kinase activation

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In response to changes in the extracellular environment, cells coordinate intracellular activities to maximize their probability of survival and proliferation. Eukaryotic cells, from yeast to mammals, transduce diverse extracellular stimuli through the cell by multiple mitogen-activated protein kinase (MAPK) cascades. Exposure of cells to increases in extracellular osmolarity results in rapid activation of a highly conserved family of MAPKs, known as stress-activated MAPKs (SAPKs). Activation of SAPKs is essential for the induction of adaptive responses required for cell survival upon osmostress. Recent studies have begun to shed light on the broad effects of SAPK activation in the modulation of several aspects of cell physiology, ranging from the control of gene expression to the regulation of cell division.

Introduction

Most cells are sensitive to osmotic imbalances and, when subjected to increases in extracellular osmolarity, lose water and shrink. To adapt to such high osmotic conditions, cells generally accumulate small organic molecules (e.g. glycerol), which allow them to balance their osmotic pressure with that of the external environment. However, osmostress not only induces the accumulation of osmolytes, but also has a great impact on cellular physiology. In yeast, for instance, it causes cytoskeletal reorganization, changes in cell-wall dynamics, alteration of ion homeostasis, metabolic adjustments and cell-cycle arrest, as well as having a huge impact on gene expression (reviewed by Hohmann, 2002). Our current knowledge confirms that many principles of osmoadaptation are conserved across eukaryotes, and therefore the use of yeasts as basic models has been of great value in elucidating the signal transduction mechanisms underlying the response to high osmolarity.

In *Saccharomyces cerevisiae*, changes in the osmolarity of the medium have been reported to affect different signalling pathways. The best-characterized signalling system by far involves the mitogen-activated protein (MAP) kinase Hog1, a relative of the p38 and c-Jun N-terminal kinase (JNK) families of stress-activated protein kinases (SAPKs). In addition to the HOG pathway, a second signal transduction pathway, mediated by cAMP-activated protein kinase A (PKA), has been shown to broadly modulate osmostress-induced gene expression in yeast. More detailed reviews of PKA involvement in osmostress signalling have been published over the years (e.g. Hohmann, 2002). We therefore focus on the role of SAPKs in osmostress, in both yeast and mammals.

Signal transduction by SAPKs

MAP kinase (MAPK) cascades are common signalling modules found in both higher and lower eukaryotic cells and are composed of three consecutively activated tiers of kinases: MAPKKK, MAPKK and MAPK. Eukaryotic organisms contain multiple MAPK families organized into discrete cascades, and two major pathways that are activated by environmental and genotoxic stress have been identified in mammals. The SAPKs, including the JNKs and the p38 MAPKs, are important components of these pathways. A prototype of the SAPK family is the yeast Hog1 MAPK, which specifically responds to increased extracellular osmolarity and is required for cell survival under these conditions. Conservation of the stress MAPK cascades between yeast and humans is indicated by the fact that individual kinases in the yeast pathway can be replaced by the corresponding human enzymes.

Osmostress sensors. It was proposed that, in mammalian cells, osmotic shock causes activation of JNK by non-specific clustering and internalization of cell surface receptors such as the epidermal growth factor (EGF) receptor (Rosette and Karin, 1996). However, in yeast, specific osmosensing devices seem to be responsible for detecting changes in osmolarity. The HOG

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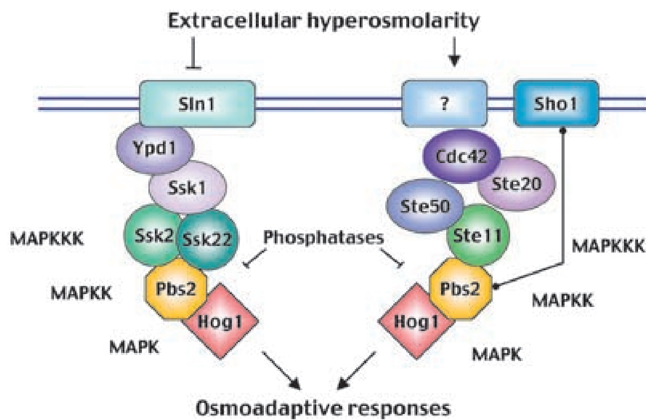

Fig. 1. Schematic diagram of the yeast HOG pathway. Pbs2 integrates signals from two major independent upstream osmosensing mechanisms, which leads to the activation of specific MAPKKKs. Under osmostress, activated Pbs2 activates the Hog1 MAPK, which induces a set of osmoadaptive responses.

pathway is activated predominantly by two independent mechanisms that lead to the activation of either the Ssk2 and Ssk22 or the Ste11 MAPKKKs, respectively (Figure 1). The first mechanism involves a 'two-component' osmosensor, composed of the Sln1-Ypd1-Ssk1 proteins. The Sln1 transmembrane protein has intrinsic histidine kinase activity and is a homologue of bacterial two-component signal transducers. Using a phospho-relay mechanism involving the Ypd1 and Ssk1 proteins, Sln1 is able to control the activity of Ssk1, which in turn interacts with and regulates the Ssk2 and Ssk22 MAPKKKs and subsequent Pbs2 activation (Posas *et al.*, 1998). Pbs2 activation can also be achieved by a second, independent mechanism (Figure 1) that involves the transmembrane protein Sho1, the MAPKKK Ste11, the Ste11-binding protein Ste50, the Ste20 p21-activated kinase (PAK) and the small GTPase Cdc42 (Posas *et al.*, 1998; Hohmann, 2002). Activation of Pbs2 by Ste11 requires the interaction of Pbs2 with Sho1 and, although this interaction is thought to be regulated (Reiser *et al.*, 2000), the basic activation mechanism for this remains unclear (Raitt *et al.*, 2000). Other, less well-characterized osmosensing mechanisms could also be feeding signals into the HOG pathway (Van Wuytswinkel *et al.*, 2000). Since mammalian cells do not seem to have specific stress sensors similar to Sln1, determination of the sensor mechanism coupled to Sho1 could help to decipher the molecular identity of mammalian osmosensors.

SAPK pathway components and organization. The central core of the yeast HOG pathway comprises a layer of MAPKKKs (Ssk2, Ssk22 and Ste11) that are responsible for the activation of the MAPKK Pbs2. Once activated, Pbs2 phosphorylates and activates the Hog1 MAPK (Figure 1). Mammalian cells activate three different MAPKs in response to osmostress: p38, JNK and ERK5. Since the role of ERK5 is still unclear, we will concentrate on the p38 and JNK MAPK pathways (Figure 2). Both p38 and JNK are encoded by several genes, which display differential tissue distribution, substrate specificity and sensitivity to chemical inhibitors (Kültz and Burg, 1998; Kyriakis and Avruch, 2001). Moreover, activation of these MAPKs can be achieved by several MAPKKs that have overlapping activities but differential specificities. MKK3 and MKK6 activate p38 MAPKs, whereas MKK4 and MKK7 are mainly responsible for the activation of JNK; all four MAPKKs can be activated by osmostress. Three different families of MAPKKKs (MEKKs, MLKs and TAOs) regulate the activation of MAPKKs and integrate the signals from upstream components into the cascades. However, the complexity of the regulation of these enzymes makes it difficult to formulate a clear picture of the stimuli that drive their activation. One of the MAPKKKs that mediates osmostress signalling is MTK1/MEKK4. MTK1 is activated by osmostress, and the overexpression of a dominant negative MTK1 can partially block the subsequent activation of p38 (Takekawa *et al.*, 1997).

The actual mechanisms of MAPKKK activation by osmostress in mammalian cells remain largely uncharacterized, but they could be similar to the mechanisms that function in yeast. For instance, Ssk2 activation by Ssk1 involves binding to the N-terminal non-catalytic domain of Ssk2. Interestingly, binding of GADD45 proteins to the human homologue of Ssk2, MTK1, results in activation of the MAPKKK (Takekawa and Saito, 1998). Moreover, in yeast, Ste11 activation is controlled in part by Ste20 and the small G-protein Cdc42; and, in mammals, Ste20-like kinases and small G-proteins have been shown to regulate MAPKKKs (Kyriakis and Avruch, 2001).

The organization of all these components into MAPK modules has been proposed to be directed by protein–protein interactions between kinases and by scaffolding proteins. Many proteins with a scaffolding function have already been reported to interact with SAPKs (e.g. JIP1/JSAP1 for JNK, and JIP2 for p38) and with signalling components that possess intrinsic scaffolding properties (i.e. MKK4 and Pbs2). It has been proposed that scaffolding proteins could contribute to signal specificity by insulating different MAPK modules. This seems to be the case for the yeast HOG pathway, since binding of Ste11 to Pbs2 MAPKK restricts Ste11 to activating only Pbs2, and not other MAPKKs (Harris *et al.*, 2001). Thus, it is possible that specific scaffolding proteins could direct osmostress stimuli to restricted SAPK cascades. Although scaffolds could play a role in signalling specificity, MAPK substrate specificity and negative feedback loops involving, for instance, protein phosphatases may also be important (Chang and Karin, 2001).

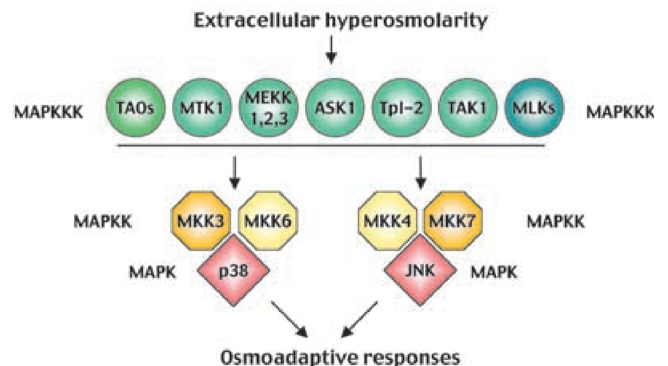

Fig. 2. Mammalian SAPK pathways. The MAPKKKs upstream of JNK and p38 belong to three broad protein kinase families differentiated by colour in the figure (TAOs, MEKKs and MLKs). There are four MAPKKs with overlapping activities but with different specificity.

Spatial organization of SAPK components. The upstream effectors of MAPK cascades seem to have specific sites of action in the cell (e.g. Cdc42), and membrane recruitment of MAPKKKs can be very important for cascade activation, as is the case for the recruitment of the MAPKKK Raf1 by the small G-protein Ras1. MAPKKs are actively excluded from the nucleus by mechanisms that involve a conserved nuclear export signal and the nucleo-cytoplasmic shuttling of MAPKs. Stimuli-dependent translocation of the MAPK ERK1 into the nucleus is mediated by its dissociation from its MAPKK and by its subsequent dimerization. Osmostress-induced phosphorylation of Hog1, rather than its kinase activity, is also essential for its nuclear accumulation (Ferrigno *et al.*, 1998; Reiser *et al.*, 1999); and, similarly to ERK1, Hog1 contains a dimerization domain, although the importance of this *in vivo* has not yet been demonstrated. However, since the concentration of Hog1 vastly exceeds that of the MAPKK, other mechanisms for controlling Hog1 localization may also come into play. For example, interactions with specific transport carriers or with nuclear retention factors may affect the nuclear accumulation of Hog1, as well as of other SAPKs (Ferrigno and Silver, 1999).

Downregulation of stress signalling. The magnitude and duration of signalling through SAPKs are critical determinants of their biological effect. Activation of Hog1 in response to mild osmolarity occurs within a minute and is extremely transient (Maeda *et al.*, 1995). This suggests that the MAPK functions as a biological switch that must be actively downregulated, both under basal conditions and during adaptation. In yeast, two major families of phosphatases interact with and inactivate Hog1: the Ser/Thr protein phosphatases type 2C (PP2C) and the protein tyrosine phosphatases (PTP). The importance of Hog1 downregulation is illustrated by the fact that simultaneous deletion of the *PTC1* and *PTP2* phosphatase genes results in inhibition of the cell proliferation that is caused by constitutive activation of the HOG pathway (Maeda *et al.*, 1993). A similar scheme is thought to operate in mammalian cells, and both PP2C and PTP have recently been shown to regulate p38 (Takekawa *et al.*, 1998; Saxena *et al.*, 1999). In addition, another family of dual-specificity phosphatases must play a key role in the regulation of MAPKs, since, for instance, the M3/6 and MKP-7 phosphatases have been shown to regulate JNK1 and p38 SAPKs (Keyse, 2000).

Adaptive responses

Downstream targets for SAPKs. Many SAPK targets have been described, both in the cytoplasm and the nucleus, which indicates that multiple cellular functions are under their control. SAPKs are proline-directed kinases. However, substrate specificity is not only defined by the targeted amino acid but also by specific docking domains present on the substrate protein and by specific substrate binding motifs in MAPKs. This interesting combination can account for the very high selectivity among MAPK subgroups (Tanoue *et al.*, 2000). The substrates that mediate the adaptive response induced by SAPKs can be classified mainly into two groups: effector kinases and transcription factors (Figure 3).

Transcriptional regulation. One of the main functions of MAPKs in response to osmostress is the regulation of gene expression. In mammalian cells, p38 controls the expression of >100 genes

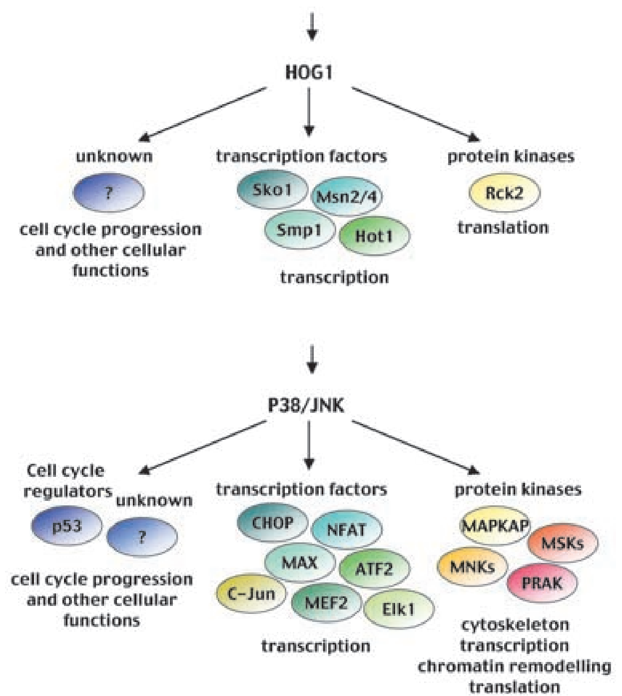

Fig. 3. Activation of SAPKs by osmostress has a great impact on cell physiology. In response to stress, the yeast Hog1 (upper panel) or the mammalian p38 and JNK (lower panel) induce diverse osmoadaptive responses.

(Ono and Han, 2000). In budding yeast, genome-wide transcription studies revealed that a large number of genes (~7%) show significant but transient changes in their expression levels after a mild osmotic shock and that the Hog1 MAPK plays a key role in much of this global gene regulation. These osmostress-regulated genes are implicated mainly in carbohydrate metabolism, general stress protection, protein production and signal transduction. This global change in transcription could account, at least in part, for the metabolic adjustments required for osmostress adaptation (Hohmann, 2002).

There is no unifying mechanism by which SAPKs and MAPKs modulate gene expression. JNK and p38 target several transcription factors directly, enhancing their ability to activate transcription (e.g. MEF2A/C, Elk1/Sap1a). Moreover, phosphorylation of c-Jun and AFT-2 by p38 and JNK kinases yields the formation of activation protein 1 (AP-1) complexes. JNK also inhibits the nuclear translocation, and therefore the function, of the activator NFAT by phosphorylation. JNK and p38 MAPKs also modulate gene expression through remodelling the structure of chromatin. p38 controls the phosphorylation of histone H3 and the high mobility group protein 14 (HMG-14) through the activation of the mitogen- and stress-activated protein kinase 1 (MSK-1) (Kyriakis and Avruch, 2001). On the other hand, acetylation of histones H2B and H4 by ATF-2 is stimulated by JNK phosphorylation (Kawasaki *et al.*, 2000). Additionally, p38 has been reported to phosphorylate the TATA-binding protein (TBP), a prerequisite for its binding to the TATA box (Carter *et al.*, 1999). Whether any of these MAPK functions control gene expression during osmostress signalling remains to be determined.

In yeast, five transcription factors have been proposed to be controlled by the Hog1 MAPK. Hot1, Smp1, Msn2 and Msn4 activate, whereas Sko1 represses or activates, different subsets of osmotic-inducible and Hog1-regulated genes (Rep *et al.*, 2000; Proft *et al.*, 2001; E. de Nadal and F. Posas, unpublished results). Sko1 is an ATF/CREB factor whose repressive activity via the Cyc8–Tup1 complex is inhibited by Hog1 in response to osmostress (Proft *et al.*, 2001). Msn2 and Msn4 are generic stress factors controlled by PKA and Hog1 by an unknown mechanism. Hot1 physically interacts with Hog1, and its binding to DNA and subsequent transactivation activity are regulated by its phosphorylation by the kinase (Rep *et al.*, 1999; Alepuz *et al.*, 2001).

In addition to the conventional MAPK role in regulating transcription factor activity by direct phosphorylation, the finding that Hog1 can associate with chromatin at promoter regions of target genes adds a new dimension to gene regulation by signalling kinases. Activated Hog1 is recruited to osmotic-inducible promoters through interaction with specific transcription factors (Alepuz *et al.*, 2001). Furthermore, recent results support a model in which promoter-localized Hog1 stimulates transcription by phosphorylation of specific components of the RNA pol II holoenzyme (E. de Nadal, P.M. Alepuz, M. Zapater, G. Ammerer and F. Posas, unpublished results). Additionally, Hog1 has been reported to bind to Sko1-dependent promoters through its interaction with Sko1, and both proteins are required for the recruitment of the SAGA histone acetylase and SWI/SNF nucleosome-remodelling complexes in response to osmostress (Proft and Struhl, 2002). These results suggest that MAPK-mediated modification of the transcription machinery at gene promoters could be a common feature of transcriptional regulation.

Post-transcriptional control of gene expression. Recent studies have highlighted roles for the MAPKs in the post-transcriptional and translational control of gene expression. Both p38 and JNK kinase pathways have been shown to contribute to cytokine/stress-induced gene expression by stabilizing mRNAs through an ARE (AU-rich element, present in 3′-UTR transcripts) targeted mechanism. AREs regulate mRNA turnover by modulating poly(A)-shortening rates and the subsequent decay of mRNA. In the yeast system, inhibition of the Hog1 pathway also leads to destabilization of the ARE-bearing transcript (Vasudevan and Peltz, 2001). In addition to ARE-mediated mRNA decay, unstable mRNAs can be targeted for rapid decay by other pathways. For instance, JNK is involved in IL-2 mRNA stabilization in activated T cells (Chen *et al.*, 2000). Although this is a very interesting mechanism by which SAPKs can regulate gene expression, its role in osmostress still needs to be characterized.

Regulation of protein synthesis. In response to increases in external osmolarity, there is a transient decrease in protein synthesis (Norbeck and Blomberg, 1998; Uesono and Toh, 2002) caused by a decrease in amino-acid uptake, repression of ribosomal protein genes and a decrease in translation efficiency. The HOG pathway appears not to be involved in the initial inhibition of translation, but rather in the reactivation of translation under stress as an adaptation mechanism (Uesono and Toh, 2002). The yeast Rck2 kinase, which resembles the mammalian CaM kinases, is regulated by Hog1 (Bilsland-Marchesan *et al.*, 2000; Teige *et al.*, 2001). Reduction of protein synthesis upon stress was similar in *hog1* and *rck2* cells, which suggests that the effects of Hog1 on translation are mediated by Rck2 kinase (Teige *et al.*, 2001). Rck2 could affect translation by directly regulating the elongation factor EF-2, but an effect on initiation factors cannot be excluded. In mammalian cells, stress-induced regulation of protein synthesis is mediated by phosphorylation of the eukaryotic initiation factor eIF4E by Mnk1 or of EF-2 by the eEF-2 kinase. Both kinases have been reported to be regulated by p38 in response to stress (Waskiewicz *et al.*, 1999; Knebel *et al.*, 2001). Thus, adaptive responses to restore protein translation could be mediated by similar mechanisms in yeast and mammals.

Cell-cycle control by SAPKs. Progression through the cell cycle is critically dependent on the presence of nutrients and stress stimuli. In response to osmostress, cells transiently modulate cell-cycle progression to allow adaptation. The role of SAPKs in cell-cycle control was first proposed in *Schizosaccharomyces pombe* for the Spc1/Sty1 MAPK (Shiozaki and Russell, 1995). The Spc1/Sty1 MAPK pathway displays strong structural similarities to the HOG pathway. However, it is activated not only by osmostress but also in response to a whole range of environmental conditions (e.g. heat shock, oxidative stress and UV light). It was initially identified as having a role in cell-cycle control, since alteration of its components, resulting in either hyperactivation or signal abrogation, resulted in cell defects, and only later was this pathway related to environmental responses (Wilkinson and Millar, 2000). More recently, a role for p38 and JNK MAPK pathways in cell-cycle progression has been reported in several organisms (reviewed by Ambrosino and Nebreda, 2001; Pearce and Humphrey, 2001). Although in mammals the role of JNK in cell-cycle control has not been clearly defined, it has been proposed that depletion of JNK1 or JNK2 suppresses cell growth under non-stress basal conditions. Moreover, the proliferation of *Jnk*$^{-/-}$ cells is reduced due to G_1–S defects caused by a decrease in AP-1 transcription. p38 has been shown to regulate G_0, G_1–S and G_2–M transitions. Depending on the cell type, p38 can induce either progression or inhibition at the G_1–S transition by differential regulation of specific cyclin levels (cyclin A or D1) as well as by pRb and p53 phosphorylation (Wilkinson and Millar, 2000; Ambrosino and Nebreda, 2001). Less is known about the involvement of SAPKs in the regulation of the cell cycle upon osmostress. Mammalian cells regularly exposed to osmotic imbalances, such as inner medullary epithelial cells (IMEs), respond to high osmolarity by arresting in G_1–S, G_2 and mitosis. To protect cells from hypertonicity, p53 inhibits DNA replication and transition from G_1 to S (Dmitrieva *et al.*, 2001), and p53 is also known to be phosphorylated in response to stress by p38 MAPK. G_2–S and M delays seem to be p53-independent. For instance, hypertonicity causes a rapid activation of the G_2–M checkpoint through activation of p38, which causes a drop in Cdc2 kinase activity.

In yeast, a similar scenario is being unravelled. Increases in external osmolarity induce a transient growth arrest that results in accumulation of cells in G_1 and G_2–M phases and a concomitant drop of cells in S phase (Alexander *et al.*, 2001). It is not clear whether the Hog1 MAPK is acting in the G_1–S delay, but it has been reported that Hog1, together with the kinase Swe1, regulates a G_2 delay by affecting yeast Cdc2 kinase activity (Alexander *et al.*, 2001; J. Clotet, X. Escoté, E. Garí, M. Aldea and F. Posas, unpublished results). It is worth noting that mutations that inactivate the SLN1 osmosensor or inactivate both the PTC1

and PTP2 phosphatases block cell growth because of inappropriate hyperactivation of the Hog1 MAPK. This could be caused by improper activation of several cell-cycle checkpoints.

Perspectives

In recent years, we have learned that stress-activated MAPK pathways play a pivotal role in osmostress signal transduction, in both yeast and mammals. Moreover, osmostress responses, which were initially thought to be limited to the modification of the expression of a small number of genes, have now been shown to be involved in many aspects of cell biology. Still, some key questions regarding osmostress signalling remain to be clarified. A major issue is the identification of the mechanisms required to detect changes in osmolarity in mammalian cells. Much is known about the components of the central core of SAPK cascades; however, the mechanisms of MAPKKK activation and how signal specificity is achieved in response to osmostress remain unclear. Control of gene expression is a major outcome of SAPK activation, and the precise regulatory mechanisms have only started to be elucidated. Future identification of SAPK targets that are specifically modified in response to osmostress will result in a better understanding of the molecular mechanisms required to survive environmental changes.

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