Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin β homologs NMD5 and XPO1

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Introduction

Eukaryotic cells have evolved a variety of mechanisms to relay information from the extracellular environment to the nucleus. One such mechanism consists of MAP (mitogen-activated protein, or microtubule associated protein) kinase cascades that typically transduce signals from plasma membrane-associated receptors to nuclear transcription factors, culminating in altered expression of target genes. One such pathway is the budding yeast high osmolarity glycerol response (HOG) pathway (Brewster et al., 1993). In this pathway, the MAP kinase HOG1 is activated following the phosphorylation of two key residues (Thr174 and Tyr176) by the dual-specificity MAP kinase kinase PBS2 (Brewster et al., 1993). Activation of PBS2 in turn requires its phosphorylation by a MAP kinase kinase kinase; this can be either STE11, or one of two partially redundant MAPKKKs, SSK2 and SSK22 (Posas et al., 1998). STE11 acts in conjunction with the transmembrane SH3 domain-containing protein SHO1, while SSK2 and SSK22 are regulated by a proaryktotic-like two component phosho-relay system consisting of SLN1, YPD1 and SK1 (Maeda et al., 1994; Posas et al., 1996). The essential features of stress-activated MAP kinase pathways are conserved from yeast to humans (Ip and Davis, 1998), but how the activation of a protein kinase cascade postulated to reside in the cytoplasm may lead to changes in gene transcription remains unclear. Presumably, some component must cross the nuclear envelope. Although it has been shown that a mammalian MAP kinase homolog can translocate into the nucleus of PC12 cells under certain conditions (Traverse et al., 1992, 1994), it remains to be shown whether this is a requirement for the transduction of the signal, and exactly how translocation is achieved.

Recent work has elucidated the basic mechanisms for the import of proteins into the nucleus. Many newly synthesized proteins that are destined for the nucleus contain a ‘classical’ nuclear localization signal (NLS). The NLS is recognized in the cytoplasm by the importin α subunit of a heterodimeric NLS receptor, and targeted to the nuclear pore complex (NPC) by the importin β subunit. Translocation across the NPC requires the activity of the small Ras-related GTP binding protein Ran. It has been suggested that the nucleotide state of Ran may be a determinant for the direction of movement (into or out of the nucleus) (Corbett et al., 1995; Koepp et al., 1996) or to regulate the loading of cargo onto nuclear transport factors (Melchior and Gerace, 1998). Recently, new homologs of importin β have been identified (Gorlich et al., 1997), and have been found to play roles in such diverse pathways as the export of tRNA (Arts et al., 1998; Kutay et al., 1998) or proteins (Stade et al., 1997; Kehlenbach et al., 1998; Toone et al., 1998) from the nucleus, or the recycling of hnRNP back into the nucleus (Pollard et al., 1996; Bonifaci et al., 1997; Fridell et al., 1997; Pemberton et al., 1997; Siomi et al., 1997; Senger et al., 1998; Truant et al., 1998). Thus, nuclear transport is now thought to involve a multiplicity of importin β homologs that serve to ferry specific cargoes in both directions across the nuclear envelope, in a manner that is regulated by Ran–GSP1.

Here, we localize the components of the HOG1 signaling module, identify the regulatory mechanisms that determine the subcellular localization of HOG1 MAPK, and charac-
terize the mechanism by which HOG1 is imported into, and subsequently exported from, the nucleus. Our data suggest a new function for the regulatory phosphorylation sites of protein kinases of the MAP kinase superfamily, and by revealing a new nuclear import pathway, shed further light on the complex mechanisms that determine the compartmentalization of eukaryotic cells.

Results

The HOG1 MAP kinase translocates transiently to the nucleus after osmotic stress whereas the MAPKK PBS2 and the MAPKKK STE11 remain cytoplasmic.

To analyze the spatial organization of the HOG1 signaling pathway in yeast, we genetically fused the open reading frames of the MAPK HOG1, the MAPKK PBS2 and the MAPKKK STE11 to the N-terminus of the green fluorescent protein (GFP). When expressed from their own promoters from a low-copy number (CEN) plasmid, each GFP fusion was able to rescue the osmosensitivity of cells lacking the corresponding wild-type gene (data not shown), demonstrating that each fusion protein is functional, and reflects the behavior of the corresponding wild-type protein. Moreover, immunoblotting of extracts from cells expressing these GFP-tagged proteins, confirmed that the proteins were expressed, and no free GFP was observed (data not shown).

Initially, we focused on the HOG1 MAPK. Microscopic examination of cells expressing HOG1–GFP revealed that HOG1 localizes throughout the cytoplasm and nucleus of unstressed cells (Figure 1B, –NaCl). When cells carrying HOG1–GFP were exposed to a brief osmotic shock (0.4 M NaCl for 2 min), HOG1 changed its distribution from being mainly cytoplasmic to accumulating completely within the nucleus (Figure 1B, +NaCl). After 30 min in the presence of NaCl, the levels of HOG1–GFP in the cytoplasm were partially restored, indicating that nuclear localization of HOG1 is a transient event. Interestingly, nuclear localization correlates with the transient HOG1 phosphorylation that is observed after osmotic stress (Figure 1A, 4G10 blot). The appearance of HOG1–GFP in the cytoplasm after 30 min of stress is the result of nuclear export, rather than new protein synthesis, since addition of the protein inhibitor cyclohexamide does not affect the observed distribution (data not shown). In addition, the levels of HOG1–GFP were found not to change during the osmotic stress response (Figure 1A, GFP blot).

We asked whether the translocation of HOG1–GFP into the nucleus was specific for osmotic stress (the only form of stress known to activate HOG1). Indeed, only osmotic stress (0.4 M NaCl or 1 M sorbitol) could induce HOG1–GFP translocation (Figure 2); 7.5% ethanol, 1 mM t-butyl hydrogen peroxide and 20 min temperature shift (37°C) all failed to drive the nuclear translocation of HOG1 (Figure 2). These data show that translocation of HOG1 into the nucleus is an integral feature of the osmotic stress response.

We next asked whether the kinases upstream of the HOG1 MAPK would co-localize with HOG1. A functional fusion of the STE11 MAPKKK and PBS2 MAPKK to GFP localized exclusively to the cytoplasm of unstressed cells, and did not relocate to the nucleus of stressed cells (Figure 3). These data indicate that the MAPK is the only element of the pathway that is relocated to the nucleus after stress.

Phosphorylation of HOG1 is required for translocation after stress.

The stress-induced nuclear translocation of HOG1 correlates with phosphorylation by PBS2 MAPKK at residues that regulate HOG1 protein kinase activity (Figure 1A). Thus, there are three potential mechanisms that may regulate the sub-cellular localization of HOG1: (i) the phosphorylation of T174/Y176 residues; (ii) the activation of HOG1 protein kinase activity; or (iii) changes in the association of HOG1 with its MAPKK, PBS2.

The localization of HOG1–GFP was analyzed in a pbs2Δ strain. We observed that the protein was both nuclear and cytoplasmic, although the nuclear signal was stronger than in wild-type cells. In marked contrast to

![Figure 1. HOG1 MAPK is transiently phosphorylated and translocated to the nucleus after osmotic stress.](image-url)
HOG1 translocates to the nucleus only under osmotic stress conditions. The hog1Δ strain (TM233) was transformed with the HOG1–GFP plasmid. Cells were grown to mid-log phase and exposed to several stresses for 5 min, to a final concentration of 0.4 M NaCl (NaCl), 1 M sorbitol (sorbitol), 7.5% ethanol (ethanol), 1 mM t-butyl hydrogen peroxide (peroxide). Cells were incubated for 20 min at 37°C for the heat stress (37°C).

Cytoplasmic localization of the PBS2 MAPKK and STE11 MAPKK. PBS2 and STE11 were tagged to GFP and transformed to their respective null mutants. Cells were grown to mid-log phase and collected before (−NaCl) or 5 min after (+NaCl) the addition of 0.4 M NaCl. GFP proteins were detected by fluorescence microscopy as described in Materials and methods.

Phosphorylation of HOG1 is required for its nuclear translocation. (A) HOG1 does not translocate to the nucleus in a pbs2Δ strain. HOG1–GFP plasmid was transformed to the pbs2Δ strain (TM261). Cells were grown to mid-exponential phase and collected before (−NaCl) or 5 min after (+NaCl) the addition of 0.4 M NaCl. GFP proteins were detected by fluorescence microscopy as described in Materials and methods. (B) The hog1(TA/YA) mutant does not translocate to the nucleus after osmo-stress. A catalytically inactive HOG1, hog1(KM), and the non-phosphorylatable form of HOG1, hog1(TA/YA), were transformed into a hog1Δ strain (TM233). Translocation to the nucleus was tested as before by addition of NaCl 0.4 M for 5 min.

Thus, we have shown that the stress-induced nuclear translocation of HOG1–GFP correlates with its phosphorylation, and that PBS2 is required for each of these events to occur. Because phosphorylation of HOG1 mediates its activation as a protein kinase, we wished to distinguish between the possibility that the protein kinase activity of HOG1 was mediating its translocation, or that the phosphorylation of the activating residues Thr174/Tyr176 was sufficient. Two mutants were constructed; hog1(TA/YA) is a mutant in which the two phosphorylation sites required for MAPK activation are mutated to Ala. This mutant cannot be phosphorylated after stress (Figure 5B) and cannot complement the osmosensitivity of a hog1Δ strain (data not shown). The second mutant, hog1(KM) can be phosphorylated at Thr174/Tyr176, but this time the phosphorylated protein is devoid of protein kinase activity due to a mutation of a Lys to a Met in the active site. This mutant is also unable to complement the osmosensitivity of a hog1Δ strain.
The hog1(TA/YA)–GFP allele failed to localize to the nucleus after osmotic stress (Figure 4B, bottom). This is consistent with the previous result that phosphorylation of HOG1 is required for its translocation into the nucleus. In contrast, hog1(KM) behaves identically to wild-type HOG1, demonstrating that the protein kinase activity of HOG1 is not required for its stress-induced translocation into the nucleus. Together, these data show that phosphorylation of HOG1, but not activation of its kinase activity, is required for HOG1 movement into the nucleus.

Dephosphorylation of nuclear HOG1 correlates with its exit from the nucleus (Figure 1). The protein phosphatases that dephosphorylate Y176 of HOG1, namely PTP2 and PTP3, have been shown to be activated by HOG1-mediated phosphorylation (Jacoby et al., 1997; Wurgler-Murphy et al., 1997). Thus, when a kinase-deficient allele of HOG1 is expressed in a hog1-deficient strain, its level of phosphorylation after stress remains higher for at least 45 min due to the lack of phosphatase activity (Figure 5B). We asked whether dephosphorylation of nuclear HOG1 is required for its return to the cytoplasm.

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We next addressed the mechanism whereby cytoplasmic HOG1 is targeted to the nucleus. First, we asked whether the classical importin α/β heterodimer was responsible for this event. Cells carrying a temperature-sensitive

Nuclear accumulation of HOG1 requires Ran–GSP1

To date, all nuclear transport events require the activity of the small GTP-binding protein Ran–GSP1. We asked whether this was also the case for the nuclear translocation of HOG1–GFP. gsp1-1 cells that carry a temperature-sensitive mutant allele of GSP1 are unable to translocate proteins carrying prototypical nuclear import signals into the nucleus at restrictive temperatures (Wong et al., 1997). Similarly, gsp1-1 cells were unable to translocate HOG1–GFP into the nucleus in response to osmotic stress after 1 h incubation at the restrictive temperature (Figure 6). The failure to translocate HOG1 was not due to a defect in the activation of the HOG kinase cascade, as HOG1 became phosphorylated in gsp1-1 cells even after shift to the restrictive temperature (data not shown). This finding confirms that the stress-induced translocation of HOG1 proceeds via a mechanism that, like all nuclear proteins studied thus far, requires Ran–GSP1.
In contrast, nmd5Δ cells completely failed to translocate HOG1 into the nucleus following stress treatment. HOG1–GFP becomes phosphorylated in nmd5Δ cells (data not shown), but completely fails to translocate into the nucleus even 30 min after stress (Figure 7A), by which time HOG1 has returned to the cytoplasm of wild-type cells (Figure 1). This genetic approach argues that the importin β homolog NMD5 is required for the complete translocation of HOG1–GFP into the nucleus of stressed cells.

**NMD5–GFP transiently translocates to the nucleus following osmotic stress**

We wished to characterize further the function of NMD5. We constructed a fusion of the NMD5 ORF to GFP, or to a triple repeat of the hemagglutinin (HA) tag, and placed these fusions under the control of the NMD5 promoter. Each of these constructs was able to rescue the phenotypes of nmd5Δ cells, including HOG1 translocation (data not shown), demonstrating that they were functional. In addition, immunoblotting revealed that the NMD5–GFP protein was expressed (Figure 8B). Microscopic examination of nmd5Δ cells expressing NMD5–GFP from CEN plasmids revealed that the protein localized throughout the cytoplasm of unstressed cells (Figure 8A). Within 2 min of stress, NMD5–GFP (but not other importin β homolog–GFP fusions, including KAP95, PSE1, SXM1 or XPO1, not shown) accumulated at the nuclear periphery and in the nucleus (Figure 8A). The NMD5–GFP fusion began to relocate to the cytoplasm within 30 min following stress. Stress was not observed to affect the levels of NMD5–GFP (Figure 8B). The correlation between NMD5 and HOG1 movement further supports the idea that HOG1 import into the nucleus may be mediated by NMD5.

**The export of dephosphorylated HOG1 from the nucleus requires a functional NES receptor**

The nuclear localization of HOG1–GFP was observed to be transient (Figure 1). Even when cells remain exposed to stress, much of HOG1–GFP returns to the cytoplasm within 30 min. Nuclear export of a number of proteins has been shown to be mediated by a leucine-rich nuclear export sequence (NES) that is recognized by an importin β homolog–GFP fusions, including KAP95, PSE1, SXM1 or XPO1, not shown) accumulated at the nuclear periphery and in the nucleus (Figure 8A). The NMD5–GFP fusion began to relocate to the cytoplasm within 30 min following stress. Stress was not observed to affect the levels of NMD5–GFP (Figure 8B). The correlation between NMD5 and HOG1 movement further supports the idea that HOG1 import into the nucleus may be mediated by NMD5.

**Discussion**

We have localized the components of the HOG1 MAP kinase cascade in *Saccharomyces cerevisiae*. Functional fusions to GFP of the MAPKK (STE11), the MAPKK (PBS2) and the MAP (HOG1) allowed all three protein kinases to be localized to the cytoplasm (Figures 1 and 3).
Regulated nuclear exchange of the HOG1 MAP kinase

Fig. 8. (A) NMD5–GFP relocates to the nuclear periphery following stress. Cells expressing NMD5–GFP under the control of its own promoter were observed by fluorescence microscopy before (0'), and 2 and 30 min after the addition of 0.4 M NaCl. (B) Immunoblotting analysis reveals that NMD5–GFP is expressed as a protein of ~147 kDa, whose abundance does not change following stress.

Fig. 9. Nuclear export of HOG1–GFP in stress-adapted cells requires the NES receptor XPO1. Wild-type cells, or cells carrying a temperature-sensitive allele of the importin β homolog XPO1–CRM1 (xpo1-1) were transformed with the HOG1–GFP plasmid. Cells were incubated at the restrictive temperature (37°C) for 1 h, and exposed to osmotic shock (0.4 M NaCl) for the indicated times.

Neither STE11 nor PBS2 were observed to undergo altered subcellular localization in stressed cells. In contrast, HOG1–GFP rapidly and reversibly translocated into the nucleus of osmotically stressed cells (Figure 1). These data indicate that activated HOG1 serves to relay the stress signal to the nucleus.

We have defined the minimum cis-acting requirements for the translocation of HOG1. The phosphorylation of the regulatory threonine and tyrosine residues in the TGY motif of HOG1 is necessary and sufficient for its nuclear translocation. Because a kinase-impaired mutant of HOG1 still enters the nucleus in stressed cells, HOG1 kinase cannot be directly responsible for activating its own nuclear import. We suggest that phosphorylation of HOG1 by PBS2 either makes HOG1 available to the nuclear translocation machinery or that HOG1 continuously shuttles between the nucleus and the cytoplasm in unstressed cells, and T174/Y176 phosphorylation creates...
a binding site that would trap HOG1 in the nucleus. Our observation that a fraction of HOG1–GFP is nuclear in unstressed cells is consistent with the idea that HOG1 may continuously shuttle between the nucleus and the cytoplasm, although we have no independent evidence suggesting that this is the case. However, none of the nuclear export mutants tested showed nuclear accumulation of HOG1 in unstressed cells.

We wished to learn how phosphorylated HOG1 enters the nucleus. Macromolecules that cross the nuclear envelope do so accompanied by one or more members of the importin β family, and translocation of the transport complex through the nuclear pore complex requires the activity of Ran–GSP1, a Ras-related GTP-binding protein (Gorlich and Mattaj, 1996). As predicted, the translocation of HOG1 requires the activity of yeast Ran. However, nuclear import of HOG1 was independent of the function of any of the importins so far implicated in nuclear protein import, namely importin α or β (Figure 7), KAP104 or MTR10 (not shown). Of all the importin β homologs that we tested, only one was required for HOG1 import, namely NMD5. In addition, we observed that NMD5 also relocates to the nucleus following stress, with the same kinetics as HOG1. Thus, we propose that NMD5 is a specific nuclear import receptor for active HOG1, although it remains possible that NMD5 has a role in the transport of other macromolecules. Moreover, a direct molecular interaction between NMD5 and HOG1 remains to be demonstrated, and it is possible that NMD5 has an alternative role, such as the release of HOG1 from a cytoplasmic anchor.

We noted an apparent subcytoplasmic localization of HOG1–GFP in stressed, but not unstressed, nmd5Δ cells. These structures co-localize with actin patches (P.Ferrigno and F.Posas, unpublished observations). The significance of this colocalization is unclear. In mammalian cells, there appears to be cross-talk between the actin cytoskeleton and stress-activated protein kinase pathways (e.g. Alberts and Treisman, 1998), and we are currently investigating a potential link between the cytoskeleton and the stress response.

We have also shown that the export of HOG1 from the nucleus requires the activity of an importin β homolog; screening through a panel of importin β mutants revealed that only XPO1 mutants failed to export HOG1 from the nucleus after stress. Stade et al. (1997) have identified XPO1 (also known as CRM1) as a specific receptor for leucine-rich nuclear export sequences (NESs), and recent work has shown that XPO1 homologs mediate the nuclear export of proteins in a range of species (e.g. Engel et al., 1998; Toone et al., 1998). Although to date we have failed to co-immunoprecipitate XPO1 and HOG1, we suggest that XPO1 mediates the export of HOG1 to the cytoplasm of cells adapted to stress.

Our data confirm and considerably extend several recent studies on the translocation of HOG1-homologs into the nucleus. In Schizosaccharomyces pombe, it has been shown that the HOG1 homolog Spc1 also accumulates transiently in the nucleus in response to osmotic stress, and in a manner that requires its activating kinase Wis1 (Gaits et al., 1998). Interestingly, these investigators have shown that the nuclear accumulation of phosphorylated Spc1 is maintained at least in part by the tethering of Spc1 to its target, the transcription factor Atf1. Clearly, once the nuclear target(s) of HOG1 becomes known, it will be important to ask whether the tethering of HOG1 in the nucleus by target transcription factors also operates in S.cerevisae. Recently, Khokhlatchev et al. (1998) have shown that the MAP kinase ERK2 also accumulates in the nucleus when phosphorylated, in a manner that does not require its enzymatic activity, and that this accumulation is driven in part by the homodimerization of ERK2.

In summary, we propose the following model for the regulation of HOG1 kinase activity. Activation of HOG1 by PBS2 in the cytoplasm is necessary but not sufficient for the stress response. Active HOG1 needs to be translocated into the nucleus, via a process that requires the nuclear transport factor NMD5, where it presumably phosphorylates specific proteins. These comprise, at the very least, transcription factors and the tyrosine phosphatases PTP2/3 that are activated by HOG1, and in turn serve to dephosphorylate and inactivate it. Because we find that HOG1 that is trapped in the nucleus in xpo1-1 cells becomes dephosphorylated on Tyr176 (data not shown), we propose that inactivation of HOG1 occurs before it is returned to the cytoplasm. These findings may be applicable to a range of proteins, including developmentally- and cell cycle-regulated protein kinases, whose transport into the nucleus may play a key role in the regulation of their biological activity. Elucidating the molecular mechanisms whereby HOG1 interacts with the nuclear

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**Table 1. The yeast strains used in this study**

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<td>T.Maeda and H.Saito</td>
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transport machinery will likely have important implications for the control of cell growth and differentiation.

Materials and methods

Strains

The yeast strains used in this study are listed in Table I. To create the ts(51-953) using a PCR-based knockout protocol (Baudin et al., 1993). The haploid strains JTY32 and JTY35 (both the gift of David Amberg, SUNY Syracuse) were transformed with the PCR product to create the DFCI/Novartis Drug Discovery program, and by NIH grants RO1 GM36373 (to P.A.S.) and GM50909/GM56699 (to H.S). P.F. was supported by a Long Term Fellowship from the HSFO, and F.P. by a postdoctoral fellowship from the Dirección General de Investigación Científica y Técnica of the Spanish Government.

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