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The role of phosphorylation in regulating the subcellular localization of the yeast transcription factor Pho4

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The Role of Phosphorylation in Regulating the Subcellular Localization of the Yeast Transcription Factor Pho4

by

Arie Kaffman

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

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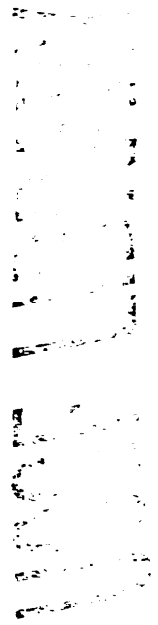
This thesis is dedicated to my parents for their unconditional support and to my wife Eve who helped me find the right balance.

Acknowledgments

I would like to thank my parents who accompanied my years in graduate school from a distance without always understanding the exact nature of my work, but always providing me with their love and support. I also want to thank my wife Eve for her patience, companionship, and her role in reminding me of other dimensions of life besides science. Special thanks to Erin O'Shea, my supervisor, teacher, and friend. I started to work with Erin in the summer of 1992 when she was a postdoctoral fellow in R. Tjian's lab and I was an undergraduate student at Berkeley. Erin taught me how to be a good bench-scientist, how to evaluate data critically, and how to combine creativity with "going for the experimental jugular". I want to thank Erin for all of this and more, because without her I would probably not be sitting here writing these lines. I also want to thank members of the O'Shea lab who made my years in graduate school such a fun and exciting experience. I am also grateful for the following scientific magazines: Science, Nature, Genes and Development and the Annual Review in Cell and Developmental Biology for providing me their permissions to reprint my work in this thesis.



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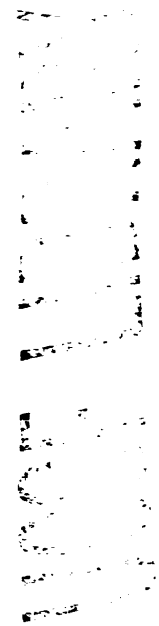

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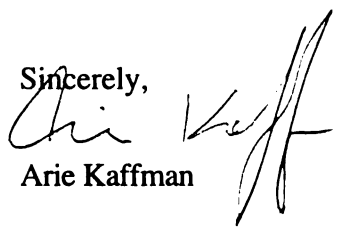

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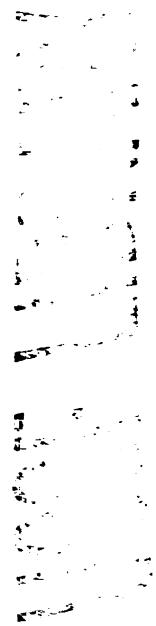

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The Role of Phosphorylation in Regulating the Subcellular Localization of the Yeast Transcription Factor Pho4

Arie Kaffman

Abstract

Budding yeast are able to monitor the environment for the presence of inorganic phosphate and to induce a transcriptional response that allows them to survive under conditions of limited phosphate availability. Induction of such phosphate-responsive genes requires the activity of the basic helix-loop-helix transcription factor Pho4. In conditions of high levels of phosphate in the medium, the cyclin-dependent kinase (Cdk)-cyclin complex Pho85-Pho80 is active and phosphorylates Pho4. Phosphorylation of Pho4 causes it to translocate from the nucleus to the cytoplasm thereby terminating transcription of phosphate-responsive genes. When phosphate is depleted from the medium the Pho85-Pho80 kinase activity is inhibited by the cyclin-dependent kinase inhibitor Pho81, and an as yet unidentified phosphatase dephosphorylates Pho4. Unphosphorylated Pho4 accumulates rapidly in the nucleus in low phosphate and induces expression of genes that help yeast to scavenge inorganic phosphate from the medium.

The goals of my thesis were (1) to identify the machinery responsible for the change in the subcellular localization of Pho4, and (2) to understand how phosphorylation of Pho4 regulates its subcellular localization. Here we show that Pse1/Kap121 is the nuclear-import receptor of Pho4 and that Msn5 is the nuclear-export receptor of Pho4. We also demonstrate that phosphorylation causes a change in the subcellular localization of Pho4 by enhancing its affinity for its export receptor and at the same time reducing its affinity for its import receptor. Thus, phosphorylation of Pho4 triggers its removal from the nucleus and also prevents its re-entry into the nucleus. We propose that such dual control of the export and the import rate of Pho4 provides an efficient and rapid way to control gene expression.

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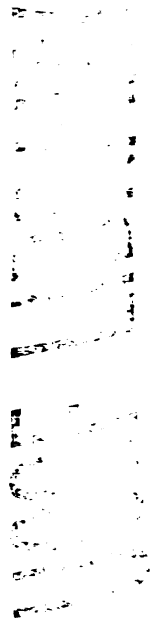
REGULATION OF NUCLEAR LOCALIZATION:

a Key to a Door*



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KEY WORDS: nuclear import, nuclear export, regulated nuclear localization, phosphorylation, review.

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CONTENTS

ABSTRACT

Information can be transferred between the nucleus and the cytoplasm by translocating macromolecules across the nuclear envelope. Communication of extracellular or intracellular changes to the nucleus frequently leads to a transcriptional response that allows cells to survive in a continuously changing environment. Eukaryotic cells have evolved ways to regulate this movement of macromolecules between the cytoplasm and the nucleus such that the transfer of information occurs only under conditions where a transcriptional response is required. This review focuses on the ways in which cells are able to regulate movement of proteins across the nuclear envelope and the significance of this regulation for controlling diverse biological processes.

INTRODUCTION

About this Review

Eukaryotic cells control many biological processes by regulating the movement of macromolecules into and out of the nucleus. Our understanding of the mechanism underlying nuclear transport has increased dramatically in the past five years, and many comprehensive reviews have been written to describe these advances (Corbett & Silver 1997, Gorlich & Mattaj 1996, Mattaj & Englmeier 1998, Moroianu 1998, Nigg 1997, Weis 1998). However, relatively little attention has been given to the impact that these advances have had on our understanding of the regulation of nuclear transport. Three purposes guide us in writing this review. First, we wish to describe several examples of biologically important problems in which control of nuclear translocation is an important

regulatory mechanism. Second, we want to emphasize common themes shared by many of these examples in an attempt to conceptualize the main issues involved in regulated translocation. Third, we want to re-examine some of the well-characterized examples of regulated translocation in light of new advances in the field. This review is not intended to be a comprehensive survey of regulated translocation [for other reviews see (Feldherr 1998, Jans et al 1998, Jans & Hubner 1996, Karin & Hunter 1995)], but rather a description of selected examples of biological problems, from which we wish to illustrate how regulated translocation is used to control a cellular response.

Nuclear Translocation, a Brief Review

All known transport between the nucleus and the cytoplasm occurs through the nuclear pore complex (NPC), a large structure spanning the nuclear envelope (Fabre & Hurt 1997, Feldherr & Akin 1990a). Proteins can enter the nucleus either by diffusion or by signal-mediated transport. The structural constraints of the NPC dictate that only proteins smaller than ~40 kD are able to enter the nucleus by passive diffusion (Davis 1995, Pante & Aebi 1996). However, many small proteins such as histones (Schwamborn et al 1998) are imported by a signal-mediated pathway, probably because signal-mediated import is more efficient and more amenable to regulation than diffusion.

Signal-mediated nuclear import requires energy, physiological temperature, a nuclear localization signal (NLS), and soluble transport machinery (see below). The import process can be described by three steps: (1) docking at the NPC; (2) translocation; and (3) nuclear deposition of the cargo (Fig 1a). During docking, cargo in the cytoplasm binds to a soluble import receptor known as importin, and the complex is then targeted to the NPC in a process that does not require physiological temperature or energy. Translocation, the movement of the importin-cargo complex through the NPC, is a poorly understood process that requires the small-GTPase Ran, GTP, and physiological temperature. Upon entering the nucleus the cargo-importin complex is dissociated by

binding of Ran loaded with GTP (Ran-GTP) to importin. This step concludes the nuclear import process - the cargo is deposited in the nucleus and importin can be recycled back to the cytoplasm for another round of transport [reviewed in (Mattaj & Englmeier 1998, Moroianu 1998, Weis 1998)].

Two of the best characterized import signals are the SV40 large T-antigen NLS (also known as the classical NLS) and the nucleoplasmin bipartite NLS. The classical NLS contains a stretch of basic amino acids, whereas the bipartite NLS is composed of two basic stretches separated by a ten amino acid spacer [for review see (Jans & Hubner 1996)]. A protein carrying the classical or the bipartite NLS is bound by a cytoplasmic receptor consisting of importin α and importin β. Importin α belongs to a divergent family of proteins that serve as adaptors able to bridge the binding between proteins carrying different NLS sequences (including the classical and the bipartite NLSs) and importin β (Mattaj & Englmeier 1998). Importin β targets the complex to the NPC, and the complex is then translocated into the nucleus and is dissociated by binding of Ran-GTP to importin β (Adam & Adam 1994, Adam & Gerace 1991, Adam et al 1989, Gorlich et al 1995a, Gorlich et al 1994, Gorlich et al 1995b, Moroianu et al 1995, Rexach & Blobel 1995, Weis et al 1995).

Protein export shares many similarities with protein import. A cargo bearing a nuclear export signal (NES) binds a soluble export receptor, known as exportin, in the nucleus. Unlike importins, which bind their cargoes in the absence of additional proteins, all known exportins bind their cargoes only in the presence of Ran-GTP (Fig 1b). The trimeric complex consisting of the cargo, exportin, and Ran-GTP is then rapidly translocated to the cytoplasm, where it dissociates upon Ran-GTP hydrolysis induced by the cytoplasmic Ran-GTPase activating protein (Ran-GAP) and its accessory protein RanBP1 [reviewed in (Gerace 1995, Mattaj & Englmeier 1998, Nakielny & Dreyfuss 1997, Wozniak et al 1998)]. The best characterized export pathway utilizes a leucine-rich NES, which is bound by the export receptor Crm1 in the presence of Ran-GTP in the

nucleus, and the trimeric complex is then exported to the cytoplasm (Fornerod et al 1997, Fukuda et al 1997a, Kudo et al 1997, Neville et al 1997, Ossareh-Nazari et al 1997, Stade et al 1997).

Recent data have suggested that the Ran-GTPase cycle plays an instrumental role in dictating the directionality of transport across the nuclear envelope (Gorlich et al 1996, Izaurralde et al 1997b). The cytoplasmic localization of the Ran-GAP (Hopper et al 1990, Mahajan et al 1997) and the nuclear localization of the guanine nucleotide exchange factor (Ran-GEF) (Ohtsubo et al 1989) predicts a high concentration of Ran-GTP in the nucleus and a low concentration of Ran-GTP in the cytoplasm (Gorlich 1997, Koepp & Silver 1996) (Fig 1a and Fig 1b). This gradient is thought to contribute to directionality by regulating the stability of the transport receptor-cargo complexes; importin-cargo complexes form in the cytoplasm and dissociate in the nucleus, whereas exportin-cargo-Ran-GTP complexes form in the nucleus and dissociate in the cytoplasm (see Fig 1a and Fig 1b).

In recent years it has become clear that multiple pathways exist for protein import and export. In budding yeast, a family of 13 proteins have been identified that share sequence homology with importin β (Fornerod et al 1997, Gorlich et al 1997). To date, seven importin β members have been shown to function as import receptors, whereas four others function as export receptors (Table 1). Even more importin β homologues are expected in mammalian cells. The reason for the existence of multiple pathways for nuclear import and export is currently unknown, and understanding the functional relevance of such diversity represents an important challenge for the field.

Recently, several import receptors have been shown to recognize and import more than one kind of NLS (Jakel & Gorlich 1998). The existence of multiple pathways for nuclear transport, some of which are able to transport cargoes using different localization signals, predicts that many protein sequences can function as an NLS or NES. Previously, a single stretch of basic amino acids on a protein was predicted to function as its NLS; however, as will be discussed later, this might not be the case. In addition, not all NLS

sequences containing a stretch of basic amino acids utilize the classical NLS pathway (Kaffman et al 1998b, Rout et al 1997, Schlenstedt et al 1997). The same is true for export; the absence of a leucine-rich NES, or the inability to block Crm1-mediated export, does not rule out the possibility that the protein is actively exported. Thus, it is currently impossible to determine the localization signal or the transport pathway responsible for the subcellular localization of a protein simply by looking at its primary amino acid sequence.

Issues Concerning Regulated Translocation

Four issues appear as recurrent themes in many of the examples we will discuss. These issues we believe represent some of the most important points to consider when studying regulated localization, and are therefore summarized below.

ASSESSING A CHANGE IN STEADY STATE LOCALIZATION OF A PROTEIN

Steady state localization of a protein is determined by its relative rates of nuclear import and export. If the rate of import exceeds the rate of export, the protein will be localized to the nucleus at steady state. In contrast, if the rate of export is greater than the rate of import, the protein will be localized to the cytoplasm. A change in the rate of import (Guiochon-Mantel et al 1996) and/or export (Kaffman et al 1998a) can lead to a shift in the steady-state localization of a protein. Before the elucidation of the mechanism by which proteins can be actively exported from the nucleus and the realization that this process can also be subject to regulation, regulated localization was assumed to be due to regulated import. However, with the recognition that both import and export can be subject to regulation, it has become essential to experimentally observe import in the absence of export (or vice versa) in order to determine which rate is subject to regulation [for some good experimental examples see (Beals et al 1997b, Klemm et al 1997, Lee et al 1996)].

SHUTTLING VS. NON-SHUTTLING PROTEINS Shuttling proteins continuously move from the cytoplasm to the nucleus and back. The steady-state localization of these proteins reflects a dynamic process of nuclear entry and exit. Two categories of shuttling proteins have been described. One class composed of carrier proteins, such as some heterogeneous nuclear ribonucleoproteins particles (hnRNP), presumably are exported to the cytoplasm bound to RNA, and are then re-imported into the nucleus for another round of transport (Lee et al 1996, Michael et al 1995). A second class of shuttling proteins is not thought to function as carriers, but instead uses shuttling as a way to regulate their activity. These proteins are localized to the cytoplasm at steady state because their export is more efficient than their import. Export of these proteins is blocked under conditions when the activity of these proteins is required in the nucleus (Yan et al 1998, Yang et al 1998). Maintaining such proteins in the cytoplasm apparently establishes a futile cycle, as they continuously move in and out of the nucleus. As discussed later, this mode of regulation might be important for coordinating nuclear and cytoplasmic events, and also offers a simple, reversible, and rapid mode for regulating nuclear activity.

INACTIVATION OF A SIGNAL Many signals that enter the nucleus require a mechanism for turning them off under the appropriate conditions. For example, a transcription factor that is translocated into the nucleus under a specific set of conditions has to be inactivated under conditions in which its activity is no longer required. Protein degradation (Tebb et al 1993), nuclear export (Kaffman et al 1998a), and ligand-dependent inactivation (Madan & DeFranco 1993), are mechanisms used to terminate such nuclear activity.

REDUNDANCY For many proteins, regulation of nuclear localization represents only one level in which their activity is controlled (Arenzana-Seisdedos et al 1997, Jin et al 1998, Komeili & O'Shea 1999, Moll et al 1991, Park et al 1995, Picard et al 1988). As

discussed below, this redundancy may serve to increase the robustness of these switches and it might also provide additional levels in which environmental and cellular inputs can be integrated [see (Ferrell 1998) for an interesting discussion].

The Use of Regulated Localization in Biological Systems

Cells utilize regulated nuclear localization in at least three different ways. First, cells can use regulated localization as a molecular switch to control transcription of genes, or to regulate transitions in the cell cycle. This is the most familiar way in which regulated localization is used, and most of the examples in this review demonstrate this type of regulation. Second, regulated localization can be used as a timing device by providing a delay between the synthesis of a protein and its ability to act in the nucleus. Such a delay in nuclear activity is thought to be important in producing robust oscillation in biological clocks (Van Gelder & Krasnow 1996). Third, regulated localization is used to ensure proper maturation of many biological activities. Some examples include the production of mRNA, tRNA, or the assembly of ribosomal particles (Izaurralde & Adam 1998). To ensure that a maturation step occurs prior to export, the export machinery transfers only mature particles to the cytoplasm. We examine the regulated export of tRNA as an example of this mode of regulated localization.

MECHANISMS OF REGULATED LOCALIZATION

Several steps in the movement of proteins across the nuclear envelope could be targeted for regulation. (1) The cargo itself can be modified in a way that affects its ability to bind the import or export receptor. (2) The cargo-receptor complex can be tethered to an insoluble cellular component, thereby preventing it from binding the NPC. (3) The activity of the soluble transport machinery can be regulated. (4) The NPC itself might be modified

in a way that affects its transport properties. As much more is known about regulating the cargo-receptor association it is discussed below in much more detail.

Regulating Cargo-Receptor Complex Formation

Regulating the affinity of the cargo for its receptor is achieved in most cases either by phosphorylation of the cargo, or by intermolecular association of the cargo with accessory proteins. These two modes of regulation are not mutually exclusive as they can be used sequentially to regulate nuclear localization (see MAPK below for an example). As described later, phosphorylation and intermolecular association with accessory proteins can either enhance or decrease the affinity of a cargo for its receptor.

PHOSPHORYLATION The regulated localization of Pho4, NF-AT, Swi5 and cyclin B illustrates how phosphorylation can affect the affinity of a cargo for its transport receptor. These examples are presented with some additional background information intended to provide biological context to emphasize the importance of regulated translocation in these processes.

Pho4 When budding yeast are grown in phosphate-rich medium, the CDK-cyclin complex Pho85-Pho80 is active (Schneider et al 1994) and phosphorylates the transcription factor Pho4 on five serine residues (O'Neill et al 1996) (Fig 2a), causing its rapid nuclear export (Kaffman et al 1998a). As cells deplete phosphate from the medium, the kinase activity of the Pho85-Pho80 complex is inhibited (Schneider et al 1994), and an unidentified phosphatase dephosphorylates Pho4. Dephosphorylation of Pho4 causes it to accumulate in the nucleus (O'Neill et al 1996) and to induce transcription of genes that allow yeast to grow under conditions of limited inorganic phosphate availability (Lenburg & O'Shea 1996, Oshima 1997).

Msn5, an importin β homologue (Fornerod et al 1997, Gorlich et al 1997), is the export receptor for Pho4; it is required for export of Pho4 in vivo and binds directly to Pho4 in vitro (Kaffman et al 1998a). Remarkably, Msn5 binds exclusively to phosphorylated Pho4 (Kaffman et al 1998a), providing an explanation for the selective export of phosphorylated Pho4 from the nucleus (Fig 2b). The Msn5-mediated export of Pho4 explains why Pho4 is exported even though it lacks a leucine-rich NES, and demonstrates that the absence of a leucine-rich NES on a protein does not rule out the possibility that it is actively exported. Phosphorylation of two serine residues on Pho4 is necessary and sufficient to promote its nuclear export in vivo, and to trigger binding to Msn5 in vitro (Komeili & O'Shea 1999) (Fig 2a). It is currently unknown how phosphorylation of Pho4 triggers its association with Msn5, but two models have been proposed (Kaffman et al 1998a). In the first model, a phosphorylation-induced conformational change in Pho4 exposes a binding site for Msn5. In the second model, Msn5 binds specifically to a phosphopeptide within Pho4. These two models are not mutually exclusive, as Msn5 may be able to recognize more than one binding site on Pho4.

Msn5 mediates nuclear export of other proteins such as Far1 (M Peter, personal communication), Msn2, Msn4 (C Schueller, personal communication), and Mig1 (M Johnston, personal communication). Interestingly, phosphorylation seems to be a prerequisite for nuclear export in all these cases (M Peter, C Schueller, and M Johnston, personal communication). As Msn5 is required for the phosphorylation-induced export of seemingly unrelated proteins and binds specifically to phosphorylated Pho4, it is tempting to speculate that Msn5 may be an export receptor dedicated to phosphorylated proteins. Such an export receptor might be recruited to phosphorylated proteins in a manner analogous to recruitment of SH2 domains to peptides containing phosphorylated tyrosines. The appeal of this model is that it provides a simple and general mechanism to explain how phosphorylation can promote nuclear export of a large group of proteins that share very little sequence homology.

Pho4 is imported into the nucleus by the importin β homologue Pse1/Kap121 (Fig 2c). Pse1 is required for Pho4 import in vivo and binds directly to Pho4 in vitro (Kaffman et al 1998b). The NLS of Pho4 contains a basic stretch of amino acids (Kaffman et al 1998b), demonstrating that a basic stretch of amino acids is not a unique feature of the classical import pathway. This observation raises the question of what information within the NLS determines the specificity of a cargo for a certain import pathway.

Phosphorylation of Pho4 reduces its affinity for Pse1 (Kaffman et al 1998b). Phosphorylation of a site within the Pho4 NLS (Fig 2a) is necessary and sufficient to reduce the affinity of Pho4 for Pse1 in vitro, and substitution of serine with aspartic acid at this site impedes import of Pho4 in vivo (Komeili & O'Shea 1999). Thus, our studies suggest that phosphorylation of Pho4 triggers its export and also blocks its reimport (Fig 2b). This dual regulation of import and export is not unique to Pho4 and has been reported for other proteins such as Msn2 (C Schueller, personal communication), NF-AT (Beals et al 1997a, Beals et al 1997b) and NF-kB (Arenzana-Seisdedos et al 1997, Henkel et al 1992).

Remarkably, phosphorylation of Pho4 also regulates its transcriptional activity. Phosphorylation of a site within a region of Pho4 required for association with the transcription factor Pho2 (Hirst et al 1994) (Fig 2a) prevents it from binding Pho2 (Komeili & O'Shea 1999). Pho2 is required for the transcriptional induction of several phosphate-responsive genes, including the gene encoding the secreted acid phosphatase *PHO5* (Oshima 1997) (Fig 2c). Phosphorylation of Pho4 on the serine in the Pho2 binding domain is sufficient to block *PHO5* expression in high phosphate conditions (Komeili & O'Shea 1999). This additional level of regulation explains why Pho4 mutants that cannot be exported do not activate expression of *PHO5* in high phosphate conditions.

In summary, the phosphorylation state of Pho4 dictates its subcellular localization by promoting its association with its export receptor and also by inhibiting its affinity for its import receptor. In addition, phosphorylation inhibits the Pho2-dependent transcriptional

activity of Pho4. The observation that regulation of either transcriptional activity or nuclear localization is sufficient to control the activity of Pho4 raises the question as to the need for such redundant modes of regulation. This redundancy is not unique to Pho4 [see (Beg & Baldwin 1993, Jin et al 1998, Park et al 1995, Picard et al 1988)] and therefore might represent an important aspect of regulating the activity of proteins. Redundancy provides tighter transcriptional control; Pho4 mutants with a defect in either localization or transcriptional regulation do not turn off *PHO5* expression as efficiently as wild-type Pho4 (Komeili & O'Shea 1999). In addition, localization might be important in regulating Pho4 transcription from promoters that do not require Pho2 for transcriptional activation.

NF-AT Phosphorylation of the transcription factor NF-AT is thought to affect its subcellular localization by modulating both its import and export rates. Stimulation of the T-cell receptor (TCR) leads to activation of a signal transduction pathway, resulting in the production of numerous cytokines and surface molecules which are required for a proper immune response [for review see (Crabtree & Clipstone 1994, Rao et al 1997)]. Stimulation of the TCR causes an elevation in cytosolic Ca^{2+} levels, which activate the phosphatase calcineurin, leading to dephosphorylation of the transcription factor NF-AT (Loh et al 1996a, Loh et al 1996b, Ruff & Leach 1995). A complex consisting of dephosphorylated NF-AT and calcineurin is then translocated to the nucleus, allowing for transcriptional induction of genes required for T-cell activation (Shibasaki et al 1996) (Fig 3b). Some of the most potent immunosuppressive drugs, such as cyclosporin A and FK506, act by inhibiting the ability of calcineurin to dephosphorylate NF-AT, thereby preventing the nuclear accumulation of NF-AT (Crabtree & Clipstone 1994).

Four members of the NF-AT family have been described: NF-AT1, NF-AT2/NF-ATc, NF-AT3, and NF-AT4 [using the nomenclature proposed in (Rao et al 1997)]. These proteins share high sequence similarity throughout their DNA binding domain, but reduced similarity (30-40% identity) in their NF-AT-homology region (NHR) (Luo et al 1996a).

The NHR domain is located at the N-terminus, is required to bind calcineurin, and is thought to play a major role in controlling the subcellular localization of these proteins (Fig 3a). The presence of several family members sharing limited sequence similarity complicates the analysis of the mechanism responsible for regulating their subcellular localization, because conflicting results can reflect differences between family members or modifications in the experimental system used. For example, two NLS sequences were identified for NA-FT2 (Beals et al 1997a), but only one NLS was identified for NF-AT1 (Luo et al 1996b), and NF-AT4 (F. McKeon, personal communication). In addition, one leucine-rich NES was identified for NF-AT2 (Klemm et al 1997), but two different NES sequences, located in the N-terminus, were recently reported for NF-AT4 (Zhu & McKeon 1999). Further studies are required to determine if conflicting results in the literature are due to different mechanisms used to regulate subcellular localization of different family members or due to differences in the experimental approaches used.

Studies of NF-AT2 suggest that phosphorylation inhibits the rate of import by inducing an intramolecular conformational change that makes its two NLS sequences inaccessible for binding by the import machinery (Beals et al 1997a) (Fig 3a and Fig 3b). The inactivation of both NLS sequences, one at the N-terminus and the other at the C-terminus, is required for the cytoplasmic localization of NF-AT2. In addition, fragments containing the C- and N-termini can bind in trans, suggesting that the two domains can interact in the intact protein. Moreover, binding of the C- and N-termini requires phosphorylation of the N-terminal fragment and a wild-type NLS sequence in the C-terminal fragment, suggesting that intramolecular association requires phosphorylation at the N-terminus and an intact NLS at the C-terminus (Beals et al 1997a). However, a more direct measure of NLS accessibility, such as the ability to bind its import receptor or accessibility to NLS-specific antibodies, is required to determine whether the two NLS sequences are inaccessible in phosphorylated NF-AT.

Two models have been proposed to describe how export of NF-AT is regulated. The first model proposes that phosphorylation enhances nuclear export of NF-AT, and that under inducing conditions calcineurin maintains NF-AT in its unphosphorylated state, leading to a decrease in its rate of export (Beals et al 1997b). The second model argues that direct binding of calcineurin to NF-AT prevents association of NF-AT with its export receptor, thereby decreasing the rate of the nuclear export of NF-AT (Zhu & McKeon 1999).

Three observations support the first model and suggest that phosphorylation of NF-AT enhances its rate of export. First, overexpression of glycogen synthase kinase 3 (GSK3), which phosphorylates NF-AT, enhances its rate of export and this enhancement requires multiple phosphorylation sites on NF-AT (Beals et al 1997b). Second, lithium chloride, which blocks GSK3 kinase activity, slows export of NF-AT in vivo (Klemm et al 1997). Third, a leucine-rich NES is required for rapid nuclear export of NF-AT2 in vivo (Klemm et al 1997), and nuclear export of NF-AT2 in vitro requires the export receptor Crm1 (Kehlenbach et al 1998). Interestingly, deletion of the leucine-rich NES slows export but does not abolish export of NF-AT2 in vivo (Klemm et al 1997), suggesting that NF-AT2 may also be exported in vivo by a mechanism that is independent of its leucine-rich NES. This NES-independent export seems to require phosphorylation since addition of lithium chloride abolishes export of NF-AT2 lacking its leucine-rich NES (Klemm et al 1997).

The second model in which binding of calcineurin blocks binding of the export receptor is supported by the following observations. First, when Ca^{2+} levels are lowered calcineurin is inactivated and presumably dissociates from NF-AT (Shibasaki et al 1996) (Fig 3b). Second, nuclear accumulation of NF-AT4 can be blocked by overexpressing the export receptor Crm1, and this inhibition of nuclear accumulation requires two NES sequences (NES1 and NES2) at the N-terminus of NF-AT4 (Zhu & McKeon 1999). Third, calcineurin and Crm1 bind NF-AT via these two NES sequences and binding of

Crm1 and calcineurin to NF-AT4 are mutually exclusive events (Zhu & McKeon 1999). Fourth, co-overexpression of activated calcineurin inhibits the cytoplasmic accumulation of NF-AT4 that is observed when Crm1 is expressed alone. Moreover, a catalytically inactive calcineurin mutant that can still bind NF-AT4 is also able to prevent the Crm1-mediated cytoplasmic localization of NF-AT4, indicating that the phosphatase activity of calcineurin is not required for this effect (Zhu & McKeon 1999). Based on these results, the authors proposed that direct binding and masking of NES sequences by calcineurin inhibits the association of NF-AT4 with its export receptor, leading to nuclear localization of NF-AT4 (Fig 3b). The appealing aspect of this model is that it provides a simple explanation for the observation that NF-AT and calcineurin are imported to the nucleus as a complex, and more generally provides a paradigm for how calcium-dependent association between two proteins can be used to regulate subcellular localization. However, it should be noted that all the above studies were done using overexpressed proteins and it is possible that under these conditions phosphorylation plays only a minor role in regulating the export of NF-AT.

Although NF-AT is used in this section to demonstrate how phosphorylation regulates its subcellular localization the exact role that phosphorylation plays in controlling the subcellular localization of NF-AT still needs to be determined.

Swi5 The regulated localization of the transcription factor Swi5 illustrates how cell cycle-dependent phosphorylation might be used to regulate association of a protein with its import receptor. Cell division in budding yeast is asymmetric, and gives rise to a large mother cell and a smaller daughter cell. Haploid budding yeast have two mating types, a and α, that are interchangeably switched in mother cells after each cell division. Mother cells are able to switch mating type because they are able to induce expression of the endonuclease HO which is required for mating-type switching; daughter cells are unable to induce HO and therefore unable to switch mating type (Herskowitz 1988, Nasmyth 1993).

The transcription factor Swi5 is required to induce expression of the endonuclease HO, and is therefore required for mating-type switching (Nasmyth 1993). Swi5 is expressed during S phase, mitosis, and G2 (Nasmyth et al 1987) and is restricted to the cytoplasm during these phases of the cell cycle (Nasmyth et al 1990). At the end of mitosis Swi5 translocates into the nuclei of both mother and daughter cells (Nasmyth et al 1990), but induces HO expression only in mother cells (Nasmyth 1983), presumably due to daughter-specific inhibition of its activity (Bobola et al 1996, Sil & Herskowitz 1996). Soon after its entry into the nucleus, Swi5 is rapidly degraded (Nasmyth et al 1990, Tebb et al 1993). This tight regulation of Swi5 activity ensures that HO expression, and mating type switching, occur only at a specific point during the cell cycle.

Swi5 has an NLS that resembles the bipartite NLS of nucleoplasmin, and a 50 amino acid peptide containing this NLS is sufficient to target a β-galactosidase fusion protein to the nucleus in a cell cycle-dependent manner (Moll et al 1991). Swi5 is phosphorylated in a cell cycle-dependent manner by the cyclin-dependent kinase Cdc28 (Moll et al 1991), which is the yeast homologue of mammalian Cdc2. Cdc28 phosphorylates Swi5 on three serines; two of these serines lie in close proximity to the NLS, and the third is more than 100 amino acids N-terminal to the NLS. Mutations in any two of the phosphorylation sites cause partial nuclear localization, and mutation of all three sites is required for full nuclear localization of Swi5 during G2 (Moll et al 1991). These data indicate that the effect of phosphorylation of these sites is cooperative and is required for the cytoplasmic localization of Swi5. Based on this data, it was proposed (Moll et al 1991) that phosphorylation of sites in the vicinity of the basic NLS prevents binding of the import machinery to Swi5, leading to its cytoplasmic localization. Reduction in the kinase activity of Cdc28, which occurs during exit from mitosis, allows for dephosphorylation and nuclear entry of Swi5.

Three issues are worth noting with regard to Swi5 regulated localization. First, although Swi5 regulated localization is considered a classic example for how

phosphorylation prevents binding of the import machinery to an NLS, it was never formally shown that phosphorylation reduces the affinity of Swi5 for its import receptor, nor has its import receptor been identified. Second, the cooperativity observed between the three phosphorylation sites in regulating the subcellular localization of Swi5 has not been explained. Third, HO expression is not induced in daughter cells, even when Swi5 is constitutively nuclear, due to other layers of HO transcriptional regulation [reviewed in (Amon 1996)]. This is another example where subcellular localization represents only one level for regulating protein function. The importance of Swi5 subcellular localization is that it provides a paradigm for how the subcellular localization of a protein is regulated by phosphorylation in a cell cycle-dependent manner.

Cyclin B The regulated localization of cyclin B illustrates how phosphorylation reduces the affinity of a shuttling protein for its export receptor, leading to its nuclear accumulation. Eukaryotic cells regulate entry into mitosis by modulating the activity of a mitosis-promoting factor (MPF). MPF is a kinase composed of two subunits, a cyclin-dependent kinase (CDK) and a cyclin, both of which are required for its kinase activity. In mammalian cells MPF consists of Cdc2 (the CDK) and cyclin B. The transition from G2 to mitosis is associated with an increase in kinase activity, presumably driving cellular changes such as chromosome condensation, nuclear envelope breakdown, and formation of a mitotic spindle. The kinase activity of MPF is tightly regulated by inhibitory phosphorylation on conserved residues of the CDK subunit. This regulation is thought to allow cells to enter mitosis only after they have completed DNA replication, or managed to repair DNA damage detected prior to mitosis [reviewed in (Lew & Kornbluth 1996, Morgan 1997)]. However, a Cdc2 mutant that cannot be phosphorylated on the conserved inhibitory phosphorylation sites does not cause premature entry into mitosis in mammalian cells, even though it exhibits high levels of kinase activity (Jin et al 1996). This data suggested that entry into mitosis might be regulated by an additional mechanism.

The observation that cyclin B undergoes rapid nuclear entry at the beginning of mitosis (Bailly et al 1992, Gallant & Nigg 1992, Lehner & O'Farrell 1990, Ookata et al 1992) suggested that MPF activity might also be regulated by subcellular localization. Cyclin B contains a leucine-rich NES required to maintain it in the cytoplasm during G2 (Hagting et al 1998, Toyoshima et al 1998, Yang et al 1998). Conditions that lead to constitutive nuclear localization of cyclin B, such as the addition of an extra NLS or inhibition of its nuclear export, cause premature mitosis only when the kinase activity of MPF is elevated at the same time (Jin et al 1998, Toyoshima et al 1998). Taken together, these observations suggest that the regulation of MPF kinase activity and the regulation of its subcellular localization both contribute to proper control of the G2-to-M transition.

Nuclear export of cyclin B is regulated in a cell cycle-dependent manner. The activity of the NES on cyclin B is negatively regulated by phosphorylation on four conserved serines adjacent to the NES. Xenopus cyclin B mutants that cannot be phosphorylated are localized to the cytoplasm and are unable to support oocyte maturation (Li et al 1995, Li et al 1997). This defect could be overcome by adding an additional NLS to cyclin B (Li et al 1997), suggesting that steady-state nuclear localization during mitosis is required for oocyte maturation. Mutants carrying glutamic acid substitutions that mimic phosphorylation are constitutively nuclear (Li et al 1997). Moreover, a physical interaction between cyclin B and its export receptor Crm1 has been demonstrated in Xenopus extracts (Yang et al 1998). Binding was abolished when glutamic acid was substituted for serine in cyclin B (Yang et al 1998), consistent with the idea that phosphorylation reduces the affinity of cyclin B for Crm1. As these binding experiments were done in an extract, it is not known whether additional proteins present in the extract mediate the interaction between Crm1 and cyclin B. Thus, at the onset of mitosis, phosphorylation of serines in the vicinity of the NES of cyclin B decreases its affinity for the export receptor Crm1, thereby leading to its nuclear accumulation.

Cyclin B does not appear to have a classical NLS and recent data have shown that it can bind importin β directly, even in the absence of importin α (Moore et al 1999). Moreover, importin β is sufficient to drive cyclin B import in vitro (Moore et al 1999). These results demonstrate that many sequences can possibly serve as NLSs and that inspection of the primary sequence of a protein cannot reveal the import pathway used by the protein to enter the nucleus. The identification of importin β as the import receptor of cyclin B should help in determining whether the import rate of cyclin B is also regulated and how this regulation is achieved. Interestingly, cyclin B seems to bind importin β at a different site than the site bound by importin α (Moore et al 1999). A similar mode of binding has been reported for several ribosomal proteins (Jakel & Gorlich 1998), suggesting that a single importin might be able to bind and transport more than one substrate in each round of translocation.

Cyclin B represents another example of how phosphorylation of a cargo affects its binding to the translocation machinery, leading to a change in its subcellular localization. Two points are worth noting with regard to cyclin B localization: (1) The ability of MPF to induce mitosis is regulated by controlling its kinase activity and subcellular localization (Jin et al 1998, Toyoshima et al 1998), illustrating again that subcellular localization represents only one of several levels of regulation. (2) During interphase, cyclin B shuttles continuously between the cytoplasm and the nucleus and its cytoplasmic steady-state localization is due to very efficient export mediated by Crm1. Cyclin B accumulates in the nucleus if its NES is mutated (Hagting et al 1998, Li et al 1997, Toyoshima et al 1998, Yang et al 1998) or if cells are treated with leptomycin B, an inhibitor of Crm1-mediated export (Hagting et al 1998, Toyoshima et al 1998, Yang et al 1998). These observations indicate that a block in export of cyclin B is sufficient to cause its nuclear accumulation. It was therefore proposed (Yang et al 1998) that the shuttling of cyclin B between the nucleus and the cytoplasm might serve to coordinate nuclear events, such as DNA replication, with cytoplasmic processes such as the assembly of mitotic spindle.

COMPLEX FORMATION Another way in which nuclear localization can be regulated is through intra- and intermolecular interactions. Association of the cargo with itself (ERK2) (Khokhlatchev et al 1998), with an RNA (Mei2) (Yamashita et al 1998), or with other proteins [NF-kB (Baeuerle & Henkel 1994), GR (Guiochon-Mantel et al 1996), PKA (Fantozzi et al 1994)] are described in this chapter to illustrate how complex formation can influence the ability of the cargo to bind to the soluble transport machinery. In some cases, complex formation is required for nuclear targeting, whereas in others, complex disassembly is required for nuclear localization. Complex formation or disassembly can be regulated in many different ways, such as by phosphorylation-dependent degradation (Piette et al 1997), or by binding to a ligand (Picard & Yamamoto 1987).

MAPK Numerous signal transduction cascades transduce signals from the plasma membrane through the cytoplasm and into the nucleus (Karin & Hunter 1995). Many signaling pathways utilize a three-tiered kinase cascade involving a mitogen-activated protein kinase (MAPK). MAPK is activated in the cytoplasm by phosphorylation on conserved threonine and tyrosine residues, causing it to translocate into the nucleus. A block in translocation of the activated MAPK leads to a defect in signal transduction (Brunet et al 1999, Dickens et al 1997), which argues that translocation is required for proper signal transduction. In the nucleus, activated MAPK phosphorylates downstream targets to induce the appropriate transcriptional response (Lewis et al 1998, Robinson & Cobb 1997). MAPK is activated by a cytoplasmic kinase, referred to as MEK. The cytoplasmic localization of MEK requires a leucine-rich NES at its N-terminus (Fukuda et al 1996). MEK in turn is phosphorylated and activated by a membrane-associated kinase, referred to as MEKK [for reviews (Lewis et al 1998, Robinson & Cobb 1997)] (Fig 4).

The extended family of MAPKs is divided into two large sub-families. The extracellular signal-regulated kinases (ERKS), which include ERK1 and ERK2, are

involved mainly in cell proliferation and differentiation. The stress-activated protein kinases (SAPK), which include p38 and the Jun N-terminal kinase (JNK), are activated in response to cellular stresses such as UV irradiation, heat, and high osmolarity (Lewis et al 1998, Robinson & Cobb 1997).

Much progress has been made recently towards understanding the mechanism by which activation of the MAPK leads to its rapid nuclear localization. Studies using yeast have shown that for the p38 homologues, Hog1 in budding yeast and Spc1 in fission yeast, phosphorylation on the activating tyrosine and threonine residues is necessary for nuclear translocation; mutants that cannot be phosphorylated on these sites are unable to enter the nucleus (Ferrigno et al 1998, Gaits et al 1998). However, the kinase activity of MAPK is not required, as mutants lacking kinase activity are translocated to the nucleus (Ferrigno et al 1998, Khokhlatchev et al 1998). Phosphorylation of ERK2 induces it to form homodimers, and phosphorylated ERK2 mutants that fail to dimerize do not enter the nucleus (Khokhlatchev et al 1998). Based on these results it was proposed that phosphorylation of ERK2 triggers its dimerization, which is required for its nuclear localization (Fig 4).

Several mechanisms have been proposed to explain the phosphorylation-induced nuclear translocation of MAPK including: (1) release from a cytoplasmic anchor, thought to be MEK; (2) enhancement of import; and (3) inhibition of export. Data consistent with the release from MEK comes from studies in mammalian cells where overexpression of the Xenopus MAPK leads to its nuclear translocation, but coexpression of Xenopus MEK maintains it in the cytoplasm (Fukuda et al 1997b). Moreover, a 60 amino-acid region from MEK containing the MAPK binding site and the NES is necessary and sufficient to maintain overexpressed MAPK in the cytoplasm (Fukuda et al 1997b). Additionally, phosphorylation of MAPK decreases its affinity for MEK (Fukuda et al 1997b, Gaits et al 1998).

Phosphorylation may regulate other MAPKs by a different mechanism since the ability to localize MAPK to the cytoplasm seems specific for this MEK-MAPK pair; other unrelated MEKs were not able to retain the Xenopus MAPK in the cytoplasm (Fukuda et al 1997b). In addition, phosphorylation of Spc1 in fission yeast reduces its affinity for Wis1 (Gaits et al 1998) (its MEK), but a deletion of Wis1 did not result in nuclear localization of Spc1 (Gaits et al 1998), suggesting that binding to Wis1 cannot be the sole determinant of Spc1 cytoplasmic localization.

The mechanism of MAPK nuclear entry has been puzzling because no sequences resembling NLSs have been identified. The recent discovery that Hog1, the p38 homologue in budding yeast, requires the small GTPase Ran and the importin β homologue Nmd5 for its nuclear import may help to explain why MAPK does not have a classical NLS (Ferrigno et al 1998). Biochemical studies of Hog1 and Nmd5 should help establish if Nmd5 is the import receptor for Hog1 by determining whether Nmd5 binds Hog1 directly and whether phosphorylation affects the ability of the two proteins to interact.

Support for the role of phosphorylation in inhibiting the rate of export of MAPK comes from two observations. First, microinjection studies indicate that phosphorylated ERK2 is exported more slowly than unphosphorylated ERK2 (Khokhlatchev et al 1998). Second, activation of Hog1 causes its transient entry into the nucleus followed by its redistribution back to the cytoplasm. Nuclear localization of MAPK and its length of stay in the nucleus correlate with its phosphorylation state; phosphorylated protein is maintained in the nucleus, whereas dephosphorylated protein is exported (Ferrigno et al 1998, Gaits et al 1998). Dephosphorylation of Hog1 is mediated by the phosphatases Ptp2 and Ptp3 (Jacoby et al 1997, Wurgler-Murphy et al 1997), which are activated by Hog1. Entry into the nucleus of a kinase-defective Hog1 follows the same kinetics as wild-type protein, but its rate of dephosphorylation and export are much slower (Ferrigno et al 1998). These results are consistent with a model in which Hog1 activates its own dephosphorylation,

which is required for its export. The recent observation that the export receptor Crm1 is required for the export of Hog1 from the nucleus (Ferrigno et al 1998) should help in determining whether phosphorylation plays a role in the nuclear export of MAPK.

In summary, activation of MAPK allows the translocation of a signal originating at the plasma membrane into the nucleus. Phosphorylation of MAPK accomplishes three tasks: (1) it turns it into an active kinase (Ahn et al 1992); (2) it promotes dissociation from the cytoplasmic MEK (Fukuda et al 1997b, Gaits et al 1998); and (3) it promotes dimerization which is required for its nuclear localization (Khokhlatchev et al 1998) (Fig 4). Phosphorylation-induced dimerization followed by nuclear translocation is not a unique feature of MAPK and is seen also in a large family of transcription factors known as signal transducers and activators of transcription (STATs) (Ihle 1996, Sekimoto et al 1997). An understanding of the mechanism by which phosphorylation and dimerization allow nuclear translocation of MAPK requires more studies, but it clearly represents an important way to control the subcellular localization of numerous proteins.

NF-kB Localization of NF-kB is regulated by controlling its association with the transport machinery using intermolecular association. NF-kB is a heterodimeric transcription factor composed of the Rel A (p65) and the p50 subunits. NF-kB belongs to an evolutionarily-conserved family of transcriptional activators that also includes the mammalian Rel A oncogene and the Dorsal protein from Drosophila. NF-kB is activated by numerous stimuli, enabling it to induce transcription of a large number of genes involved in the immune response. The swiftness with which NF-kB responds to a vast number of environmental stimuli, and its ability to activate a large array of genes, is thought to demand tight control over its transcriptional activity [for reviews (Baeuerle & Henkel 1994, Baldwin 1996, Beg & Baldwin 1993, Piette et al 1997, Siebenlist et al 1994)]. This is achieved by maintaining NF-kB in an inactive form in the cytoplasm in a stable complex with the small NF-kB inhibitor, IkB. IkB inactivates NF-kB by restricting its access to the

nucleus (Arenzana-Seisdedos et al 1997, Beg et al 1992, Henkel et al 1992, Zabel et al 1993) and by inhibiting its DNA binding activity (Baeuerle & Baltimore 1988a, Baeuerle & Baltimore 1988b). Cytoplasmic localization of NF-kB is accomplished by the ability of IkB to both block import (Beg et al 1992, Henkel et al 1992, Zabel et al 1993) and to promote export (Arenzana-Seisdedos et al 1997). Different environmental stimuli activate a kinase that phosphorylates IkB on two serine residues and targets it for rapid degradation [(Rothwarf et al 1998, Woronicz et al 1997, Zandi et al 1997) and references therein]. Degradation of IkB allows for the rapid translocation of NF-kB into the nucleus and the onset of a transcriptional response.

The ability of IkB to block import has been demonstrated by showing that antibodies directed specifically against the NLS of Rel A and p50 fail to recognize the complex when IkB is bound, but are able to recognize NF-kB in its absence (Zabel et al 1993). In addition, mutations in the NLS of the Rel A subunit abolish its ability to bind IkB (Ganchi et al 1992), consistent with a model in which IkB binding masks the NLS on Rel A. Recently two independent studies have reported the crystal structure of IkB bound to NF-kB (Huxford et al 1998, Jacobs & Harrison 1998). The structure shows that the NLS of the Rel A (p65) subunit is induced to form an α helix when bound by IkB, allowing several of its residues to make contact with IkB (Jacobs & Harrison 1998). Since the classical NLS binds importin α in its extended conformation (Conti et al 1998), such a change in the secondary structure of the NLS might be sufficient to prevent its recognition by the import receptor. Details of the mechanism by which the p50 NLS is masked cannot be determined from the structure.

NF-kB bound by IkB is unable to bind DNA (Baeuerle & Baltimore 1988a, Baeuerle & Baltimore 1988b), and addition of IkB to NF-kB complexed with DNA results in dissociation of the NF-kB-DNA complex (Zabel & Baeuerle 1990), indicating that DNA and IkB binding to NF-kB are mutually exclusive. Additionally, it was shown that IkB contains a leucine-rich NES and that microinjection of IkB into the nucleus enhances NF-

kB export (Arenzana-Seisdedos et al 1997). IkB transcription is induced by NF-kB and new protein synthesis is required for inactivation of NF-kB (Baeuerle et al 1988, Sun et al 1993). Thus, IkB seems to play an important role in terminating the NF-kB transcriptional response by triggering dissociation of NF-kB from DNA and promoting its nuclear export.

In a manner similar to MAPK, nuclear localization of NF-kB is controlled by regulating its association with other proteins (IkB), which in turn controls its access to the soluble transport machinery. The ability of an accessory protein like IkB to control several activities of a transcription factor makes it an efficient molecular switch; such a tight switch may be important for controlling potent transcription factors such as NF-kB. Since IkB is degraded upon activation of NF-kB, it must be resynthesized to turn off the transcriptional activity of NF-kB. This requirement for new protein synthesis makes NF-kB inactivation rather slow.

The NF-kB related protein Dorsal is held in the cytoplasm by Cactus which shares high homology to other IkB family members (Siebenlist et al 1994). The rate of Dorsal entry into nuclei is also controlled by phosphorylation of a site in the vicinity of the NLS. Phosphorylation of this site has been shown to enhance the rate of Dorsal nuclear import in vivo and in vitro by increasing the affinity for its import receptor (Briggs et al 1998). Moreover, flies carrying a mutant Dorsal protein that cannot be phosphorylated on this site give rise to embryos with a defect in dorso-ventral axis formation (Briggs et al 1998), suggesting that phosphorylation of this site plays an important role in the proper development of Drosophila embryos. The regulated nuclear translocation of Dorsal demonstrates how multiple inputs, such as unmasking of the NLS mediated by degradation of Cactus and phosphorylation-induced import, might be integrated to regulate this process.

Mei2 The Mei2 protein must bind a small RNA in order to enter the nucleus and induce meiosis (Yamashita et al 1998). This observation suggests that complex formation between

the RNA and the protein plays an important role in regulating the nuclear localization of Mei2.

Diploid cells in fission yeast switch from a mitotic cell cycle to meiosis when starved for nitrogen (Yamamoto 1996). This transition is regulated by the RNA binding protein Mei2, which is required for both meiosis I and premeiotic DNA synthesis (Beach et al 1985, Bresch et al 1968, Shimoda et al 1987, Watanabe & Yamamoto 1994). Mei2 entry into the nucleus at the beginning of meiosis is required for cells to undergo meiosis (Watanabe et al 1997, Yamashita et al 1998). For Mei2 to enter the nucleus it must bind a small polyadenylated RNA, named mei-RNA (Yamashita et al 1998). Cells lacking mei-RNA do not support nuclear entry of Mei2 and cannot enter meiosis (Yamashita et al 1998). However, addition of an NLS to Mei2 allows cells to enter meiosis even in the absence of mei-RNA (Yamashita et al 1998), suggesting that the sole function of mei-RNA in initiating meiosis is to support nuclear entry of Mei2. In addition, Mei2 mutants that are unable to bind mei-RNA cannot enter the nucleus, arguing that the RNA binding activity of Mei2 is required for its import (Yamashita et al 1998). Finally, mei-RNA requires Mei2 protein for its own nuclear import, suggesting that the two enter the nucleus as a complex (Yamashita et al 1998). However, complex formation is probably insufficient for nuclear entry, since in haploid cells starved for nitrogen levels of both Mei2 protein and mei-RNA are elevated, but both are maintained in the cytoplasm (Yamashita et al 1998). Cytoplasmic localization of Mei2 and mei-RNA in haploid cells starved for nitrogen requires phosphorylation of Mei2, since a mutant Mei2 that cannot be phosphorylated is nuclear and activates meiosis in starved haploid cells (Watanabe et al 1997).

Taken together, the data are consistent with a model in which both dephosphorylation of Mei2 and complex formation between Mei2 and mei-RNA control the ability of Mei2 to bind the transport machinery, lead to its nuclear accumulation, and induce meiosis. Since phosphorylation does not seem to affect Mei2 binding to mei-RNA (Watanabe et al 1997), the mechanism by which phosphorylation regulates the subcellular

localization of Mei2 is unclear. It will be interesting to determine if phosphorylation of Mei2 and its association with mei-RNA regulate their nuclear accumulation by controlling association of the complex with an import receptor and/or export receptor.

In summary, regulated nuclear entry of Mei2 is yet another example in which complex formation is required for nuclear entry. The unique feature in Mei2 regulated import is that the complex is composed of RNA and a protein. Obligatory complex formation between RNA and proteins prior to movement across the nuclear envelope is also observed in nuclear import and export of certain ribonucleoproteins particles (RNPs) (Izaurralde et al 1997a, Lee et al 1996, Mattaj & De Robertis 1985), and thus may represent an ancient mechanism utilized by cells to regulate transport through the nuclear pore.

Period and Timeless The regulated nuclear entry of Period and Timeless represents a unique example of how regulated nuclear localization is used by cells to construct biological clocks. The requirement of the two proteins to form a complex prior to their movement into the nucleus serves as another example of how intermolecular association can be used to regulate nuclear entry.

Circadian rhythms are daily changes observed in the behavior and physiology of many organisms. These changes are controlled by internal clocks, which share a central component known as the oscillator. The oscillator is composed of a protein or a protein complex that oscillates with the same daily periodicity, and whose oscillation is required for the formation of daily rhythms. All oscillatory systems studied to date are characterized by a transcriptional negative feedback loop, where the oscillator protein shuts off transcription of its own mRNA [for reviews (Dunlap 1996, Van Gelder & Krasnow 1996, Young 1998)]. However, oscillation of such a simple negative feedback loop will dampen over time until they stop altogether (Goodwin 1963, Leloup & Goldbeter 1998, Van Gelder & Krasnow 1996). Biological clocks circumvent this problem by introducing a delay

between RNA accumulation and the transcriptional inhibition caused by the oscillator protein (Curtin et al 1995, Van Gelder & Krasnow 1996).

The Period (Per) and the Timeless (Tim) proteins are required for circadian rhythms in Drosophila, and have been proposed to form the functional subunits of the Drosophila oscillator (Young 1998). The delay between Per and Tim synthesis and their ability to repress their own transcription is thought to occur by regulating the nuclear translocation of Per and Tim (Fig 5). The Per protein accumulates in the cytoplasm several hours before it is triggered to enter the nucleus (Curtin et al 1995), and its nuclear entry requires the presence of Tim (Vosshall et al 1994). In addition, mutations in Per that lead to a longer period of oscillation have reduced affinity for Tim (Gekakis et al 1995, Huang et al 1995) and enter the nucleus at later time points (Curtin et al 1995), suggesting that the affinity of Per for Tim determines the rate of nuclear entry and the period length.

Several studies have provided insight into the mechanism of Per and Tim regulated localization. The presence of both proteins is required for nuclear entry, since expression of each protein alone leads to its cytoplasmic accumulation (Hunter-Ensor et al 1996, Saez & Young 1996, Vosshall et al 1994). Deletion analysis revealed a domain in each of these proteins that is responsible for their cytoplasmic localization; deletion of this cytoplasmic localization domain (CLD) leads to nuclear accumulation of Per and Tim even when each is expressed alone (Saez & Young 1996). These data suggest that the CLD on each protein is required for cytoplasmic localization, and that coexpression of the two proteins relieves the block in nuclear accumulation caused by these domains. The CLD on Per has been shown to bind Tim (Saez & Young 1996), suggesting that complex formation might mask the CLD on Per, leading to nuclear localization of the complex (Fig 5). Interestingly, nuclear accumulation of the coexpressed Per-Tim complex is slower compared to nuclear import of an individually expressed, CLD-deleted protein (Saez & Young 1996). This observation is consistent with the idea that the slow formation of a productive complex which is capable of entering the nucleus provides a delay necessary to establish robust oscillation. The

mechanism by which the CLD causes cytoplasmic localization requires further studies, but at least for Per might not occur via active export, since exposure to light causes rapid degradation of Tim but is not associated with rapid export of Per from the nucleus (Hunter-Ensor et al 1996, Zeng et al 1996).

Recently *DOUBLETIME* was identified as another gene required for circadian rhythms in Drosophila (Price et al 1998). Doubletime (Dbt) is essential for viability (Price et al 1998), and shares homology with casein kinase epsilon (Kloss et al 1998). Dbt binds Per (Kloss et al 1998) and is required for its phosphorylation (Price et al 1998). Phosphorylation of Per is thought to destablize it (Price et al 1998), providing a delay in its cytoplasmic accumulation and subsequent nuclear entry (Fig 5). Larva lacking Dbt activity localize hypophosphorylated Per constitutively to the nucleus and do not show oscillation in Tim or Per RNA (Price et al 1998), consistent with the notion that such a delay is an essential component for establishing robust oscillation.

The regulated nuclear entry of Per and Tim provides another example of how complex formation is used to regulate nuclear localization. Regulated localization in this case provides a delay between the synthesis of the oscillator protein and its ability to repress its own transcription, thought to be important for proper functioning of biological clocks.

PKA Association of the catalytic subunit of the cyclic AMP dependent protein kinase holoenzyme (PKA) with accessory proteins determines its ability to translocate into the nucleus. The regulated localization of the catalytic subunit is described here because much is known about this system, and the regulated localization of the catalytic subunit is thought to play an important role in the formation of long-term memory.

PKA is a quaternary complex composed of two regulatory (R) and two catalytic subunits (C) [for a review see (Taylor et al 1990)]. PKA activity is required for many cellular processes, but appears to play a central role in coordinating cell growth and

metabolism in response to a variety of environmental stimuli. The kinase activity of C is inhibited when bound to R in the holoenzyme. Additionally, R restricts localization of C in two ways. First, the quaternary complex is too big to diffuse into the nucleus (Adams et al 1991, Fantozzi et al 1994, Nigg et al 1985). Second, the regulatory subunit is tethered and targeted to different subcellular domains by binding to a family of proteins known as protein A kinase anchoring proteins (AKAPs) (Lester & Scott 1997). AKAP localizes PKA near its downstream targets, which seems to increase the specificity and efficiency of PKA-mediated kinase activity (Lester et al 1997, Lester & Scott 1997). An increase in cyclic-AMP (cAMP) levels in the cytoplasm leads to dissociation of C from R, allowing for the formation of an active kinase (Taylor et al 1990). Free C is small enough (~40 kD) to diffuse into the nucleus (Harootunian et al 1993, Nigg et al 1985) and is able to phosphorylate downstream targets such as cyclic-AMP responsive element binding protein (CREBP) (Karin & Hunter 1995). Diffusion-mediated nuclear entry is uncommon among examples of regulated nuclear translocation, probably because it is slow compared to signal-mediated nuclear entry and more difficult to regulate. Nuclear C can be inactivated in a cAMP-dependent or -independent manner. When cAMP levels decline the catalytic subunit, which continuously diffuses in and out of the nucleus, is trapped in the cytoplasm because of its high affinity for R (Fantozzi et al 1994). However, such an inactivation mechanism is rather slow. C can also be actively exported from the nucleus by binding to the small thermostable protein kinase inhibitor, PKI (Wen et al 1995). Binding to PKI inactivates the kinase activity of C and exposes a leucine-rich NES on PKI (Wen et al 1995). The leucine-rich NES is bound by Crm1 leading to the active export of the C-PKI complex (Fornerod et al 1997, Wen et al 1995).

The biological role of PKI in regulating the activity of PKA is not fully understood, but the low levels of nuclear PKI are thought to provide a threshold which ensures that only elevated cAMP levels will cause nuclear entry of C (Fantozzi et al 1994). This allows

low levels of cytoplasmic cAMP to activate C in the cytoplasm, and high levels of cAMP to activate it in the nucleus and the cytoplasm.

Regulation of the nuclear localization of C seems to play an important role in long-term facilitation (LTF) (Bacskai et al 1993). LTF is thought to represent a cellular response equivalent to the formation of a long-term memory at the organismal level (Abel & Kandel 1998). LTF is elicited by a strong stimulus of serotonin which is required for sufficient elevation of cAMP levels, leading to nuclear translocation of the catalytic subunit of PKA (Bacskai et al 1993). Nuclear translocation of C allows it to phosphorylate the nuclear-localized CREBP, leading to transcriptional activation of genes required for LTF (Abel & Kandel 1998). Recently, it has been shown that phosphorylation of CREBP is necessary and sufficient for LTF (Bartsch et al 1998). These data suggest that controlling the ability of the catalytic subunit to diffuse and enter the nucleus may be a key event responsible for LTF and long-term memory formation.

In summary, nuclear entry of the catalytic subunit of PKA is determined by its association with other proteins such as the regulatory subunit and PKI. Association of C with the regulatory subunit is determined by the cytoplasmic level of cAMP, which in turn is controlled by different environmental stimuli.

Glucocorticoid Receptor Regulated localization of the glucocorticoid receptor (GR) illustrates how ligand binding allows a cargo to bind its import receptor, presumably by disrupting association of the cargo with accessory proteins. GR belongs to the steroid hormone receptor family, and is responsible for the coordination and regulation of cellular responses such as stress and metabolic homeostasis. In unstimulated cells GR is inactive in the cytoplasm and is complexed with a number of proteins such as Hsp90 and the immunophilin Hsp56. Binding of these proteins accomplishes three tasks important for the regulation of GR: (1) restricting GR localization to the cytoplasm; (2) inhibiting its DNA binding activity; and (3) maintaining GR in a state of high affinity for its ligand. Binding

of GR to its ligand causes the dissociation of these accessory proteins from GR, allowing for rapid nuclear translocation and the activation of gene expression [for reviews (Guiochon-Mantel et al 1996, Mangelsdorf et al 1995, Tsai & O'Malley 1994)]. Upon ligand withdrawal, the ligand is released from GR causing dissociation of GR from chromatin (Reik et al 1991), thereby terminating the ligand-induced transcriptional response. GR is exported back to the cytoplasm in a process that is currently poorly understood (Madan & DeFranco 1993, Savory et al 1999, Yang et al 1997). The slow movement of the ligand-free GR from the nucleus to the cytoplasm (Sackey et al 1996) suggests that the steady-state cytoplasmic localization of ligand-free GR, in unstimulated cells, is probably not due to rapid export.

GR contains two NLS sequences that differ in their import properties. The central region of GR contains the DNA binding domain adjacent to a functional NLS (NL1), whereas the ligand binding domain (LBD) is found at the C-terminus and is equipped with a second NLS (NL2) (Picard & Yamamoto 1987) (Fig 6a). The two NLS sequences behave differently with respect to their ligand-dependent activities. NL1 resembles a bipartite NLS, and is required for the rapid nuclear localization of GR induced by association of GR with a ligand (Savory et al 1999). Fusion of a heterologous protein to NL1 leads to constitutive nuclear localization that is ligand-independent (Picard & Yamamoto 1987). In addition, importin α binds GR and this binding requires NL1, suggesting that NL1 directs import of GR via the classical import pathway (Savory et al 1999). The observations that no sequence resembling a classical NLS can be identified in the LBD and that NL1 is required for importin α to bind GR suggest that the import activity of NL2 is not mediated by the classical import pathway. Interestingly, NL2 acts as a ligand-dependent NLS (Picard & Yamamoto 1987); GR with mutations in NL1 translocates into the nucleus only in the presence of agonist, even though both agonist and antagonist ligands displace Hsp90 from the LBD (Savory et al 1999). These observations suggest

that binding of agonist, but not antagonist, causes a conformational change in the LBD that activates NL2, leading to import that is not mediated by importin α.

The mechanism proposed for maintaining GR in the cytoplasm is controversial. Several studies indicate that import of GR is regulated by masking its NLS. Removal of the LBD renders GR constitutively nuclear even in the absence of a ligand (Picard & Yamamoto 1987), suggesting that the presence of the LBD inhibits the activity of NL1 in the ligand-free GR. In addition, binding of a ligand causes the dissociation of Hsp90 from this domain (Scherrer et al 1993), and this release correlates with the accessibility of NL1 to NL1-specific antibodies (Scherrer et al 1993, Urda et al 1989). Based on these studies it has been proposed that binding of ligand to the LBD unmasks NL1 triggering import of GR (Picard & Yamamoto 1987, Scherrer et al 1993, Urda et al 1989) (Fig 6b). However, in vitro binding assays demonstrate that the affinity of importin α for GR is similar in the presence or the absence of a ligand, indicating that binding of importin α to GR may not be inhibited by the presence of Hsp90 (Savory et al 1999). Based on their results the authors proposed that a mechanism distinct from masking of the NLS is responsible for the cytoplasmic localization of GR. Further studies are required to establish whether importin α is required for the rapid import of GR, and to determine whether association between GR and importin α is regulated by a ligand in vivo. If NLS-masking is not a major determinant in maintaining GR in the cytoplasm, the mechanism responsible for its subcellular localization needs to be identified.

GR is a good example of how nuclear localization can be regulated in response to ligand binding. To achieve this, accessory proteins associate with GR and maintain it in the cytoplasm in a state that cannot bind DNA but that has high-affinity for its ligand. Binding the ligand allows GR to enter the nucleus and to bind DNA, demonstrating again that regulated nuclear localization represents only one level for controlling the activity of a transcription factor.

OTHERS The two categories presented so far demonstrate how phosphorylation and complex formation regulate localization of several proteins. Two interesting examples which do not fit well within the above two categories are grouped together in this chapter. We hope that this chapter will serve as a reminder of the diverse ways in which cargo-receptor interactions can be regulated.

Export of tRNA Most RNAs are transcribed and processed in the nucleus and then exported to the cytoplasm. Rel Aatively little is known about the mechanism by which RNA is exported from the nucleus, and even less is known about how properly matured RNA is selectively exported from the nucleus (Lee & Silver 1997, Nakielny et al 1997). Recent progress in studying the export of transfer RNA (tRNA) has begun to shed some light on this process.

Transfer RNA is synthesized in the nucleus as a precursor, where it undergoes several maturation steps prior to export, including 5' and 3' end processing (Deutscher 1995), and in some cases intron removal (Westaway & Abelson 1995). Only mature tRNA is exported to the cytoplasm (Wolin & Matera 1999), as the presence of unprocessed tRNA in the cytoplasm is thought to impair translation.

Export of tRNA has been shown to be mediated by exportin-t. Exportin-t binds many forms of tRNAs in the presence of Ran loaded with GTP and is required for the export of tRNA from Xenopus oocytes (Arts et al 1998a, Arts et al 1998b, Kutay et al 1998). Deletion of Los1, the closest homologue of exportin-t in budding yeast, causes tRNA accumulation in the nucleus, but cells are still viable (Hellmuth et al 1998). These data suggest that, at least in yeast, tRNA can also be exported in a Los1-independent manner. The ability of exportin-t to bind tRNA directly provides a mechanism for the export of tRNAs, but does not address the issue of how mature tRNA is preferentially exported.

A partial answer came from studies demonstrating that exportin-t forms a stable complex with tRNA having matured ends, but fails to bind tRNA with unprocessed ends (Arts et al 1998b, Kutay et al 1998, Lund & Dahlberg 1998). Since only tRNA with matured ends is exported, these data are consistent with a model in which selective export is achieved by preferential binding of exportin-t to mature tRNA. However, exportin-t binds equally well to spliced and unspliced tRNA (Arts et al 1998b), indicating that selective export of spliced tRNA is probably not achieved by preferential binding of exportin-t to spliced tRNA. Proposals for the mechanism responsible for maintaining unspliced tRNA in the nucleus are somewhat controversial. Lund et al. have demonstrated that splicing of tRNA occurs before end-processing and that tRNA is aminoacylated in the nucleus; a block in aminoacylation in the nucleus inhibits export of tRNA (Lund & Dahlberg 1998). Based on their data, they proposed that sequential processing of tRNA ensures selective export of matured tRNA; splicing precedes end-processing, which occurs prior to aminoacylation, which is necessary for export. In contrast, Arts et al. did not observe a dramatic decrease in the rate of nuclear export of tRNA that could not be aminoacylated (Arts et al 1998b). From their data they proposed that unspliced tRNA is retained in the nucleus by binding to a nuclear factor that prevents its association with exportin-t.

Issues such as the role of tRNA aminoacylation in export, the levels of unspliced but end-processed tRNA in the nucleus, and the mechanism that prevents such unprocessed tRNA from leaving the nucleus in vivo await further studies. Even though many aspects of tRNA export are still not fully understood, tRNA export provides a paradigm for understanding how cells utilize regulated export to allow proper maturation of RNA.

yAP1 In response to environmental stress cells activate transcription of genes that help them to survive adverse conditions (Ruis & Schuller 1995). The transcription factors important for the stress response, yAP1 in budding yeast and its homologue pAP1 in

fission yeast, are rapidly localized to the nucleus in response to oxidative stress and exposure to a variety of toxic compounds (Kuge et al 1997, Toone et al 1998). In the nucleus yAP1 and pAP1 induce transcription of anti-oxidative genes such as thioredoxin, catalase, and genes that belong to the multi-drug-resistance family, to provide protection against toxic compounds [(Kuge & Jones 1994, Toone et al 1998) and references therein].

Cytoplasmic localization of yAP1 in unstressed cells requires a small region known as the cysteine-rich domain (CRD) (Kuge et al 1997). The CRD has been shown to be necessary and sufficient for the regulated localization of yAP1. Deletion of the CRD leads to nuclear accumulation of yAP1, and fusion of the CRD to a heterologous protein is sufficient to impose regulated localization on this protein in response to oxidative stress (Kuge et al 1997). The CRD contains three conserved cysteines that are required for its proper function (Kuge et al 1997). These observations led to the proposal that the cysteines act as an oxidation sensor able to control CRD activity and the subcellular localization of yAP1 (Kuge et al 1997). It was later shown that the CRD contains a leucine-rich NES and that the cytoplasmic localization of yAP1 requires the activity of the export receptor Crm1 (Kuge et al 1998, Toone et al 1998, Yan et al 1998). Moreover, the affinity of the CRD for Crm1 increases under reducing conditions in vivo (Kuge et al 1998, Yan et al 1998) and in vitro and requires the conserved cysteines (Yan et al 1998). However, since all the binding experiments were done using crude extracts it is not known if this redox-regulated interaction requires other proteins.

These observations suggest that yAP1 shuttles rapidly between the nucleus and the cytoplasm, and that its cytoplasmic steady-state localization is due to very efficient export mediated by Crm1. In the presence of oxidative stress, the CRD presumably acts as a redox-sensor whose oxidation inhibits the interaction between yAP1 and Crm1, leading to rapid nuclear accumulation of yAP1 and activation of the anti-toxic transcriptional response.

There are two particularly interesting features of the regulated subcellular localization of yAP1. First, the CRD in yAP1 apparently contains a redox-transducer that couples the redox state in the cell to the activity of the NES, and thus to nuclear export. Second, localization of yAP1 seems to be regulated primarily at the level of its export rate, because blocking Crm1-mediated export or mutating the NES on yAP1 is sufficient to cause nuclear accumulation, even in the absence of stress (Kuge et al 1998, Toone et al 1998, Yan et al 1998). Constant shuttling between the cytoplasm and the nucleus creates a futile cycle and therefore might not be a very cost-effective way to regulate subcellular localization. The advantage of this type of regulation might be its simplicity, since it relies on regulating export only. This differs from the coordinated regulation of import and export observed with Pho4 (Kaffman et al 1998a), NF-kB (Arenzana-Seisdedos et al 1997), and NF-AT (Klemm et al 1997).

Cytoplasmic Anchoring

In this form of regulated localization, a receptor-cargo complex can form but cannot be targeted to the NPC because the cargo is tethered to an insoluble cellular component. This mechanism has been proposed for several cargoes. In some of these cases, such as cyclin B (Hagting et al 1998, Toyoshima et al 1998, Yang et al 1998) and MAPK (Fukuda et al 1997b), localization was later shown to occur by regulated export. For other examples, such as Xenopus nuclear factor 7 (Xnf7) (Li et al 1994) and JNK-interacting protein 1 (JIP-1) (Dickens et al 1997), the possibility of regulated nuclear export was never ruled out, and the mechanism by which cytoplasmic tethering occurs has not been determined.

Some of the best characterized examples of cytoplasmic retention include the sterol response element binding protein (SREBP) (Wang et al 1994), Notch (Struhl & Adachi 1998), cyclic-AMP protein kinase A (PKA) (Fantozzi et al 1992, Harootunian et al 1993, Lester & Scott 1997), and β-catenin [(Fagotto et al 1996), see below for more details].

SREBP and Notch are integral membrane proteins found in the ER and plasma membrane, respectively, of unstimulated cells. Upon stimulation, both proteins undergo proteolytic cleavage to produce a soluble fragment that enters the nucleus and can activate transcription (Struhl & Adachi 1998, Wang et al 1994). This type of regulation is irreversible because the mature soluble fragment cannot be anchored back to the cytoplasm by the same mechanism. As mentioned earlier, the catalytic subunit of PKA is retained in the cytoplasm by binding to the regulatory subunit, forming a complex that is too big to diffuse into the nucleus (Fantozzi et al 1992, Harootunian et al 1993) and anchoring it to AKAP proteins attached to insoluble structures within the cell (Lester & Scott 1997).

One issue that a cytoplasmic-anchoring mechanism has to address is how to ensure that cytoplasmic tethering will occur prior to nuclear import. In the case of membrane proteins, such as SREBP and Notch, this occurs by a translation-mediated insertion of the proteins into the ER membrane (Struhl & Adachi 1998, Wang et al 1994). In the case of the catalytic subunit (C) of PKA, nuclear import occurs by diffusion (Harootunian et al 1993), which is relatively slow, and cytoplasmic localization of C is also assisted by active export mediated by PKI (Wen et al 1994, Wen et al 1995). Thus, it seems likely that cytoplasmic anchoring will be used in combination with other forms of regulated localization, such as nuclear export or NLS masking, in order to improve its efficiency.

More examples are needed to demonstrate how anchoring in the cytoplasm is accomplished, and how it is established prior to import. Such studies will provide additional information to assess the significance of this mechanism in regulating nuclear localization.

β-catenin/Armadillo The regulated localization of β-catenin provides an interesting example of how cytoplasmic anchoring and cytoplasmic instability are combined to coordinate nuclear localization and activity of this protein (Fig 7). β-catenin in vertebrates and its homologue Armadillo in Drosophila are multi-functional proteins that play an

important role in the Wnt-signaling pathway [for reviews see (Brown & Moon 1998, Cadigan & Nusse 1997)]. In Xenopus, β-catenin plays an important role in body-axis formation and its overexpression induces double-axis formation with two-headed tadpoles (McMahon & Moon 1989). β-catenin is able to serve at least two different functions. First, it is an adaptor linking cadherins, which are surface membrane proteins involved in cell adhesion, to the actin cytoskeleton [reviewed in (Kemler 1993)]. Second, β-catenin is also required for coactivating transcription with the lymphoid enhancer factor 1 (LEF1/TCF) (Behrens et al 1996, Huber et al 1996, Molenaar et al 1996), which presumably induces a transcriptional program required for cell-fate determination and dorso-ventral axis formation (Brannon et al 1997).

The Wnt protein is a secreted glycoprotein that binds a membrane-bound receptor on the target cell and activates a signal transduction cascade leading to translocation of β-catenin from the cytoplasm into the nucleus. In the nucleus, β-catenin associates with LEF1 and this complex activates transcription of genes that are required for cell-fate determination [reviewed in (Brown & Moon 1998, Cadigan & Nusse 1997)] (Fig 7). Two mechanisms are thought to ensure that β-catenin is maintained in the cytoplasm in unstimulated cells. First, β-catenin is tethered to membrane-bound cadherins (Fagotto et al 1996, Kemler 1993). Second, the pool of free, cytoplasmic β-catenin is maintained at a low level by its constitutive degradation mediated by the proteasome (Cadigan & Nusse 1997) (Fig 7). This is a good example of how cytoplasmic anchoring and degradation in the cytoplasm can act together to prevent nuclear entry of a protein.

Several observations suggest that cadherins are able to regulate the signaling activity of β-catenin. Newly synthesized E-cadherin, still localized to the endoplasmic reticulum, is able to tether β-catenin to the membrane (Ozawa & Kemler 1992), allowing for an immediate decrease in the free cytoplasmic β-catenin upon synthesis of E-cadherins. In addition, overexpression of C-cadherin inhibits β-catenin signaling and leads to redistribution of β-catenin from the cytoplasm to the plasma membrane (Fagotto et al

1996). However, a mutant β-catenin that cannot bind C-cadherin is not redistributed to the plasma membrane, and its signaling activity is not inhibited when coexpressed with C-cadherin (Fagotto et al 1996). Together, these results suggest that physical association between C-cadherin and β-catenin is required for tethering β-catenin to the membrane, and that cytoplasmic anchoring plays a role in regulating the signaling activity of β-catenin.

The mechanism by which activation of the Wnt pathway leads to stabilization of β-catenin is not fully understood but two players have been implicated. Glycogen synthase kinase 3β (GSK3β) activity is required for destabilization of β-catenin [reviewed in (Cadigan & Nusse 1997)], and activation of the Wnt pathway causes a two-fold reduction in GSK3β kinase activity (Cook et al 1996). However, it is still unclear whether GSK3β kinase activity is directly responsible for destabilization of β-catenin (Cadigan & Nusse 1997). The second player is the adenomatous polyposis coli protein (APC), which is a tumor suppressor involved in colon carcinomas and certain melanomas [for review see (Polakis 1997)]. Mutations in APC correlate with an increase in β-catenin stability, localization of β-catenin to the nucleus (Munemitsu et al 1995), and induction of transcription (Korinek et al 1997, Morin et al 1997). The activities of GSK3β and APC are both required for the constitutive degradation of β-catenin by the proteasome. Inhibition of the proteasome stabilizes β-catenin and is associated with nuclear accumulation of β-catenin (Aberle et al 1997, Orford et al 1997, Salomon et al 1997), suggesting that stabilization of β-catenin is sufficient to activate the pathway. The current data is consistent with a model in which Wnt signaling leads to a decrease in the activities of GSK3β and APC, causing the stabilization of β-catenin, and its translocation into the nucleus (Fig 7).

Two observations have led researchers to propose that β-catenin enters the nucleus by associating with LEF1: (1) β-catenin lacks a sequence resembling a classical NLS; (2) wild type LEF1 coexpressed with β-catenin is able to induce nuclear localization of β-catenin, but LEF1 lacking a domain required for the association with β-catenin is unable to

cause the nuclear translocation of β-catenin (Behrens et al 1996, Molenaar et al 1996). However, mutants in the Drosophila homologue Armadillo that are defective in binding to dTCF (the Drosophila homologue of LEF1) are still able to enter the nucleus upon Wnt signaling (Orsulic & Peifer 1996, van de Wetering et al 1997), suggesting that association with TCF is not required for the nuclear entry of β-catenin in Drosophila embryos. In an attempt to resolve this issue the import of β-catenin was studied using an in vitro import assay. Import of β-catenin requires energy, but does not require addition of exogenous proteins, and addition of cytosol completely eliminates import of β-catenin (Fagotto et al 1998). Based on: (1) sequence similarity between β-catenin and other members of the importin family (Malik et al 1997, Peifer et al 1994); (2) the ability of β-catenin to dock on the nuclear envelope in the absence of exogenous proteins; (3) the ability of β-catenin to bind purified Nup1 (a yeast nuclear pore protein); and; (4) the observation that addition of exogenous Ran did not enhance import (similar observations were reported for trasport receptors) it was proposed that β-catenin is imported by a novel pathway similar to the way in which soluble transport receptors are translocated into the nucleus (Fagotto et al 1998). These data are consistent with a model in which β-catenin is able to be imported independently of LEF1 but does not rule out the possibility that association of β-catenin with LEF1 is necessary for efficient import in the presence of cytosol. Further studies are required to determine what activities in the cytosol are responsible for inhibiting the import of β-catenin in vitro, to understand the role of LEF1 in nuclear import of β-catenin, and to determine if β-catenin can serve as a soluble transport receptor capable of importing other cargoes into the nucleus.

Several points are worth noting with regard to the regulated localization of β-catenin. First, localization of β-catenin is probably regulated by the combined action of cytoplasmic anchoring mediated by cadherins and cytoplasmic degradation promoted by GSK3β and APC. Second, translocation of β-catenin to the nucleus seems to occur by a

novel import pathway. Third, inappropriate regulation of β-catenin stability triggers its nuclear localization and correlates with certain forms of cancer.

Regulating the Soluble Transport Machinery

The soluble transport machinery, which includes importins, exportins, and components of the Ran GTPase cycle, could also be targeted for regulation. Data to support this mode of regulation comes from several studies that are mainly correlative in nature. The lack of detailed understanding emphasizes the need for more experimental work aimed at elucidating the mechanism underlying this mode of regulation.

The matrix (M) protein from Vesicular Stomatitis Virus (VSV) inhibits protein import and nuclear export of almost all forms of RNA (Her et al 1997). VSV is an RNA virus that replicates in the cytoplasm (Weck & Wagner 1978, Weck & Wagner 1979). Inhibition of nuclear export reduces the concentration of cellular mRNA in the cytoplasm, and thus may enhance the transcriptional and translational efficiency of viral RNA (Black et al 1994), supporting efficient establishment of viral infection (Her et al 1997). The mechanism by which the M protein is able to inhibit transport is currently unknown, but because of its global inhibitory effect on nuclear transport, it was proposed to do so by inhibiting the Ran GTPase cycle (Her et al 1997). If this is the case, the M protein seems to act catalytically, as it inhibits transport at a concentration that is 1000-fold lower than the concentration of Ran (Her et al 1997).

Another example of the regulation of the transport machinery is the change in localization of importin α in response to cell cycle position. In Drosophila, the importin α homologue Pendulin enters the nucleus at the onset of mitosis with kinetics similar to those observed for cyclin B (Kussel & Frasch 1995, Torok et al 1995); a similar phenomenon has been observed in fission yeast (Matsusaka et al 1998). This regulated nuclear entry occurs mainly in proliferating cells and is thought to be important in regulating cell proliferation (Kussel & Frasch 1995, Torok et al 1995). The mechanism responsible for

this change in localization is not known, and might be related to the ability of importin α to be phosphorylated (Torok et al 1995). However, this change in localization suggests that the relative import or export rates of Pendulin are subject to cell cycle regulation, possibly providing a mechanism to block import of a large number of cargoes during mitosis.

A similar phenomenon was reported in budding yeast, where the steady state localization of the import receptor Nmd5 has been observed to change from being mainly cytoplasmic to mainly nuclear in response to osmotic shock (Ferrigno et al 1998). As mentioned earlier, Nmd5 is required for the regulated nuclear entry of the MAPK Hog1 when cells are exposed to high osmolarity (Ferrigno et al 1998). The significance of the change in Nmd5 localization is yet to be established, but is consistent with a model in which the activity of Nmd5 is regulated in response to changes in environmental conditions.

Both the mechanism by which the soluble transport machinery is regulated and the significance of this regulation in controlling subcellular localization of other proteins require more experimental work. However, it is tempting to speculate that this might represent an important mode of regulation, as it provides a simple way to coordinately control transport of large number of cargoes. This mode of regulation might be useful in regulating cellular responses such as differentiation or proliferation that require coordinated regulation of numerous proteins.

Regulation of the NPC

Results from several studies have suggested that the transport properties of the NPC itself might be regulated. The ciliated unicellular organism *Tetrahymena thermophila* provides a unique system to study this phenomenon because it has two nuclei, the macronucleus and the micronucleus, that share the same cytoplasm (Gorovsky 1973). The two nuclei serve distinct cellular functions, and share some proteins, but each nucleus also contains a unique subset of proteins (Gorovsky 1986). Microinjection of histone H1,

which resides in the macronucleus, into the cytoplasm results in exclusive targeting of this protein to the macronucleus (White et al 1989). In contrast, microinjection of histone H4, which is found in both nuclei, targets it to the micronucleus in dividing cells, and the macronucleus in non-dividing cells (White et al 1989). As these two nuclei share the same soluble transport machinery it was proposed that the asymmetric distribution of proteins is due to different transport properties of the NPC in the two nuclei (White et al 1989).

In another example, quiescent cells show a slower rate of nuclear import of large gold particles coated with nucleoplasmin, compared to proliferating cells. Interestingly, smaller gold particles are imported with same efficiency in both types of cells, indicating that difference in import rates was size-specific (Feldherr & Akin 1990b, Feldherr & Akin 1991). To determine if this reflects a change in the activity of the NPC, a heterokaryon consisting of proliferating and quiescent cells was obtained by fusing the two cells together. Injection of large gold particles into the cytoplasm of this heterokaryon show that import into the nucleus of proliferating cells is significantly more efficient compared to import into the quiescent nucleus (Feldherr & Akin 1993). As the two nuclei share the same soluble machinery, it was proposed that the NPC is modified to allow more efficient import into the nucleus of proliferating cells (Feldherr & Akin 1993). The observation that the difference in import is size-dependent further supports the notion that the activity of the NPC might be regulated (Feldherr & Akin 1993). Based on these studies it was proposed that such size-specific global regulation of transport may be important in restricting nuclear export of large particles, such the ribosomal subunits and other RNPs, allowing quiescent cells to maintain a low metabolic state (Feldherr & Akin 1993).

Recently a component of the NPC in budding yeast, Nup53, has been shown to be phosphorylated during mitosis (Marelli et al 1998). This cell cycle-dependent phosphorylation correlates with the inability of the importin β homologue Pse1 to associate with the nuclear pore (Marelli et al 1998). Since Nup53 seems to be important for the ability of Pse1 to bind the NPC and to transport cargo (Marelli et al 1998), these results

suggest that phosphorylation of Nup53 during mitosis regulates import mediated by Pse1 in a cell cycle-dependent manner. As import mediated by Pse1 is not completely blocked in cells lacking Nup53 (Marelli et al 1998) the significance of this regulation still needs to be determined. Similar studies are needed to: (1) characterize examples where the NPC is modified in response to cellular signals; (2) establish how these modifications affect transport properties; and (3) determine the significance of this type of regulation in controlling biological processes.

MAIN CHALLENGES AND FUTURE DIRECTIONS

Although the nuclear transport field has seen tremendous progress in understanding the movement of proteins into and out of the nucleus, many questions remain regarding the regulation of this process. These studies can be grouped into at least three broad lines of investigation.

Further characterization of biological pathways that utilize regulated localization Many cellular processes have been shown to utilize regulated localization, but very few examples exist in which the import and export receptors required for these processes have been identified. Identification of import and export receptors should help in determining whether cargo-receptor interactions are regulated. In addition, further characterization of cytoplasmic anchoring, the regulation of the soluble transport machinery, and the regulation of NPC-transport properties is required to determine the roles that these mechanisms play in regulating nuclear transport.

Mechanistic studies This line of work attempts to understand the mechanism by which regulation is achieved. For example, further studies are necessary to determine how phosphorylation triggers import or export of proteins. More specifically, it will be interesting to know whether certain phosphopeptides are sufficient to recruit import and export receptors in a manner analogous to recruitment of SH2 domains by peptides

containing phosphorylated tyrosines. It will also be of interest to determine how complex formation, such as in Per/Tim, ERK2, STATs or Mei2, is used to regulate nuclear localization.

Conceptual issues These studies should try and address more teleological questions regarding regulated localization. Some examples include: (1) What dictates whether import and/or export is subject to regulation? It will be interesting to determine whether any rules exist that can predict which rate is more likely to be regulated for a given cellular response. (2) What role do shuttling proteins play in regulated localization? As was proposed for cyclin B, shuttling proteins might be used to communicate cytoplasmic and nuclear events, allowing cells to respond as a single unit to environmental changes or to cell cycle position. (3) Why does regulated localization represent only one level of control for most proteins? It is possible that the control over nuclear localization is inherently leaky and therefore requires additional levels of regulation. It is also possible that multiple levels of control, of cellular processes, are generally required to construct robust molecular switches. Finally, the presence of multiple layers of regulation may affect the nature of the switch, changing its output from a graded response to a more biphasic ON/OFF type of switch.

It is our hope that by recognizing the importance of regulated nuclear localization in many biological processes, a better understanding will emerge leading to the development of new therapeutic tools that target this process to control disease.

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FIGURE LEGENDS

Figure 1. Schematics of nuclear import and export. **a.** Nuclear import can be divided into three steps: (1) docking; (2) translocation; (3) deposition of cargo in the nucleus. **b.** Nuclear export requires the formation of a trimeric complex composed of exportin, Ran-GTP, and the cargo. Note that the Ran-GTP gradient allows the formation of export complexes in the nucleus, import complexes in the cytoplasm, and the dissociation of these complexes in the cytoplasm and the nucleus, respectively.

Figure 2. Phosphorylation regulates the subcellular localization of Pho4. **a.** Domains and phosphorylation sites (P) in Pho4 important for regulating its transcriptional activity. **b.** In high-phosphate conditions Pho4 is phosphorylated in the nucleus; phosphorylation of Pho4 triggers its association with its export receptor Msn5 and blocks reimport by decreasing its affinity for Pse1. **c.** In low-phosphate conditions unphosphorylated Pho4 binds to its import receptor, Pse1, and is translocated into the nucleus. In the nucleus, Pho4 associates with Pho2 and the complex induces expression of phosphate-responsive genes.

Figure 3. The subcellular localization of NF-AT is regulated by phosphorylation and calcium-induced masking of its NES, mediated by calcineurin. **a.** Schematic of localization-signal sequences and phosphorylation sites (P) important for regulating the subcellular localization of NF-AT2. NF-AT homology region (NHR); DNA binding domain (DBD). **b.** Phosphorylation is thought to enhance nuclear export of NF-AT, and to block nuclear import of NF-AT by masking its two NLS sequences. In addition, calcium-induced binding of calcineurin to the NES in NF-AT prevents binding of the export receptor Crm1 to NF-AT, thereby blocking its export.

Figure 4. Activation of MAPK causes its entry into the nucleus. Phosphorylation of MAPK by the cytoplasmic MEK causes: (1) activation of MAPK kinase activity; (2) release from MEK; (3) dimerization; and (4) nuclear accumulation. Nuclear entry of activated MAPK leads to phosphorylation of downstream targets, leading to induction of a transcriptional response.

Figure 5. A delay in nuclear entry of Per and Tim is required for persistent oscillation of the biological clock of Drosophila. Per and Tim enter the nucleus as a complex. The formation of a Per/Tim complex that can enter the nucleus is thought to be regulated by: (1) degradation of Per; and (2) CLD sequences on Per and Tim that prevent their nuclear localization. Upon entering the nucleus, Per and Tim act together to inhibit their own transcription.

Figure 6. Two distinct NLS sequences in GR mediate its entry into the nucleus. **a.** Schematic of functional domains and NLS sequences in GR. **b.** Association of GR with accessory proteins (illustrated here with Hsp90) determines its subcellular localization. Binding of a ligand (L) to the LBD causes dissociation of these accessory proteins, leading to nuclear accumulation of GR. Nuclear import of GR is mediated by two NLS sequences with distinct import properties.

Figure 7. Nuclear entry of β-catenin is governed by the combined action of cytoplasmic anchoring to cadherins and the degradation of β-catenin in the cytoplasm. Degradation is promoted by GSK3β and APC. Wnt signaling inhibits the cytoplasmic degradation of β-catenin, leading to its accumulation in the cytoplasm and entry into the nucleus. In the nucleus, β-catenin associates with the transcription factor LEF1, and together this complex activates transcription of genes that are important for cell-fate determination.

Table 1 Importins and exportins in the yeast *Saccharomyces cerevisiae*

Importins	**Examples of cargoes**	**References**
Importin β/Kap 95 with importin α	SV40 large T antigen	(Enenkel et al 1995, Shulga et al 1996)
Kap104	Nab2; Nab4	(Aitchison et al 1996)
Yrb4/Kap 123	L-25	(Rout et al 1997, Schlenstedt et al 1997)
Pse1/Kap 121	L-25; Pho4	(Kaffman et al 1998b, Rout et al 1997, Schlenstedt et al 1997)
Nmd5	Hog1	(Ferrigno et al 1998)
Sxm1	Lhp1	(Rosenblum et al 1997)
Mtr10	Npl3	(Pemberton et al 1997)
Exportins		
Los1	tRNA	(Hellmuth et al 1998)
Crm1/Xpo1	leucine rich NES; yAP1	(Kuge et al 1998, Stade et al 1997, Yan et al 1998)
Msn5/Ste21	Pho4, Far1, Msn2, Msn4, and Mig1	(Kaffman et al 1998a), Peter M., Schueller C., and Johnston M., personal communication
Cse1	importin α	(Hood & Silver 1998, Kunzler & Hurt 1998, Solsbacher et al 1998)
Unknown function		
Lph2	Not known	
HRC1004	Not known	
Pdr6	Not known	

FIGURE 1

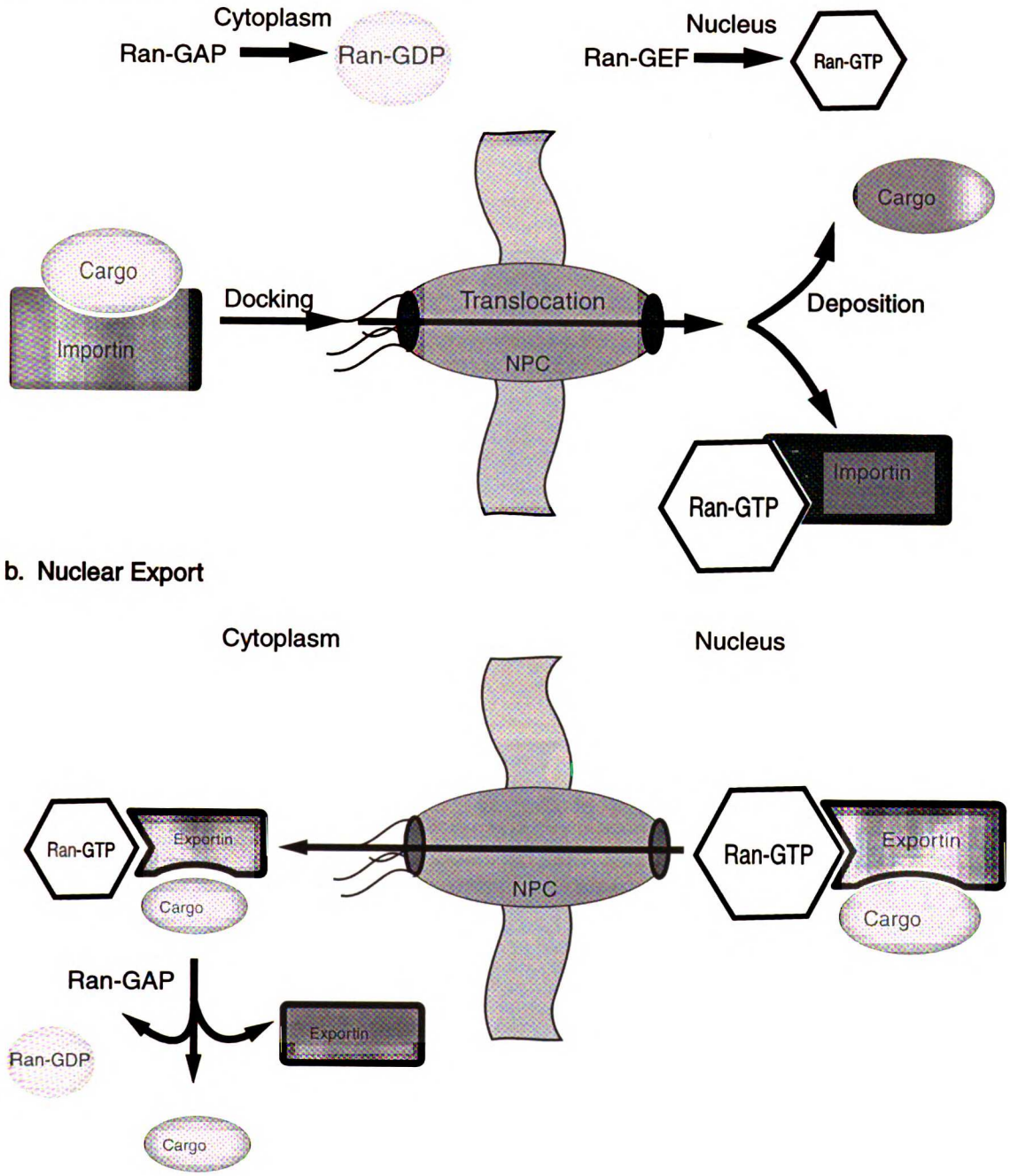

a. Nuclear Import
Cytoplasm
Ran-GAP
Ran-GDP
Nucleus
Ran-GEF
Ran-GTP
Cargo
Importin
Docking
Translocation
NPC
Cargo
Deposition
Importin
Ran-GTP
b. Nuclear Export
Cytoplasm
Nucleus
Ran-GTP
Exportin
Cargo
NPC
Ran-GTP
Exportin
Cargo
Ran-GAP
Exportin
Ran-GDP
Cargo

FIGURE 2

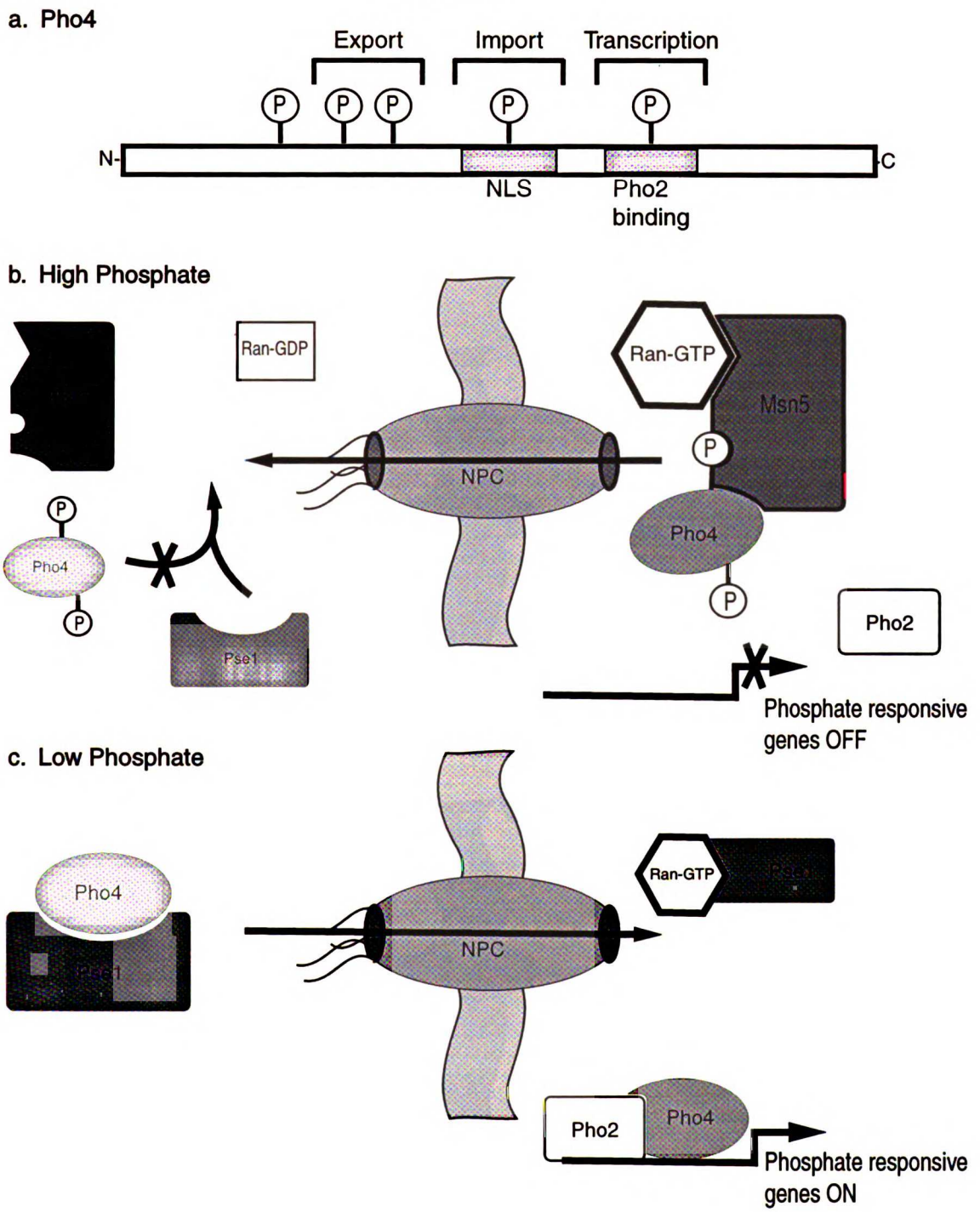

a. Pho4
Export
Import
Transcription
P
N-
C
NLS
Pho2
binding
b. High Phosphate
Ran-GDP
Ran-GTP
Msn5
NPC
Pho4
Pse1
Pho2
Phosphate responsive
genes OFF
c. Low Phosphate
Pho4
Ran-GTP
NPC
Pho2
Pho4
Phosphate responsive
genes ON

FIGURE 3

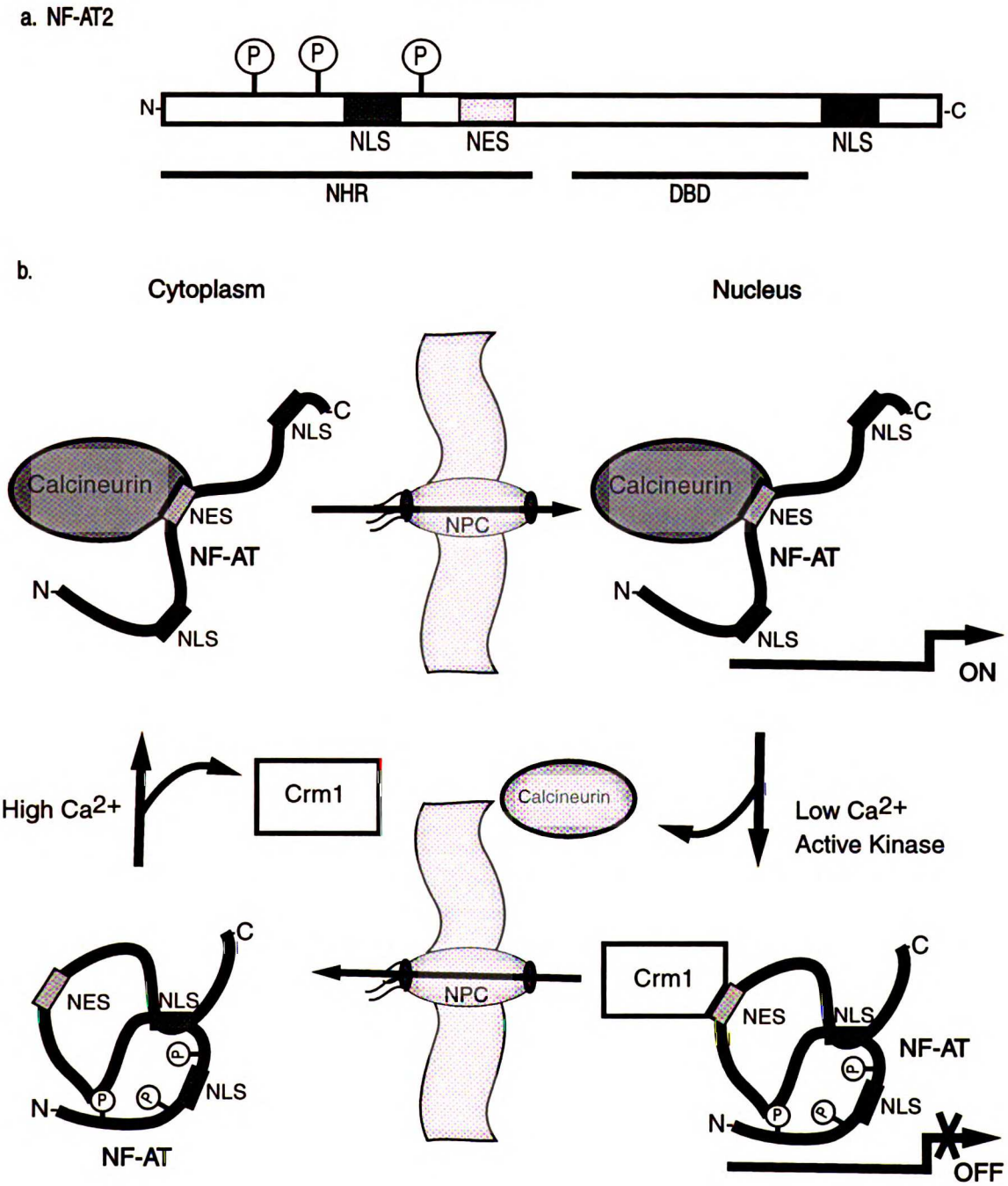


FIGURE 4

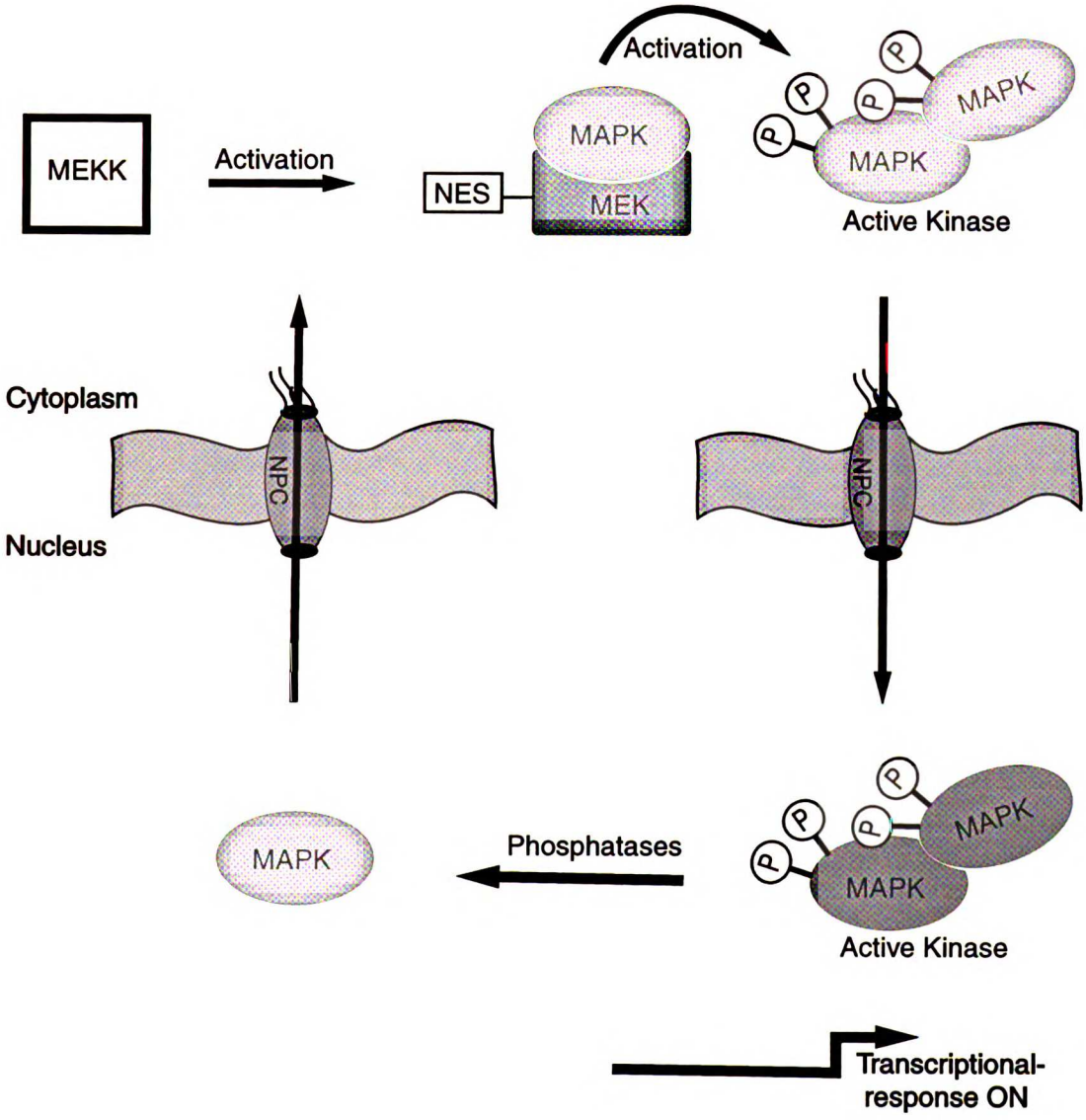
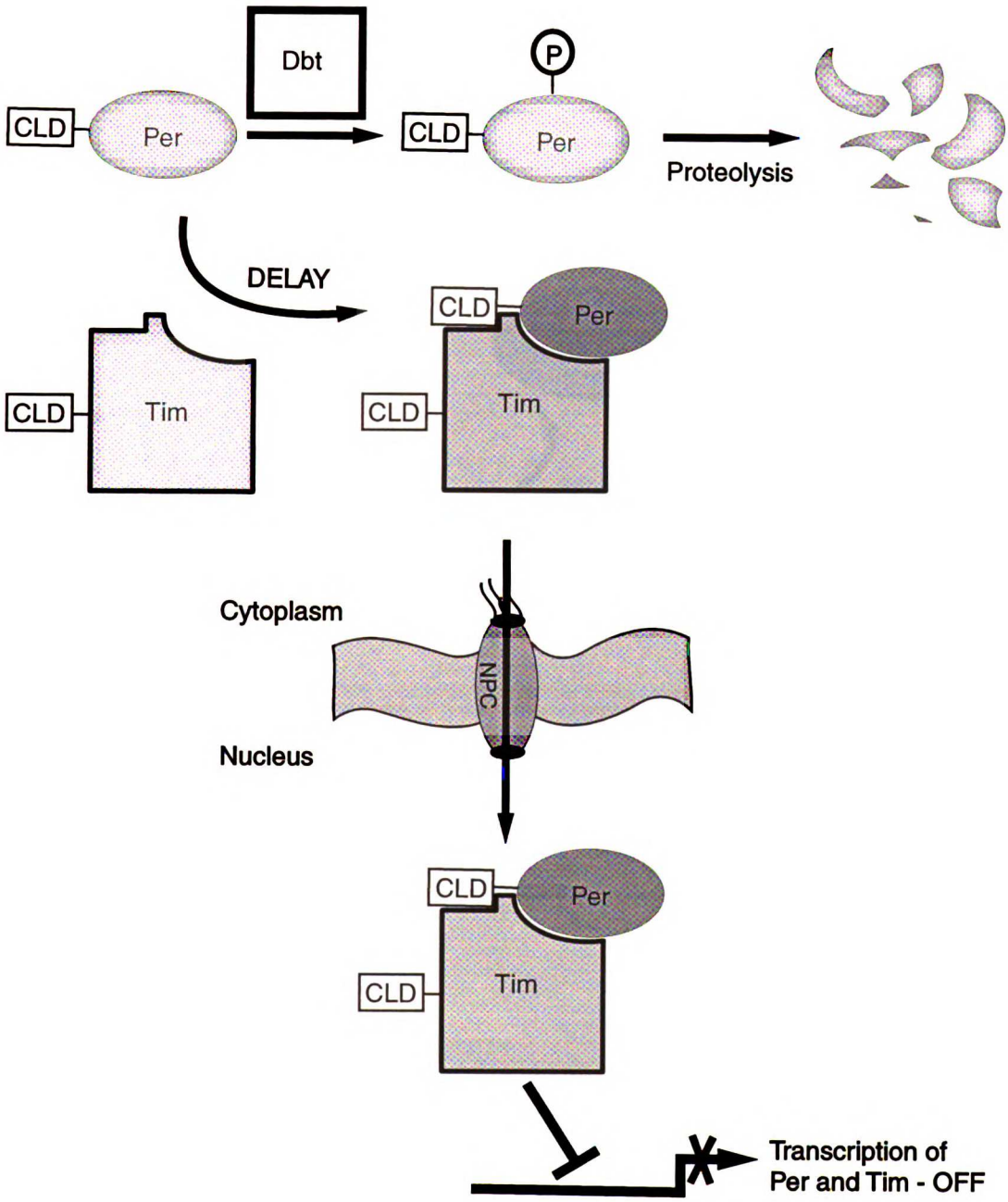

Activation
MAPK
MAPK
P
MEKK
Activation
NES
MAPK
MEK
Active Kinase
Cytoplasm
NPC
NPC
Nucleus
MAPK
Phosphatases
MAPK
MAPK
Active Kinase
Transcriptional-
response ON

FIGURE 5


Dbt
P
CLD
Per
CLD
Per
Proteolysis
DELAY
CLD
Tim
CLD
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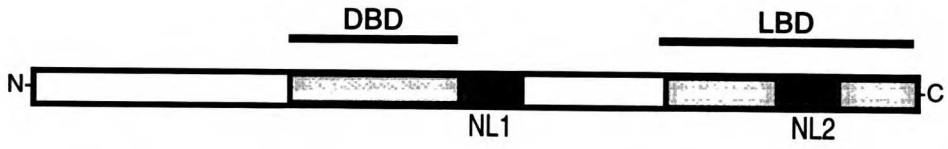
CLD
Tim
Cytoplasm
NPC
Nucleus
CLD
Per
CLD
Tim
Transcription of
Per and Tim - OFF

FIGURE 6

a. Glucocorticoid receptor



b.

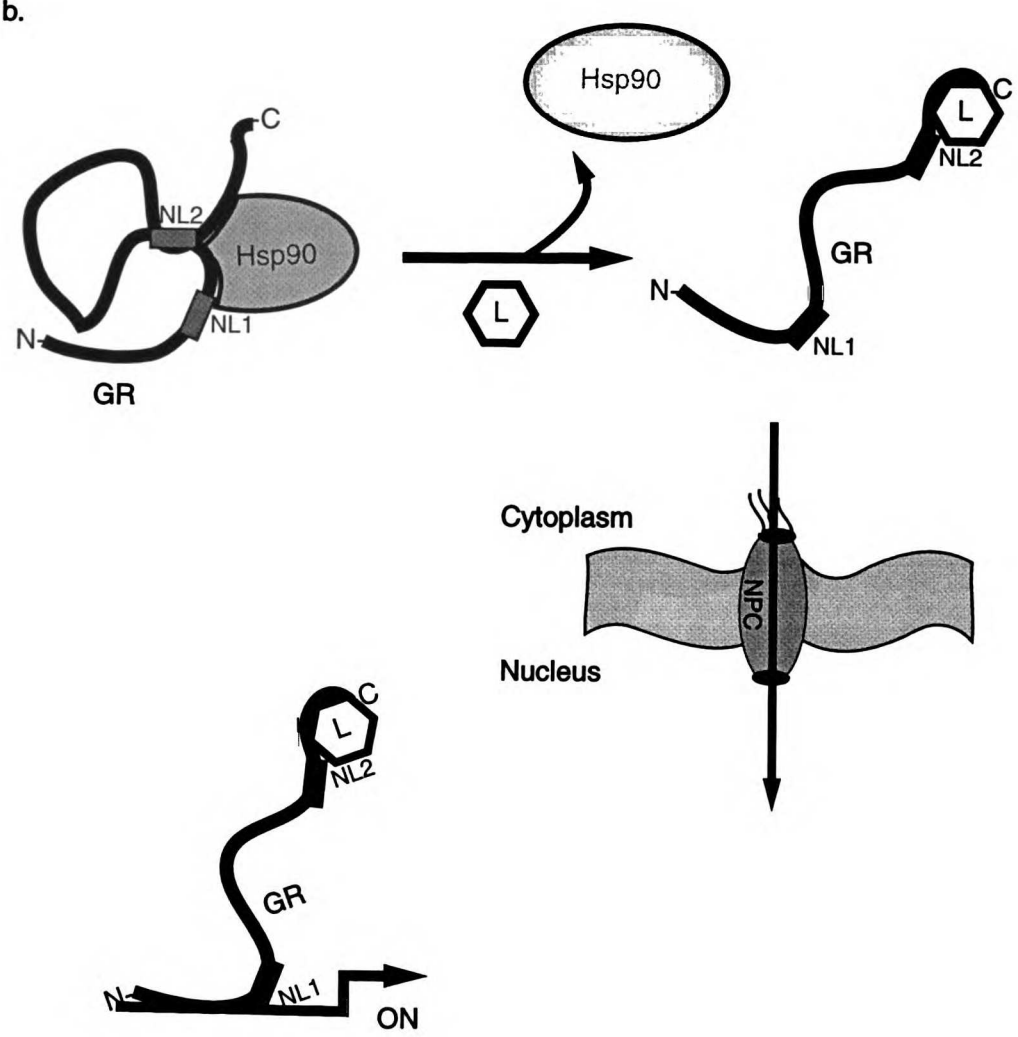
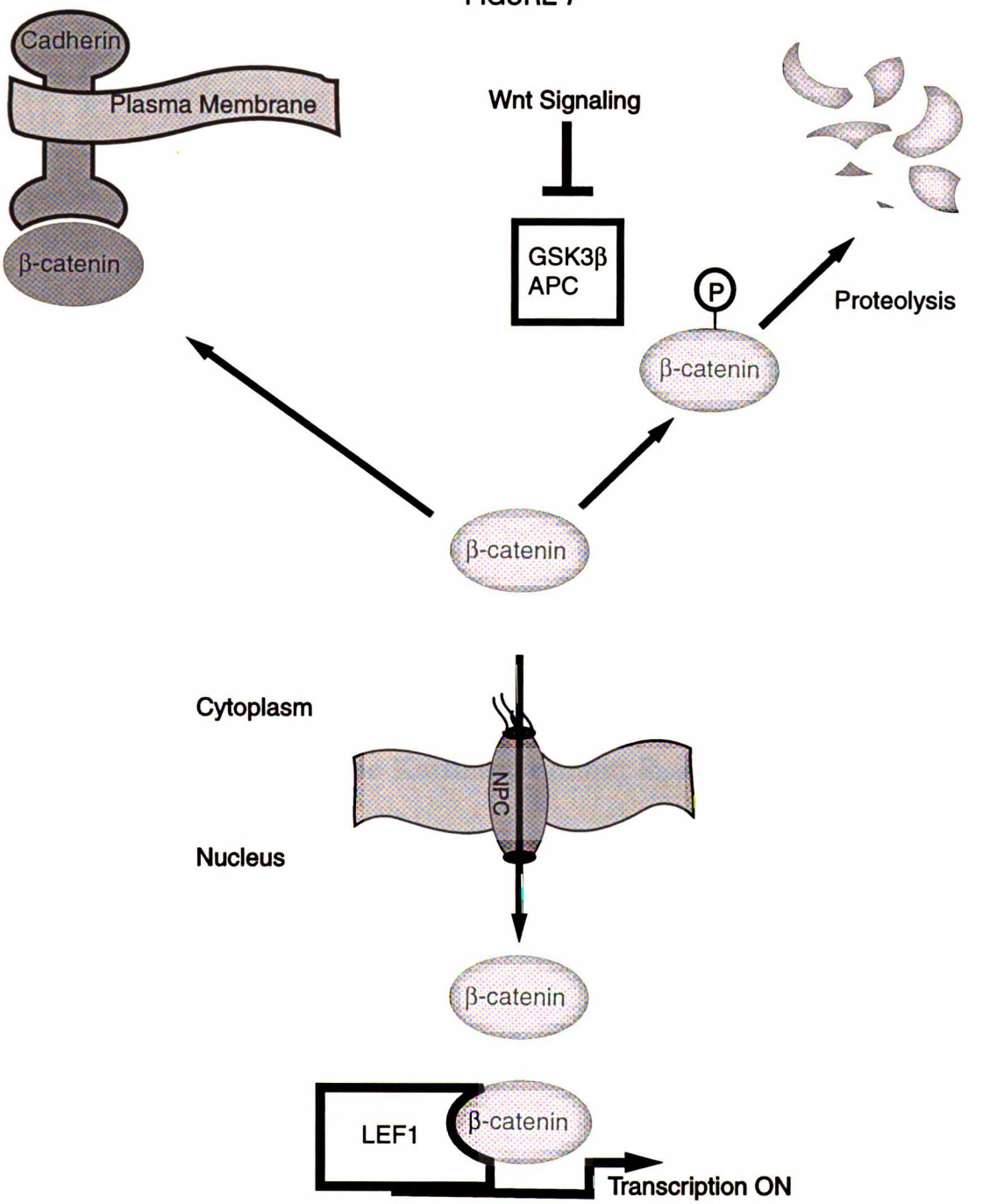


FIGURE 7


Cadherin
Plasma Membrane
β-catenin
Wnt Signaling
GSK3β
APC
P
Proteolysis
β-catenin
β-catenin
Cytoplasm
NPC
Nucleus
β-catenin
LEF1
β-catenin
Transcription ON

Chapter 1

Regulation of PHO4 Nuclear Localization by the PHO80-PHO85 Cyclin-CDK Complex

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Credits: In this paper E. Jolly and I mapped the phosphorylation sites in Pho4 (Fig 2) and I made the initial observation that unphosphorylatable Pho4 ($Pho4^{SA}$) expresses *PHO5* even when yeast cells are grown in high phosphate. E. O'Neill made the observation that the subcellular localization of Pho4 changes in response to phosphate levels (Fig 1) and that phosphorylation of Pho4 is required for its cytoplasmic accumulation in high phosphate (Fig 4a and 4b). Reprinted with permission from: "O'Neill EM, Kaffman A, Jolly ER, O'Shea EK. 1996. Regulation of PHO4 nuclear localization by the PHO80-PHO85 cyclin-CDK complex. *Science* 271:209-212". Copyright 1996 American Association for the Advancement of Science.

PHO4, a transcription factor required for induction of the *PHO5* gene in response to phosphate starvation, is phosphorylated by the PHO80-PHO85 cyclin-CDK complex when yeast are grown in phosphate-rich medium. Here it is shown that PHO4 is concentrated in the nucleus when yeast are starved for phosphate and is predominantly cytoplasmic when yeast are grown in phosphate-rich medium. The sites of phosphorylation on PHO4 are identified and it is shown that phosphorylation is required for full repression of *PHO5* transcription when yeast are grown in high phosphate. Thus, phosphorylation of PHO4 by PHO80-PHO85 turns off *PHO5* transcription by regulating the nuclear localization of PHO4.

PHO5 transcription is tightly repressed when *Saccharomyces cerevisiae* are grown in phosphate-rich medium and is induced more than 100-fold when yeast are starved for phosphate (1). Transcriptional induction of *PHO5* requires the transcription factor PHO4, and correlative evidence suggests that PHO4 activity is negatively regulated by phosphorylation (2). When yeast are grown in phosphate-rich medium, the PHO80-PHO85 cyclin-CDK (cyclin-dependent kinase) complex phosphorylates PHO4 (2) and transcription of *PHO5* is repressed. When yeast are starved for phosphate, the kinase activity of the PHO80-PHO85 complex is down-regulated by the CDK inhibitor PHO81 (3). This leads to the appearance of an underphosphorylated form of PHO4 (2) which, in combination with a second transcription factor PHO2, binds to the *PHO5* promoter and activates *PHO5* transcription (4).

We wished to determine how changes in phosphate availability affect PHO4 function. Phosphate starvation does not have a large effect on PHO4 stability; no difference in the protein level of PHO4 is observed between yeast grown in low versus high phosphate medium (2). Because PHO4 occupies its binding sites in the *PHO5* promoter in vivo under inducing, but not repressing conditions (5), the phosphate signal is likely to affect PHO4 function at the level of DNA binding or some step prior to that, such

as nuclear localization. Preliminary data suggest that phosphorylated and unphosphorylated PHO4 have a similar affinity for DNA (6).

We therefore examined the subcellular localization of PHO4 in wild-type cells grown in low or high phosphate medium. PHO4 is concentrated in the nucleus when yeast are starved for phosphate and is largely cytoplasmic when yeast are grown in phosphate-rich medium (Fig. 1, A-D). In *pho80Δ* and *pho85Δ* strains, in which PHO4 is not phosphorylated (2) and which express *PHO5* constitutively (1), PHO4 was concentrated in the nucleus, even when the strains were grown in phosphate-rich medium (6). In contrast, PHO4 was predominantly cytoplasmic in a *pho81Δ* strain grown in high or low phosphate conditions (6). In this strain, PHO4 is phosphorylated even when yeast are starved for phosphate (2), and *PHO5* expression is uninducible (1). These data demonstrate that the PHO signal transduction pathway is required for properly regulated nuclear localization of PHO4.

Our next goal was to determine whether phosphorylation was required for the regulation of PHO4 activity. We therefore wished to identify the residues of PHO4 that are phosphorylated by PHO80-PHO85. Several observations guided our efforts to identify these sites of phosphorylation: (i) Phosphoamino acid analysis indicated that PHO4 is phosphorylated exclusively on serine residues (Fig. 2A); (ii) PHO4 has no perfect matches to the CDK phosphorylation site consensus Ser/Thr-Pro-X-Lys/Arg (7), where X is any amino acid, but does contain six Ser-Pro dipeptide sequences (Fig. 2B). We tested whether these six Ser-Pro dipeptides are sites of phosphorylation by PHO80-PHO85. The observation that the PHO80- and PHO85-dependent phosphorylation sites observed in vitro and in vivo are similar (2) allowed us to use PHO4 phosphorylated in vitro by PHO80-PHO85 for the experiments described here. We generated a mutated version of PHO4 with six serine to alanine changes in the Ser-Pro dipeptides, called $PHO4^{SA1-6}$. We analyzed this mutant by tryptic phosphopeptide analysis which revealed that all of the major phosphopeptides were missing from the $PHO4^{SA1-6}$ map (Fig. 2C; compare $PHO4^{SA1-6}$ to

PHO4^{WT}). These data suggest that the major sites of phosphorylation on PHO4 are among the six Ser-Pro dipeptides.

To determine which among these six dipeptide sequences are sites of phosphorylation, we analyzed a series of six mutants, each with a single serine residue mutated to alanine (PHO4^{SA1} - PHO4^{SA6}). Comparison of the tryptic phosphopeptide map of wild-type PHO4 with those of mutants PHO4^{SA1} and PHO4^{SA5} indicated that no peptides were missing from the phosphopeptide maps of these mutants (Fig. 2C). In contrast, the tryptic phosphopeptide maps of mutants PHO4^{SA2}, PHO4^{SA3}, PHO4^{SA4}, and PHO4^{SA6} (Fig. 2C) have at least one altered or missing phosphopeptide (8), suggesting that Ser-Pro dipeptides 2, 3, 4, and 6 are sites of phosphorylation by PHO80-PHO85 and that mutation of serine to alanine prevents phosphorylation of that site. An alternative explanation is that mutation of serine to alanine at one or more of these sequences affects phosphorylation of a different serine.

To distinguish between these explanations, we used a second approach which exploits the observation that CDKs phosphorylate threonine as well as serine (7). Six PHO4 mutants were generated, each containing a single serine to threonine change in Ser-Pro dipeptides 1 through 6 (PHO4^{ST1} - PHO4^{ST6}), and analyzed by phosphoamino acid analysis (Fig. 2A). Wild-type PHO4 is phosphorylated only on serine (Fig. 2A); thus, if a phosphorylation site is mutated to threonine, phosphoamino acid analysis should reveal both phosphoserine and phosphothreonine. Phosphothreonine is present in PHO4^{ST1}, PHO4^{ST2}, PHO4^{ST3}, PHO4^{ST4}, and PHO4^{ST6}, but not in PHO4^{ST5} (Fig. 2A). Thus, both the tryptic phosphopeptide analysis and phosphoamino acid analysis suggest that Ser-Pro dipeptides 2, 3, 4, and 6 are sites of phosphorylation. In the case of Ser-Pro dipeptide 1, some phosphothreonine was detected in the phosphoamino acid analysis of mutant PHO4^{ST1}, yet no difference was observed between the tryptic phosphopeptide maps generated with PHO4^{SA1} and wild-type PHO4 (9).

To determine whether Ser-Pro dipeptide 1 is a site of phosphorylation, we generated two additional mutants, PHO4^{SA2346} and PHO4^{SA12346}, in which four or five of the serine residues were changed to alanine. These two mutants, in addition to PHO4^{SA1-6} and wild-type PHO4, were subjected to phosphorylation in vitro by PHO80-PHO85 and the total amount of phosphate incorporated into each protein was determined and compared to wild-type PHO4 (Fig. 3). PHO4^{SA12346} and PHO4^{SA1-6} each incorporated 4% of the phosphate incorporated by wild-type PHO4, whereas PHO4^{SA2346} incorporated 9%. From these data, we conclude that Ser-Pro dipeptide 1 is a site of phosphorylation and that Ser-Pro dipeptides 1, 2, 3, 4, and 6 represent the predominant sites of phosphorylation by PHO80-PHO85 (10). This result is consistent with mass spectrometry data which demonstrate that a considerable fraction of wild-type PHO4 phosphorylated in vitro contains five phosphate groups (11).

To test directly whether phosphorylation of PHO4 is required for it to be localized in the cytoplasm, we examined the subcellular localization of the PHO4^{SA12346} mutant expressed from a plasmid in a *pho4D* strain grown in phosphate-rich medium (Fig. 4, A and B). We observed that all PHO4^{SA12346} visible above background levels was concentrated in the nucleus. By contrast, wild-type PHO4 expressed from a plasmid in the same strain was predominantly cytoplasmic (6). These data demonstrate that phosphorylation of PHO4 is required for it to be localized in the cytoplasm under repressing (high phosphate) conditions.

To determine whether the regulated nuclear localization of PHO4 is sufficient for proper regulation of *PHO5* expression, we examined acid phosphatase activity in yeast expressing the PHO4^{SA12346} mutant. This strain expresses *PHO5* constitutively in phosphate-rich medium (Fig. 4C), though the level of *PHO5* expression is only 10% of the fully induced level observed with wild-type PHO4 in phosphate-depleted medium (12). We also examined the ability of PHO4^{SA12346} to induce *PHO5* expression under conditions of phosphate starvation. This mutant form of PHO4 activated *PHO5* expression

to 42% of the level observed for wild-type PHO4 (Fig. 4C), suggesting that this mutated form of PHO4 is mildly defective as a transcriptional activator. These data suggest that whereas phosphorylation of PHO4 is important for full repression of *PHO5*, the PHO pathway may also control another aspect of PHO4 activity or *PHO5* regulation.

We have described an important mechanism by which the activity of the transcription factor PHO4 is regulated by the PHO80-PHO85 cyclin-CDK complex in response to extracellular phosphate levels. A different mechanism was proposed, based on data obtained from two-hybrid analysis of PHO4 and PHO80 (13), in which PHO80 binds to PHO4 and masks its transcription activation domain. We believe that although the PHO4-PHO80 interaction may regulate PHO4 activity under certain conditions, it is not a physiologically relevant mechanism for the following reasons: (i) PHO4 is not bound to its sites in the *PHO5* promoter in vivo when yeast are grown in high phosphate medium (5). If PHO4 is not bound to the *PHO5* promoter under these conditions, an interaction that masks its activation domain is not likely to be relevant for *PHO5* regulation. (ii) The two-hybrid experiments were performed with highly overexpressed PHO4, a situation that leads to accumulation of PHO4 in the nucleus (6) and to constitutive expression of *PHO5* in high phosphate medium (14). We believe that the masking model may be relevant only under conditions that lead to the accumulation of PHO4 in the nucleus in high phosphate medium, such as when PHO4 is overexpressed or in yeast expressing the $PHO4^{SA12346}$ mutant.

This type of masking may be responsible for the incomplete derepression of *PHO5* (only 10%) observed with yeast expressing $PHO4^{SA12346}$ grown in phosphate-rich medium. Furthermore, the ability of PHO80 to interact with PHO4 could be regulated by the CDK inhibitor PHO81, which would explain the observation that yeast expressing $PHO4^{SA12346}$ are still partially responsive to phosphate starvation.

The mechanism regulating PHO4 localization is similar to the mechanism by which the yeast transcription factor SWI5 is regulated by the CDK CDC28 (15), suggesting that CDKs may regulate the subcellular localization of many different proteins by

phosphorylation. In contrast to PHO4, the physiological relevance of regulated nuclear localization of SWI5 is unclear, because a mutated form of SWI5 that is in the nucleus at all stages of the cell cycle does not cause inappropriate expression of the SWI5 target gene *HO* (15).

A paucity of physiologically relevant cyclin-CDK substrates has hampered efforts to determine the mechanisms by which these important kinases regulate cell cycle events. This description of how PHO80-PHO85 acts to regulate the activity of PHO4 provides an important insight into how phosphorylation by cyclin-CDK complexes can affect protein function.

Figure Legends

Fig. 1. Localization of the PHO4 protein. Indirect immunofluorescence was performed with PHO4 antiserum to determine the subcellular localization of PHO4 (16). Each pair of panels shows the same field; the left panel of each pair shows the PHO4 staining pattern, and the right panel shows DAPI staining (to visualize nuclei). The genotype of each strain (17) and the type of medium (18) in which it was grown are as follows: (**A** and **B**) a wild-type strain grown in phosphate-rich medium; (**C** and **D**) a wild-type strain grown in phosphate-depleted medium; (**E** and **F**) a *pho4Δ* strain grown in phosphate-rich medium.

Fig. 2. Identification of the sites of phosphorylation on PHO4. (**A**) Phosphoamino acid analysis (19) of wild-type PHO4 or mutant PHO4 proteins with single serine to threonine substitutions at putative phosphoacceptor residues. Analysis was performed with protein phosphorylated in vitro by PHO80-PHO85. Shown in the lower right is a schematic diagram representing ninhydrin visualization of the migration of standards used for phosphoamino acid analysis: phosphoserine (P-Ser); phosphothreonine (P-Thr); and phosphotyrosine (P-Tyr). (**B**) A schematic diagram of the PHO4 protein is shown. The amino acid position of the serine residue in each Ser-Pro dipeptide is indicated (SP1 - SP6) (20). The basic helix-loop-helix domain (bHLH), which contains both the DNA-binding and dimerization domains of PHO4, consists of residues 251-309 (21). A putative transcription activation domain consists of residues 1-109 (22). (**C**) Tryptic phosphopeptide analysis of wild-type PHO4 or mutant PHO4 proteins with serine to alanine substitutions at putative phosphoacceptor residues. Proteins were phosphorylated in vitro by PHO80-PHO85 (23). Ser-Pro dipeptides 2 and 3 are contained in the same tryptic peptide, which leads to a complicated pattern of phosphopeptides in the maps of $PHO4^{SA2}$ and $PHO4^{SA3}$ (8). Dashed lines indicate missing or altered phosphopeptides; the large dashed ellipse marks where the tryptic peptides derived from Ser-Pro dipeptides 2 and 3

were located in the wild-type map. The origin is in the lower left corner of each panel. Samples were electrophoresed in the horizontal dimension and chromatography was performed in the vertical dimension.

Fig. 3. PHO4^{SA12346} is not efficiently phosphorylated by the PHO80-PHO85 cyclin-CDK complex. Comparison of the amount of phosphate incorporated into PHO4^{SA1-6}, PHO4^{SA12346}, and PHO4^{SA2346} relative to wild-type PHO4 in an in vitro kinase assay performed using immunopurified PHO80-PHO85 kinase (24). For the negative control, wild-type PHO4 protein was used as substrate in a kinase assay performed with a mock PHO80-PHO85 immunopurification derived from an extract that did not contain epitope-tagged PHO80.

Fig. 4. PHO4^{SA12346} is localized to the nucleus in phosphate-rich medium and causes partially constitutive expression of *PHO5*. (**A**) Indirect immunofluorescence (16) performed with PHO4 antiserum on a *pho4Δ* strain transformed with a low-copy plasmid that expresses PHO4^{SA12346} under the control of the *PHO4* promoter (YCp400^{SA12346} (25)) grown in phosphate-rich medium (18). (**B**) DAPI staining of the field shown in (A) permits visualization of nuclei (16). (**C**) Yeast strains expressing no PHO4, wild-type PHO4, or PHO4^{SA12346} (26) were grown on plates containing either standard synthetic medium (high phosphate, upper panel) or phosphate-depleted medium (low phosphate, lower panel), and then overlaid with agar containing a chromagenic phosphatase substrate (27). Yeast strains expressing *PHO5* turn red by this assay. Acid phosphatase activity was also measured using a quantitative liquid phosphatase assay (28). The acid phosphatase activity for PHO4^{SA12346} is given as a percentage of that observed with yeast expressing wild-type PHO4 grown in phosphate-depleted medium.

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8. Several factors may contribute to the complex pattern of phosphopeptides in the phosphopeptide maps of mutants $PHO4^{SA2}$ and $PHO4^{SA3}$ (Fig. 2C). Ser-Pro dipeptides 2 and 3 are contained in the same tryptic peptide, which could result in the appearance of phosphoisomers (29) in the wild-type map if the protein is not fully phosphorylated. Furthermore, there is only a single trypsin cleavage site between Ser-Pro dipeptide 1 and Ser-Pro dipeptide 2 which, if not fully cleaved, could complicate the pattern. Two phosphopeptides are missing from the map of PHO^{SA4} (Fig. 2C). Loss of these two peptides is probably a result of incomplete trypsin digestion, since the seven trypsin cleavage sites carboxy-terminal to Ser-Pro dipeptide 4 are all in amino acid contexts that are not efficiently cleaved by trypsin (29).
9. The most likely explanation for this discrepancy is that Ser-Pro dipeptide 1 is used as a site, and that tryptic phosphopeptide analysis of $PHO4^{SA1}$ was uninformative because of anomalous migration of the phosphopeptide containing Ser-Pro dipeptide 1 or insolubility of this particular peptide in the buffer used to prepare the samples (29).
10. Serines 100, 114, 128, 152 and 223 of PHO4 are phosphorylated by PHO80-PHO85. Four of the five serine residues phosphorylated by PHO80-PHO85 are found in a

similar amino acid context, conforming to the consensus Ser-Pro-X-Ile/Leu. The fifth site has a threonine instead of a hydrophobic amino acid at the third residue following the phosphoacceptor.

11. E. K. O., D. A. Jeffery, D. King, unpublished data.

12. This difference in *PHO5* expression is not due to a difference in the level of PHO4 protein, as Western blotting indicates that wild-type PHO4 and $PHO4^{SA12346}$ are expressed at comparable levels in yeast grown in low or high phosphate medium (6). Additionally, SDS-PAGE analysis of $PHO4^{SA12346}$ isolated from yeast grown in high phosphate medium does not have the reduced electrophoretic mobility that is characteristic of phosphorylated wild-type PHO4 (2). This result suggests that the difference in *PHO5* expression is not likely to be due to residual phosphorylation of $PHO4^{SA12346}$. Also, we observe no difference in the ability of wild-type PHO4 and $PHO4^{SA12346}$ to interact with PHO80-PHO85 in vitro (6), suggesting that the observed *PHO5* phenotype is not due to an inability of PHO80-PHO85 to associate with the mutant PHO4. We have also tested the phenotype of yeast expressing $PHO4^{SA1}$ and $PHO4^{SA2346}$ (6), and observed that they did not express *PHO5* constitutively. The observation that expression of $PHO4^{SA2346}$ does not cause constitutive *PHO5* expression lends further support to the idea that Ser-Pro dipeptide 1 is a functionally important phosphorylation site.

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16. 25-ml cultures of yeast were grown as described (18) to OD_{600} = 0.5 - 1.5, at which time cells were fixed by addition of 3.5 ml of 37% formaldehyde directly to the culture medium. Fixation was allowed to proceed for 2 to 4 hours at room temperature, and cells were washed twice with water. Spheroplasts were prepared as follows. Fixed cells were incubated at room temperature for 10 minutes in 1 ml of

100 mM Tris-Cl pH 7.5 containing 25 mM 2-mercaptoethanol, washed once with PBS (136 mM NaCl, 2.6 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and resuspended in 25 µl PBS per OD_{600} of cells. 2 µl of zymolyase 100T (0.5 mg/ml) per OD_{600} of cells were added, and the cells were incubated at 37°C until 80 to 90% of cells appeared dark when visualized by phase contrast microscopy. Zymolyase treatment was stopped by washing three times in 1 M sorbitol with low speed (3000 rpm) centrifugation in an Eppendorf 5415C microcentrifuge. Spheroplasts were resuspended in 25 µl of 1 M sorbitol per OD_{600} of cells. Spheroplasts were placed on glass microscope slides that were first treated with poly-L-lysine (1 mg/ml) for 5 minutes, washed twice with water and air dried. Attached spheroplasts were treated with blocking buffer (PBS + 0.1% Triton-X 100, 1% bovine serum albumin) for 30 minutes at room temperature and incubated with preadsorbed primary antibody (diluted 1:200 in blocking buffer) overnight at 4°C. Spheroplasts were then washed three times with PBS, incubated with preadsorbed secondary antibody (diluted 1:200 in blocking buffer) for 1 hour at room temperature, washed three times with PBS, and mounted in Fluoromount G (Southern Biotechnology Associates, Inc.) + 22.5 ng/ml 4',6'diamidino-2-phenylindole dihydrochloride (DAPI). Primary antibody was polyclonal rabbit antiserum directed against PHO4 protein (2). Secondary antibody was rhodamine-conjugated goat anti-rabbit IgG (Boehringer Mannheim). Both secondary and primary antibodies were preadsorbed as described (30) with spheroplasts of either a wild-type strain or a strain deleted for the *PHO4* gene, respectively.

17. The wild-type strain used in this study was K699 *MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3* (30). *PHO4*, *PHO80*, *PHO81*, and *PHO85* were each deleted in this strain by standard gene replacement techniques [R. Rothstein, *Meth. Enzymol.* **194**, 281 (1991)] to generate strains EY0130 (*pho4D::TRP1*), EY0134

(*pho80D::HIS3*), EY0150 (*pho81D::TRP1*), and EY0140 (*pho85D::LEU2*), respectively.

18. High phosphate cultures were grown in standard synthetic complete medium (31) and low phosphate cultures were grown in phosphate-depleted synthetic complete medium (32). Low phosphate cultures were inoculated at OD_{600} = 0.05 - 0.2 using yeast from a log phase culture that had been grown in standard synthetic complete medium and then washed two times with sterile water to remove phosphate. High and low phosphate cultures were grown at 30°C with shaking for 8 to 16 hours to a final OD_{600} = 0.5 - 1.5 prior to analysis.

19. The procedures used for preparation of recombinant PHO4 (either wild-type or mutant) and in vitro phosphorylation of PHO4 using immunopurified PHO80-PHO85 complex are described (2). Plasmids T7-PHO4^{ST1} - T7-PHO4^{ST6} (25) were used to express the mutant versions of PHO4 in *E. coli*. Phosphoamino acid analysis was performed as described (29).

20. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

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23. The procedures used for preparation of recombinant PHO4 (either wild-type or mutant), in vitro phosphorylation of PHO4 using immunopurified PHO80-PHO85 complex, and tryptic phosphopeptide analysis are described (2). Plasmids T7-PHO4^{SA1} - T7-PHO4^{SA6}, and T7-PHO4^{SA1-6} (25) were used to express the mutant versions of PHO4 in *E. coli*.

24. The procedures used for preparation of recombinant PHO4 (either wild-type or mutant) and in vitro phosphorylation of PHO4 with immunopurified PHO80-PHO85 complex were essentially as described (2). The two changes from the published

procedure were as follows: (i) a version of PHO80 containing two copies of an epitope tag from the polyoma virus medium T antigen (sequence MEYMPME (20)) [T. Grussenmeyer, K. H. Scheidtmann, M. A. Hutchinson, W. Eckhart, G. Walter, *Proc. Natl. Acad. Sci. USA* **82**, 7592 (1985)] at its NH_2-terminus was used instead of the HA tag, and; (ii) Protein G Sepharose (Pharmacia Biotech) was used instead of Protein A Sepharose. Phosphorylated proteins were then resolved by SDS-PAGE. An equivalent amount of substrate, as judged by Coomassie blue staining and protein concentration determination, was used in each reaction. Quantitation was performed with the use of a PhosphorImager (Molecular Dynamics) and is expressed as a percentage of phosphate incorporated into wild-type PHO4 phosphorylated in vitro by PHO80-PHO85. Values represent an average of between three and five experiments. Standard deviation for each average value is shown. Plasmids T7-PHO4^{SA2346} and T7-PHO4^{SA12346} (25) were used to express the mutant versions of PHO4 in *E. coli.*

25. To generate plasmids with individual serine to threonine substitutions (T7-PHO4^{ST1} - T7-PHO4^{ST6}) or serine to alanine substitutions (T7-PHO4^{SA1} - T7-PHO4^{SA6}) mutations were introduced into T7-PHO4 (2) with site-directed mutagenesis [J. Geisselsoder, F. Witney, P. Yuckenberg, *Biotechniques* **5**, 786 (1987)]. Plasmids containing multiple phosphorylation site mutations were generated by performing site-directed mutagenesis with multiple oligonucleotides simultaneously and/or using standard molecular biology techniques to combine mutants. The plasmid YCp400 was generated by ligating a 3.2 kb Hind III-Hpa I fragment from pAC312 (21) into YCp50 (33) that had been digested with Bam HI, end-filled, and then digested with Hind III. YCp400^{SA12346} was generated by replacing an appropriate fragment from T7-PHO4^{SA12346} in YCp400.

26. The three yeast strains used were generated by transforming the *pho4D* strain (17) with each of the following three plasmids: (i) YCp50, an empty *URA3*-marked ARS-CEN vector (33); (ii) YCp400 (25), a plasmid expressing wild-type PHO4 under the

control of the *PHO4* promoter; or (iii) YCp400^{SA12346} (25), a plasmid expressing PHO4^{SA12346} under the control of the *PHO4* promoter.

27. Each strain was patched onto a plate containing standard synthetic medium (31) and allowed to grow overnight at 30°C. This plate was then replica-plated, both back onto standard synthetic medium and onto phosphate-depleted medium (32). These two plates were allowed to grow at 30°C for 12 hours, and then were overlaid with agar containing a chromagenic phosphatase substrate (34).

28. Liquid acid phosphatase assays were performed using a modification of the procedure described (34). Strains were grown until they reached OD_{600} of between 0.5 and 1.5, at which point 1 ml of culture was harvested and resuspended in 200 µl of water. 10 µl of this suspension was added to 90 µl of water and 400 µl of substrate (56.2 mg/ml p-nitrophenyl phosphate in 0.1 M sodium acetate pH 4.2). The reaction was allowed to proceed for 10 minutes at room temperature. Reactions were stopped by addition of 720 µl of saturated sodium carbonate. Cells were removed from the reactions by centrifugation, and the absorbance at 420 nm was measured. One unit of acid phosphatase is defined to be a change of 1.0 in absorbance at 420 nm per ml of yeast at a density of OD_{600} = 1.0. The strain used in these assays is wild-type for *PHO3*, a second, constitutively expressed acid phosphatase gene. The presence of PHO3 results in a low level of background phosphatase activity, which was subtracted before the percentage of wild-type PHO5 activity was calculated. The actual values determined for the acid phosphatase activity (expressed as U/OD_{600}) of these strains are: in high phosphate medium, YCp50 = 4.1 +/- 1.1, YCp400 = 3.6 +/- 0.6, YCp400^{SA12346} = 8.8 +/- 0.8; in low phosphate medium, YCp50 = 2.6 +/- 0.8, YCp400 48.3 +/- 4.8, YCp400^{SA12346} = 22 +/- 5.6. For comparison, the acid phosphatase activity of a *pho80Δ* strain is 51.3 +/- 10.2 in high phosphate medium and 59.9 +/- 14.8 in low phosphate medium.

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Figure 1

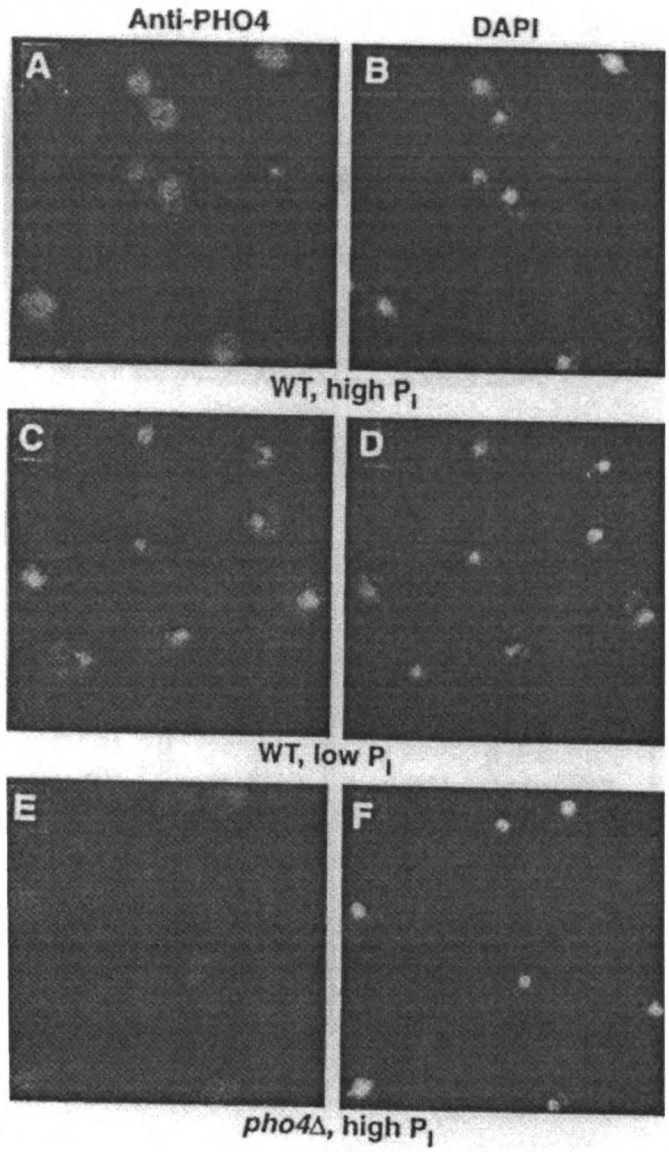


Figure 2

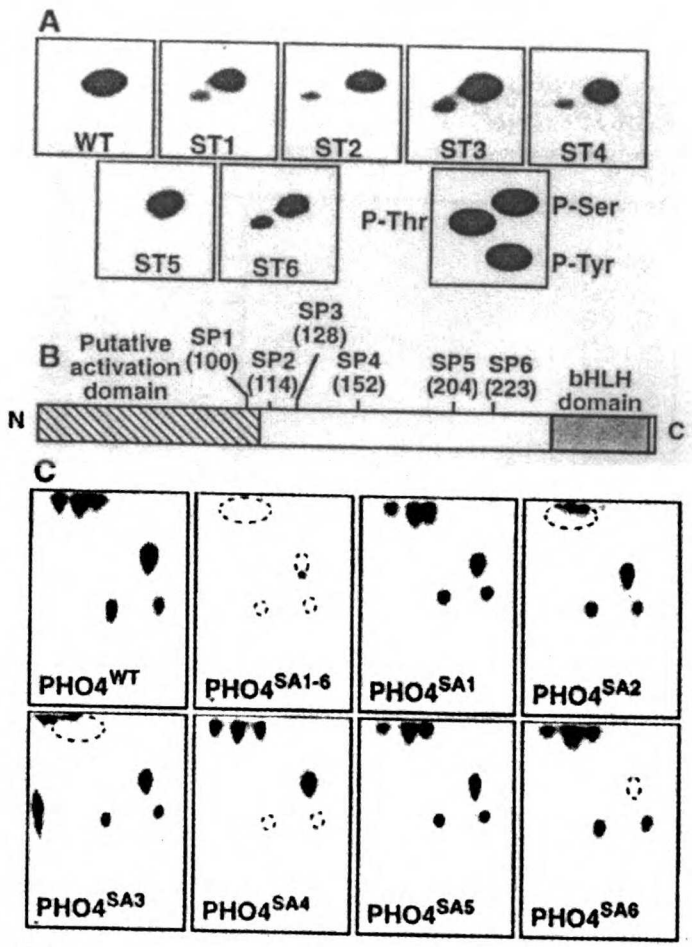

A
WT
ST1
ST2
ST3
ST4
ST5
ST6
P-Thr
P-Ser
P-Tyr
B
Putative activation domain
SP1 (100)
SP2 (114)
SP3 (128)
SP4 (152)
SP5 (204)
SP6 (223)
bHLH domain
N
C
C
PHO4WT
PHO4SA1-6
PHO4SA1
PHO4SA2
PHO4SA3
PHO4SA4
PHO4SA5
PHO4SA6

Figure 3

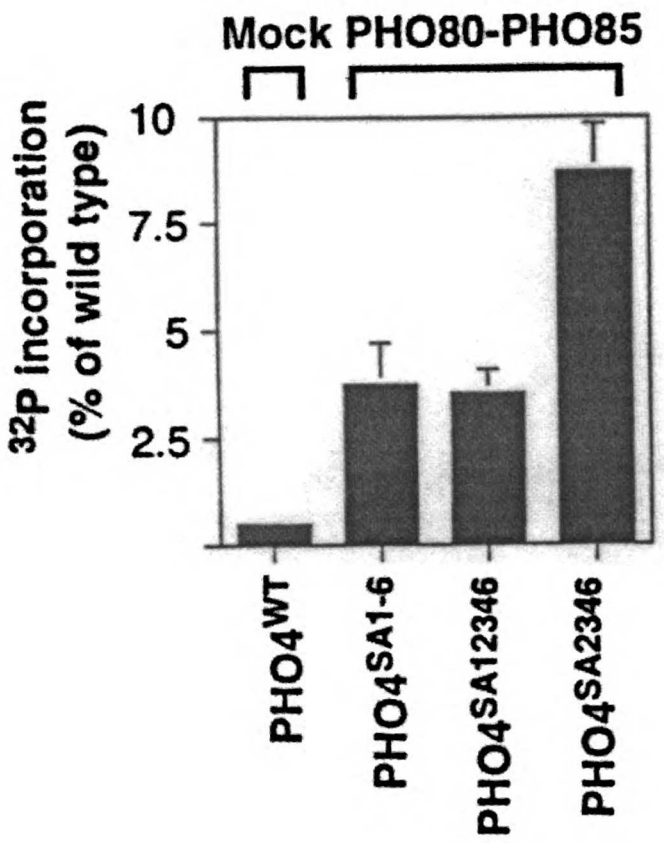
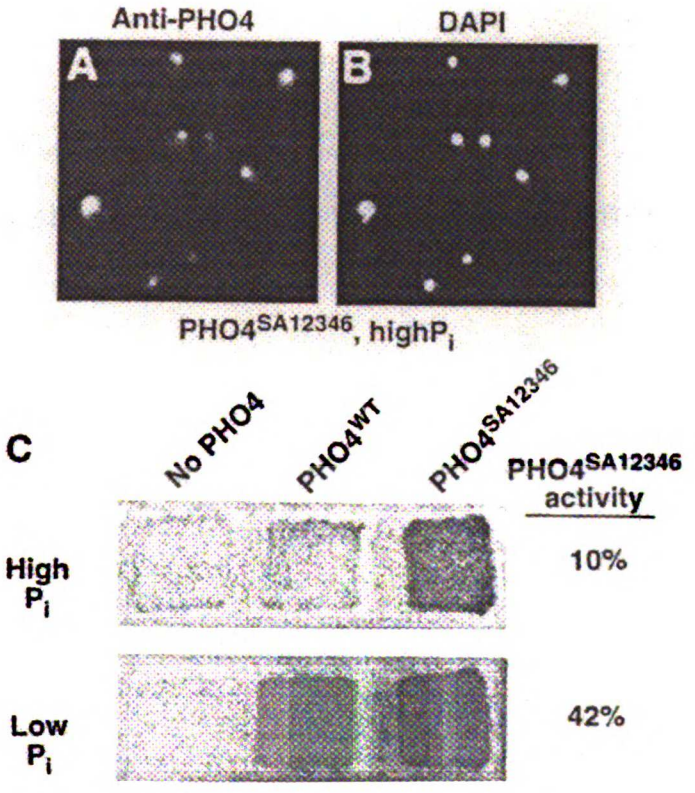

Mock
PHO80-PHO85
32P incorporation (% of wild type)
10
7.5
5
2.5
PHO4WT
PHO4SA1-6
PHO4SA12346
PHO4SA2346

Figure 4


Anti-PHO4
DAPI
A
B
PHO4SA12346, highPi
C
No PHO4
PHO4WT
PHO4SA12346
PHO4SA12346 activity
High Pi
10%
Low Pi
42%

Chapter 2

Phosphorylation Regulates Association of the Transcription Factor Pho4 with Its Import Receptor Pse1/Kap121

Running Title: Pse1 is the Nuclear Import Receptor for Pho4

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Credits: In this paper Nicole Rank made the initial observation that Pse1 is required for nuclear import of Pho4 in vivo and identified the NLS in Pho4 (Figures 1, 2 and 3), and I characterized biochemically the interaction between Pse1 and Pho4 and showed that phosphorylation of Pho4 inhibits its ability to bind Pse1 (Figures 4, 5, 6, and 7).

The transcription factor Pho4 is phosphorylated and localized predominantly to the cytoplasm when budding yeast are grown in phosphate-rich medium and is unphosphorylated and localized to the nucleus upon phosphate starvation. We have investigated the requirements for nuclear import of Pho4 and find that Pho4 enters the nucleus via a non-classical import pathway which utilizes the importin β family member Pse1/Kap121. Pse1 binds directly to Pho4 and is required for its import in vivo. We have defined the nuclear localization signal on Pho4 and demonstrate that it is required for Pse1 binding in vitro and is sufficient for *PSE1*-dependent import in vivo. Phosphorylation of Pho4 inhibits its interaction with Pse1, providing a mechanism by which phosphorylation may regulate import of Pho4 in vivo.

One way in which cells respond to extracellular signals is by modulating gene expression. This response requires the transfer of information from the plasma membrane, through the cytoplasm, and into the nucleus. Protein kinase cascades are commonly used to transduce extracellular signals, and they typically culminate in the phosphorylation of transcription factors. Phosphorylation of both transcription factors and kinases has been shown to result in regulation of their nuclear localization, suggesting that control of the subcellular localization of these proteins is important for the response to extracellular signals (Gaits et al. 1998; Khokhlatchev et al. 1998; Toone et al. 1998 and reviewed in Jans and Hubner 1996).

The subcellular localization of proteins can be controlled by regulating import into the nucleus and/or by regulating export from the nucleus. Nuclear import and export occur through the nuclear pore complex, a large macromolecular assembly of proteins embedded

in the nuclear envelope. Transport is signal-mediated, requires energy and physiological temperature, and is bidirectional. Targeting signals are recognized by soluble transport receptors which then translocate with their cargo into or out of the nucleus (reviewed in Nigg 1997).

The first targeting signal to be described was the SV40 large T antigen nuclear localization signal (NLS), consisting of a cluster of basic amino acids (Kalderon et al. 1984). The import receptor which recognizes the classical NLS is a heterodimer consisting of importin α and importin β. importin α binds directly to the NLS (Gorlich et al. 1994; Weis et al. 1995), and importin β binds to importin α, the GTPase Ran, and the nuclear pore complex (Gorlich et al. 1995a; Moroianu et al. 1995; Rexach and Blobel 1995). A complex consisting of the NLS, importin α, and importin β assembles in the cytoplasm, docks at the nuclear pore, translocates across the pore, and then is disassembled in the nucleus (Newmeyer and Forbes 1988; Richardson et al. 1988; Gorlich et al. 1995b; Imamoto et al. 1995; Rexach and Blobel 1995; Moroianu et al. 1996).

Additional signals have been defined which target proteins from the nucleus to the cytoplasm (reviewed in Nakielny and Dreyfuss 1997). The nuclear export signal (NES) contained in the HIV Rev protein and PKI, an inhibitor of cAMP dependent protein kinase A, is a small sequence rich in leucine residues (Wen et al. 1995). The leucine-rich NES is bound by the export receptor Crm1/Xpo1 and Ran-GTP in the nucleus. This complex translocates across the nuclear pore, and is disassembled in the cytoplasm (Fornerod et al., 1997; Fukuda et al., 1997; Kudo et al., 1997; Neville et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997).

A recent advance in the nuclear transport field is the discovery of multiple import and export pathways which utilize different transport receptors (reviewed in Nakielny and Dreyfuss 1997; Weis 1998). This has led to the identification of a family of transport receptors related in sequence to importin β, with the N-terminus being most conserved in a ~150 amino acid region required for binding to the small GTPase Ran (Gorlich et al.

1997). Searches of the yeast genome database revealed thirteen proteins with significant homology to importin β (Gorlich et al. 1997). Several of these importin β family members have now been shown to be import or export receptors, and it has been postulated that the remainder are transport receptors defining novel import or export pathways. Many studies are focused on determining the functions of the different importin β family members.

Ran plays an essential role in nuclear import and export; it is thought that the nucleotide state of Ran is used to impart directionality to transport processes (Gorlich et al. 1996; Izaurralde et al. 1997). The regulators of Ran are compartmentalized within the cell; RanGAP, the Ran GTPase activating protein, is localized to the cytoplasm and RCC1, the guanine nucleotide exchange factor for Ran, is exclusively nuclear (Ohtsubo et al. 1989). The localization of RanGAP and RCC1 predicts that the GTP-bound form of Ran will predominate in the nucleus. The steep gradient of Ran-GTP between the nucleus and the cytoplasm controls assembly and disassembly of transport complexes. Ran-GTP binding by importin β family members facilitates release of import receptors from their cargo in the nucleus (Rexach and Blobel 1995; Schlenstedt et al. 1997). Ran-GTP binding by importin β family members involved in export is required for the formation of export complexes consisting of the export receptor, cargo, and Ran-GTP (Fornerod et al. 1997; Kutay et al. 1997; Kutay et al. 1998). Thus, transport receptors utilize the Ran-GTP gradient as a marker for nuclear and cytoplasmic compartments.

Eukaryotic cells have taken advantage of the barrier between the cytoplasm and nucleus as a way to restrict access to the nucleus and to control the activity of regulatory proteins. Although there are many examples of regulated localization, for most systems it is not clear if import and/or export is regulated or how this regulation is achieved. Until recently, it was assumed that import into the nucleus was the step subject to regulation. The identification of multiple export pathways suggests that the localization of some proteins might be regulated at the level of export. A goal of the field is to understand the regulation of nuclear import and export. One major challenge is to understand how signal

transduction pathways influence the import and export of regulatory proteins to control their subcellular localization in response to extracellular signals.

We are studying the regulation of Pho4, a yeast transcription factor whose localization is regulated by phosphorylation in response to changes in the extracellular concentration of inorganic phosphate (O'Neill et al. 1996). Pho4 is required for phosphate starvation-specific gene expression (reviewed in Oshima 1997). When yeast are grown in phosphate-rich medium, Pho4 is phosphorylated by the Pho80-Pho85 cyclin-CDK complex (Kaffman et al. 1994) and is localized predominantly to the cytoplasm (O'Neill et al. 1996), resulting in transcriptional repression of phosphate starvation-specific gene expression. Upon phosphate starvation, the CDK inhibitor Pho81 inhibits Pho80-Pho85 (Schneider et al. 1994), leading to accumulation of unphosphorylated Pho4 in the nucleus, and transcription of phosphate-responsive genes (O'Neill et al. 1996).

We are interested in understanding how phosphorylation of Pho4 regulates its subcellular localization in response to changes in extracellular inorganic phosphate. We find that Pho4 is imported into the nucleus via a non-classical import pathway utilizing the importin β family member Pse1/Kap121. Pho4 is the first protein found to be imported exclusively by Pse1. We identify the Pho4 NLS and demonstrate that this region is both necessary for Pse1 binding in vitro and sufficient for *PSE1*-dependent nuclear import in vivo. Additionally, we demonstrate that the interaction between Pho4 and Pse1 is inhibited by phosphorylation, suggesting that import of Pho4 in vivo is regulated by phosphorylation. Our studies provide insight into the mechanism by which phosphorylation of Pho4 regulates its import, and serve as a paradigm for understanding other examples of regulated nuclear localization.

Results

Nuclear import of Pho4 is defective in a pse1-1 strain

Localization of Pho4 is regulated by phosphorylation in response to changes in the extracellular concentration of inorganic phosphate. A mechanistic understanding of the control of Pho4 localization by phosphorylation requires identification of the transport receptors that carry Pho4 into and out of the nucleus. To identify transport receptors required for import of Pho4, we examined the localization of Pho4-green fluorescent protein (GFP) in different yeast strains containing mutations in the importin β family members. In a wild-type strain, Pho4-GFP is predominantly cytoplasmic when cells are grown in phosphate-rich medium and is concentrated in the nucleus when yeast are starved for phosphate (Figure 1). In a yeast strain harboring a temperature-sensitive mutation in the essential importin β family member *PSE1* (Seedorf and Silver 1997), also referred to as *KAP121* (Rout et al., 1997), we find that Pho4-GFP does not accumulate in the nucleus upon phosphate starvation (Figure 1). The defect in Pho4 localization is observed even at the permissive temperature in a *pse1-1* strain. Other strains with mutations in the importin β family members *KAP104*, *LOS1*, *MTR10*, *SXM1*, *XPO1* and *CSE1* (Hopper et al. 1980; Xiao et al. 1993; Kadowaki et al. 1994; Aitchison et al. 1996; Seedorf and Silver 1997; Stade et al. 1997) do not show a defect in localization of Pho4-GFP upon phosphate starvation (data not shown).

The requirement of Pse1 for nuclear accumulation of Pho4 could reflect a role for Pse1 in transducing the phosphate starvation signal, or a role for Pse1 in import of Pho4. To determine if Pse1 is required for Pho4 import, we examined the localization of $Pho4^{SA}$, a Pho4 mutant containing five serine to alanine substitutions at the sites of phosphorylation by Pho80-Pho85 (O'Neill et al. 1996). $Pho4^{SA}$ is localized to the nucleus of a wild-type strain even when phosphate is abundant (O'Neill et al., 1996). Thus, $Pho4^{SA}$ can be used to assess the requirements for Pho4 import that are independent of the function of the

phosphate signal transduction pathway. In contrast to what is observed in wild-type yeast, Pho4^{SA}-GFP localizes predominantly to the cytoplasm in a *pse1-1* mutant strain (Figure 2), indicating that Pse1 is required for the import of Pho4 into the nucleus.

Previous studies have suggested that Pse1 and a closely related importin β family member Kap123 might have overlapping functions (Rout et al. 1997; Schlenstedt et al. 1997; Seedorf and Silver 1997). We wished to test if Kap123 also plays a role in the import of Pho4 by examining Pho4^{SA}-GFP localization in a *kap123Δ* strain. We find no defect in the import of Pho4^{SA}-GFP in a *kap123Δ* strain (Figure 2), indicating that this importin β family member is not required for the import of Pho4.

If the mislocalization of Pho4 in a *pse1-1* mutant strain reflects a defect in a specific import pathway rather than a defect which renders Pho4 incompetent for import, it should be possible to target Pho4 to the nucleus in a *pse1-1* mutant strain using an alternate import pathway. Strains with mutations in either *PSE1* or *KAP123* are capable of importing a reporter protein targeted to the nucleus with an NLS derived from the SV40 large T antigen (Seedorf and Silver 1997). We fused the SV40 large T antigen NLS to Pho4-GFP (Pho4-GFP-NLS) and examined its localization in a wild-type strain, a *pse1-1* strain, and a *kap123Δ* strain (Figure 2). Pho4-GFP-NLS is localized to the nucleus in each of these strains, suggesting that the defect in Pho4 import in a *pse1-1* mutant is the result of a defect in a specific import pathway. Taken together, these data suggest that Pse1 is the import receptor for Pho4.

Identification of the Pho4 NLS

To identify regions of Pho4 required for its import, we constructed several Pho4 deletion mutants as fusion proteins with GFP and analyzed their localization in a strain lacking *PHO80*. Pho4 cannot be phosphorylated in a *pho80Δ* strain, leading to its constitutive nuclear localization (Kaffman et al. 1994; O'Neill et al. 1996), and thereby simplifying analysis of the steady state localization of the fusion proteins. The NLS of many

transcriptional regulators resides within the DNA binding domain (Williams et al. 1997; Latimer et al. 1998). Mutational analysis of Pho4 demonstrates that the Pho4 DNA binding domain (Figure 3A) is neither necessary nor sufficient for nuclear localization in a *pho80Δ* strain ($Pho4_{2\text{-}247}$-GFP, $Pho4_{248\text{-}312}$-GFP_3, Figure 3B). The smallest domain necessary for Pho4 import lies within amino acids 157-164 ($Pho4_{\Delta 157\text{-}164}$-GFP, Figure 3B); however, this small domain is not sufficient for nuclear import ($Pho4_{156\text{-}171}$-GFP_3, Figure 3B). Amino acids 140-166 of Pho4 define the smallest domain of Pho4 that is both necessary ($Pho4_{\Delta 141\text{-}165}$-GFP, Figure 3B) and sufficient ($Pho4_{140\text{-}166}$-GFP_3, Figure 3B and Figure 3C) for nuclear import. These data demonstrate that Pho4 amino acids 140-166 function as an NLS in vivo (Figure 3B and Figure 3C). The $Pho4_{140\text{-}166}$-GFP_3 fusion protein is not imported into the nucleus in a *pse1-1* mutant, indicating that this fusion protein is targeted to the nucleus via the *PSE1*-dependent import pathway (Figure 3C). It is interesting to note that this domain of Pho4 contains a single site for phosphorylation by Pho80-Pho85 (Figure 3D). $Pho4_{140\text{-}166}$-GFP_3 is localized to the nucleus in both high and low phosphate media (data not shown), presumably because the peptide NLS cannot be phosphorylated by the Pho80-Pho85 kinase. These studies demonstrate that Pho4 contains a single NLS which is targeted to the nucleus in a *PSE1*-dependent manner.

Pho4 binds to Pse1 directly

If Pse1 is the import receptor for Pho4, we expect Pse1 to bind to Pho4. To test for interaction between Pho4 and Pse1, we purified a fusion protein from *E. coli* consisting of Pho4 joined to two z domains derived from Protein A ($Pho4^{WT}$-zz). $Pho4^{WT}$-zz was immobilized on IgG Sepharose and incubated with extract derived from yeast expressing Pse1-GFP. The resin was washed, bound proteins were eluted from $Pho4^{WT}$-zz, separated by SDS-PAGE, and analyzed by immunoblotting using anti-GFP antibodies. We find that $Pho4^{WT}$-zz binds Pse1-GFP (Figure 4B). As a control for the functional relevance of the Pho4-Pse1 interaction, we examined interaction of Pse1 with $Pho4_{\Delta 157\text{-}$

$_{164}$-zz, a Pho4 mutant that cannot be targeted to the nucleus (Figure 3B and 4A). No detectable interaction is observed between Pho4$_{\Delta 157\text{-}164}$-zz and Pse1-GFP (Figure 4B, lane 3). However, both Pho4$_{\Delta 157\text{-}164}$-zz and Pho4^{WT}-zz interact to the same extent with the transcription factor Pho2, known to bind Pho4 (Magbanua et al. 1997), suggesting that both proteins are properly folded (data not shown). These data indicate that Pse1 and Pho4 interact and suggest that the interaction is functionally relevant for import of Pho4.

To examine the specificity of the interaction between Pho4 and Pse1, we tested for interaction between Pho4 and the closely related importin β family members Sxm1 and Kap123 (Gorlich et al. 1997; Seedorf and Silver 1997). In yeast extract, neither Kap123-GFP nor Sxm1-GFP binds immobilized Pho4^{WT}-zz (Figure 4C). These results are consistent with the in vivo localization data (Figure 2) and suggest that the interaction between Pho4 and Pse1 is specific for this importin β family member.

Next, we wished to determine if the interaction between Pse1 and Pho4 was direct, or mediated by another protein. To test for a direct interaction between Pho4 and Pse1, we incubated Pho4^{WT}-zz with Pse1-6His, both purified from *E. coli*, in the presence of either yeast extract or bovine serum albumin. We find that Pho4^{WT}-zz binds directly to Pse1-6His and that this interaction is not potentiated by proteins in the yeast extract, indicating that Pse1 directly recognizes Pho4 (Figure 5).

The Pse1-Pho4 complex is disassembled by Ran-GTP

Studies of other import receptor-cargo interactions suggest that they form stable complexes in the cytoplasm which are then dissociated in the nucleus by binding to Ran-GTP (Rexach and Blobel 1995; Moroianu et al. 1996; Schlenstedt et al. 1997). We wished to determine if the Pho4-Pse1 complex could be dissociated by incubation with the yeast Ran homologue, Gsp1. Pse1-6His was prebound to Pho4^{WT}-zz and the Pse1-Pho4^{WT}-zz complex was incubated with either Gsp1-GTP, Gsp1-GDP, or a buffer control. Pse1 dissociates from Pho4 when incubated with Gsp1-GTP, but not when incubated with

Gsp1-GDP or buffer alone (Figure 6). The N-terminally truncated form of Pse1 (labeled as *) which lacks the Ran-binding domain (data not shown), does not dissociate from Pho4^{WT}-zz after incubation with Gsp1-GTP (Figure 6, lanes 4 to 6). These data are consistent with a model in which Pho4 binds Pse1 in the cytoplasm and is released in the nucleus by the binding of Gsp1-GTP to Pse1. These results provide further support for the functional relevance of the interaction between Pho4 and Pse1.

Phosphorylation of Pho4 regulates its association with Pse1

Since phosphorylation regulates the nuclear localization of Pho4, we sought to determine the effect of phosphorylation on the interaction of Pho4 with Pse1. Pho4^{WT}-zz was purified from *E. coli* and either phosphorylated in vitro with a yeast extract containing the Pho80-Pho85 cyclin-CDK complex or mock-phosphorylated by incubating with yeast extract in the absence of ATP. Pho4 can be efficiently phosphorylated in vitro and the sites of phosphorylation utilized in vitro and in vivo are indistinguishable (Kaffman et al. 1994). Unphosphorylated and phosphorylated Pho4^{WT}-zz were purified with IgG Sepharose beads and incubated with yeast extract containing Pse1-GFP. The resin was washed, and proteins bound to Pho4^{WT}-zz were eluted and analyzed by SDS-PAGE followed by immunoblotting with an anti-GFP antibody (Figure 7A). We find that Pho4^{WT}-zz interacts preferentially with Pse1 in its unphosphorylated state.

As a control, we performed the same experiment with Pho4^{SA}, a mutant Pho4 containing alanine substitutions at the sites of phosphorylation. We find that "phosphorylated" and mock-phosphorylated Pho4^{SA}-zz have a similar affinity for Pse1-GFP, indicating that the difference in binding we observe between phosphorylated and unphosphorylated Pho4 is the result of specific phosphorylation at the sites utilized by Pho80-Pho85 (Figure 7A, lanes 3 and 4). Moreover, phosphorylated and unphosphorylated Pho4^{WT}-zz and Pho4^{SA}-zz bind Pho85 with the same affinity (data not shown).

To determine if the preferential binding of unphosphorylated Pho4 to Pse1 is mediated by proteins in the yeast extract, we compared binding of purified Pse1-6His to purified phosphorylated and unphosphorylated $Pho4^{WT}$-zz (Figure 7B). We find that purified Pse1-6His binds preferentially to unphosphorylated Pho4, suggesting that phosphorylation of Pho4 reduces its intrinsic affinity for Pse1. These data suggest that phosphorylation of Pho4 regulates its import into the nucleus by preventing its interaction with Pse1.

Discussion

The subcellular localization of Pho4 is regulated by phosphorylation in response to changes in the extracellular concentration of inorganic phosphate. Pho4 is phosphorylated and localized predominantly to the cytoplasm when yeast are grown in phosphate-rich medium, and it is unphosphorylated and concentrated in the nucleus when yeast are starved for phosphate. We wished to determine how phosphorylation of Pho4 regulates its nucleocytoplasmic transport and to identify the machinery that moves Pho4 into and out of the nucleus. We demonstrate here that Pse1 is the import receptor for Pho4 and that phosphorylation of Pho4 directly regulates its association with Pse1, providing a mechanism by which phosphorylation may regulate import of Pho4 in vivo.

Although previous studies have suggested a function for Pse1 in nucleocytoplasmic transport, its exact role is unclear. Pse1 is an essential gene, suggesting that it transports cargo whose proper localization is required for vegetative growth (Chow et al. 1992; Seedorf and Silver 1997). Based on the following data, Pse1/Kap121 was proposed to play a role in both ribosomal protein import and in mRNA export: (1) mRNA export is defective in the *pse1-1 kap123Δ* double mutant, but not the *kap123Δ* or *pse1-1* mutant (Seedorf and Silver 1997); (2) the *kap123Δ* strain is defective in import of the ribosomal protein L25 (Rout et al. 1997; Schlenstedt et al. 1997) and that defect can be suppressed by

overexpression of *PSE1* (Rout et al. 1997); and (3) in yeast extract, Kap123 binds to the ribosomal protein L25 and Pse1 can bind to L25, but only in the absence of Kap123 (Rout et al. 1997; Schlenstedt et al. 1997). These data are consistent with Kap123 being an import receptor for ribosomal proteins and with Pse1 playing a partially redundant role in ribosomal protein import. The mRNA export defect observed in the *pse1-1 kap123Δ* double mutant may be indirect, as Pse1 clearly has a direct role in protein import.

We have demonstrated that nuclear import of Pho4 requires *PSE1*. Pho4 is the first cargo whose import is dependent exclusively on the function of Pse1. Our results, in combination with previous studies, allow us to ascribe unique and separate functions to Pse1 and Kap123, as the *kap123Δ* strain is not defective in Pho4 import, nor does Kap123 interact with Pho4. The essential role for Pse1 in transport remains unclear, because cells lacking Pho4 or exhibiting defects in Pho4 localization do not have growth defects when grown in rich medium. The identification of a 27 amino acid peptide within Pho4 that is targeted to the nucleus in a *PSE1*-dependent manner should facilitate the establishment of a consensus NLS recognized by Pse1. This consensus sequence may lead to the identification of other Pse1 cargoes, providing a better understanding of its essential role in vegetative growth.

We have shown that phosphorylation of Pho4 reduces its affinity for Pse1 and that this reduction in affinity is a direct consequence of phosphorylation and is not mediated by other proteins in yeast extract. Remarkably, the NLS in Pho4 contains a single site of phosphorylation by Pho80-Pho85. It will be of interest to determine the contribution of individual phosphorylation sites to the regulation of the interaction with Pse1. In addition, we wish to understand the structural effect that phosphorylation has on the ability of Pho4 to interact with Pse1.

The subcellular localization of many proteins, including the transcriptional regulators NF-AT, Mig1, Swi5, and Swi6, appears to be regulated by phosphorylation in response to either cell cycle position or extracellular signals (Moll et al. 1991; Sidorova et

al. 1995; Beals et al. 1997; De Vit et al. 1997). The role of phosphorylation in regulation of localization is not understood at a mechanistic level in any of these cases. The cell cycle regulated localization and phosphorylation of the yeast transcription factor Swi5 has striking parallels with the regulation of Pho4. In both systems phosphorylation of a transcription factor by a cyclin-CDK complex results in the localization of the transcription factor to the cytoplasm. For Swi5, a region of the protein containing sites of phosphorylation by the CDK Cdc28 has been shown to be both necessary and sufficient for cell cycle regulated localization (Moll et al. 1991). However, it is unclear if the regulated localization of Swi5 reflects regulation of import and/or export. Additionally, it is not clear if phosphorylation of Swi5 regulates its association with a transport receptor, with an adapter protein, or with a nuclear or cytoplasmic anchor.

Phosphorylation of a fusion protein containing the SV40 large T antigen NLS has been shown to affect its nuclear import by modulating its interaction with importin α (Jans et al. 1991; Xiao et al. 1996; Hubner et al. 1997). However, these phosphorylation events have not been shown to occur in vivo, nor have they been observed to regulate the nuclear import of full-length SV40 large T antigen. Our work provides a simple demonstration of how phosphorylation of a protein by a physiologically-relevant kinase modifies its association with a transport receptor. This change in binding affinity could regulate the rate of nuclear import, thereby leading to a change in the nucleocytoplasmic localization of the cargo.

Previously, a correlation between the phosphorylation state of a protein and its cytoplasmic compartmentalization has been interpreted as evidence for regulated import. The discovery of nuclear export pathways has led to the understanding that both nuclear import and/or export are potential points of regulation. The regulated localization of Pho4 in response to extracellular concentrations of inorganic phosphate is likely to reflect control of both import and export of Pho4. Data presented in this manuscript suggest that import of Pho4 is regulated by phosphorylation and our unpublished studies suggest that export of

Pho4 also is regulated by phosphorylation. An understanding of how phosphorylation of Pho4 leads to a change in its subcellular localization will require an experimental dissection of the effect of phosphorylation on the rates of import and export.

We propose that phosphorylation of a transcription factor in response to an environmental cue acts as a molecular switch, inhibiting import and, at the same time, promoting export. Blocking import prevents further nuclear entry of a transcription factor, while enhancing export leads to a rapid elimination of residual nuclear activity. This type of molecular switch allows cells to utilize nucleocytoplasmic localization of a transcription factor as a way to regulate gene expression in a rapid and efficient way.

Materials and methods

Yeast strains and plasmids

Yeast strains were grown in YEPD or synthetic (SD) medium supplemented with amino acids (Sherman 1991) (except in phosphate starvation assays; see below). *pho4Δ::TRP1* (EY0130) and *pho4Δ::TRP1 pho80Δ::HIS3* (EY0219) yeast strains derived from K699 (Nasmyth et al. 1990) were generated by standard gene replacement techniques (Rothstein 1991). PSY580, *pse1-1*, *kap123Δ* have been described previously (Seedorf and Silver 1997). Yeast transformations were performed by the lithium acetate method essentially as described (Guthrie and Fink 1991).

pACPHO4-GFP (EB0347) was constructed as follows. First, the *PHO4* promoter (nucleotides -323 to -1 upstream of the ATG) was amplified by PCR and subcloned into pRS316-GFP, which contains GFP^{S65T} (Heim et al. 1995) cloned into the Eco RI and Bam HI sites of pRS316 (Sikorski and Hieter 1989), to generate pPHO4pr-GFP (EB0346). The entire *PHO4* coding region amplified by PCR was then subcloned in front of *GFP* in pRS316-GFP as a Bgl II-Eco RI fragment to generate pACPHO4-GFP. pACPHO4^{SA}-GFP (EB0356) is identical to pACPHO4-GFP except there are five serine to alanine substitutions at the sites of phosphorylation by Pho80-Pho85 (O'Neill et al. 1996). pPHO4-GFP_3 (EB0757) was constructed by amplifying two additional copies of the complete GFP^{S65T} gene which were cloned into pPHO4-GFP as Mfe I-Eco RI fragments. pPHO4-GFP derivatives (pPHO4$_{2\text{-}247}$-GFP [EB0350], pPHO4$_{2\text{-}94}$-GFP_3 [EB0824], pPHO4$_{248\text{-}312}$-GFP_3 [EB0825], pPHO4$_{\Delta 95\text{-}162}$-GFP [EB0364], pPHO4$_{\Delta 172\text{-}192}$-GFP [EB0365], pPHO4$_{\Delta 141\text{-}165}$-GFP [EB0823], pPHO4$_{\Delta 157\text{-}164}$-GFP [EB0383], pPHO4$_{95\text{-}166}$-GFP_3 [EB0832], pPHO4$_{140\text{-}171}$-GFP_3 [EB0834], pPHO4$_{156\text{-}171}$-GFP_3 [EB0835], pPHO4$_{140\text{-}166}$-GFP_3 [EB0836]) were constructed by amplifying *PHO4* from pAC312 with the appropriate primers and subcloning the resulting fragments into either pACPHO4-GFP or pPHO4-GFP_3, replacing full-length *PHO4*. Further information on the

construction of Pho4-GFP derivatives is available upon request. pT7-PHO4^{WT}-zz (EB0801) was generated by amplifying two Protein A z-domains (zz) from pTL27 (Lafontaine and Tollervey 1996) and using the zz fragment was used to replace the Eco RI-Bam HI fragment, containing *GFP*, in pACPHO4-GFP. The Nco I-Bam HI fragment containing Pho4^{WT}-zz was then cloned into pET16b (Novagen) to generate pT7-PHO4^{WT}-zz. pT7-PHO4^{SA}-zz (EB0814) is identical to pT7-PHO4^{WT}-zz except there are five serine to alanine substitutions at the sites of phosphorylation by Pho80-Pho85 (O'Neill et al. 1996). pT7-PHO4$_{\Delta 157\text{-}164}$-zz (EB0769) was generated by first subcloning the Eco RI-Bam HI fragment containing zz from pPHO4-zz into pPHO4$_{\Delta 157\text{-}164}$. Next, PHO4$_{\Delta 157\text{-}164}$-zz was subcloned as an Nco I-Bam HI fragment into pET16b (Novagen). pPSE1-GFP (pPS1069) and pKAP123-GFP (pPS1070) have been described previously (Seedorf and Silver, 1997). pPSE1-6His (EB0815) was constructed by PCR amplifying *PSE1* from the yeast genome (Promega) and subcloning the fragment into the Bam HI site of pQE60 (Qiagen) to generate C-terminally His-tagged Pse1. pSH101-1 (a gift from Shai Shaham, University of California, San Francisco) consists of *GSP1* fused to 3 copies of the *myc* epitope tag under the control of the *GSP1* promoter in pRS303 (Sikorski and Hieter 1989). pT7-myc-GSP1 was constructed by amplifying *mycGSP1* from pSH101-1 and cloning the amplified fragment into the Nde I and Xho I sites of pAED4. All constructs were verified by sequencing except pPSE1-6His which was shown to complement a *pse1Δ* mutant (data not shown).

Phosphate starvation assay and microscopy

Yeast were grown overnight at 25°C in SD supplemented with amino acids (high phosphate medium) and then diluted and grown to an OD_{600} of 0.1 to 0.3. One ml of this culture was pelleted and the pellet was resuspended in 50 µl of high phosphate medium for steady state high phosphate localization of GFP derivatives. 2 µl of the high phosphate culture was placed on a microscope slide and GFP localization in live cultures was monitored by direct

fluorescence. The remaining yeast culture was pelleted by centrifugation and washed twice with 10 ml of water, once with 1 ml of water, and then resuspended in no phosphate medium (SD medium with potassium chloride replacing monobasic potassium phosphate) to an OD_{600} of 0.1 to 0.3. Yeast were grown in no phosphate medium for 30 to 45 min to starve the yeast for phosphate. One ml of phosphate-starved culture was pelleted and resuspended in 50 µl of no phosphate medium. 2 µl of the starved culture was place on a microscope slide and GFP localization was monitored. All images documenting GFP localization were collected with a CCD camera using identical settings (Photometrics).

Recombinant protein expression and purification

Pse1 was expressed in *E. coli* with a 6His tag at the C-terminus. Bacterial cells (strain SG13009) containing the pPSE1-6His expression vector were grown in LB + 100 µg/ml carbenicillin + 25 µg/ml kanamycin to an OD_{600} of 0.6 and expression was induced with 1 mM IPTG for 3 hr at room temperature. Cells were resuspended in lysis buffer (30 mM Tris-Cl pH 8, 500 mM NaCl, 0.05% Tween-20, and 2 mM β-mercaptoethanol) containing 30 mM imidazole and protease inhibitors (1 mM PMSF, 2 mM benzamidine, 1 µg/ml pepstatin A, 1 µg/ml leupeptin), lysed by sonication, and then cleared by centrifugation at 17,000 rpm in an SS34 rotor for 20 min. The lysate containing the Pse1-6His fusion protein was loaded on a 1 ml chelating HiTrap column (Pharmacia) at 1 ml/min in lysis buffer containing 30 mM imidazole and protease inhibitors and eluted with a 30 to 1000 mM imidazole gradient. Eluted proteins were dialyzed against 100 mM NaCl, 30 mM Tris-Cl pH 8. After dialysis, sorbitol was added to a final concentration of 250 mM and the purified protein was frozen in small aliquots and stored at -80°C. Approximately 90% of the purified material contains an equal mixture of full length Pse1-6His and a truncation product lacking part of the N-terminus. To express Myc-Gsp1, *E. coli* (strain BL21) containing pT7-mycGsp1 were grown in LB + 100 µg/ml carbenicillin to an OD_{600} of 0.6 and expression was induced with 0.4 mM IPTG for 3 hr at room temperature. Cell pellet

from 3 L of culture was resuspended in 40 ml D-50 (20 mM KH_2P0_4 pH 6.6, 10% glycerol, 2 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 5 μM GTP) containing protease inhibitors (1 mM PMSF, 2 mM benzamidine, 1 μg/ml pepstatin A, 1 μg/ml leupeptin), sonicated, and then spun 20 min in an SS34 rotor at 17,000 rpm. Extract was loaded onto a 10 ml SP Sepharose HR (Pharmacia) column at 2 ml/min and eluted with a 50 to 1000 mM KCl gradient. Fractions containing Myc-Gsp1 were precipitated using 60% ammonium sulfate, resuspended in 1.5 ml F-50 (30 mM Hepes-KOH pH 7.7, 8.7% glycerol, 2 mM $MgCl_2$, 5 μM GTP, 50 mM KCl) and loaded onto a Sephacryl S100 (Pharmacia) column equilibrated in F-50. Fractions containing Myc-Gsp1 were concentrated on a 2 ml Bio-Scale Q column (Bio-Rad) and the purified protein (> 95% pure) was frozen in small aliqouts. To produce Pho4-zz fusion proteins, *E. coli* (strain BL21) containing the Pho4-zz expression vector were grown to OD_{600} of 0.6 in LB + 100 μg/ml carbenicillin and induced with 0.4 mM IPTG for 3 hr at room temperature. Cell pellet from 1 L of culture was resuspended in 15 ml IgG buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20, 5 mM $MgCl_2$), sonicated, and spun for 1 hr at 60,000 x g. Sucrose was added to a final concentration of 250 mM and the high speed supernatant was frozen in 0.5 ml aliquots. Pho4-zz proteins were purified by diluting this high speed supernatant to ~2 mg/ml in buffer B 0.1 (20 mM Pipes-KOH pH 6.8, 1 mM EDTA, 10% glycerol, 100 mM NaCl) plus protease inhibitors (1 mM PMSF, 2 mM benzamidine, 1 μg/ml pepstatin A, 1 μg/ml leupeptin) and loaded onto a 1 ml SP Sepharose HiTrap column (Pharmacia) at 1 ml/min and eluted with a 100 to 1000 mM NaCl gradient. Fractions containing Pho4-zz (~90% pure) were then frozen in small aliqouts.

Yeast extract preparation

Yeast strain Y57 was grown in 300 ml of synthetic high phosphate medium to an OD_{600} of 1, harvested, and washed with 20 ml of H_20. Cells were resuspended in 2 ml IgG buffer containing protease inhibitors (1 mM PMSF, 2 mM benzamidine, 1 μg/ml pepstatin A, 1

μg/ml leupeptin) and transferred to a 2 ml screw cap eppendorf tube containing approximately 1 ml of 0.5 mm acid washed glass beads. Trapped air was removed and cells were lysed in a mini bead beater (BioSpec Products) using 5 x 1 min strokes with a 1 min resting period on ice. The supernatant was transferred to a new eppendorf and cleared by spinning 2 x 20 min at 4°C in a microfuge at 14,000 rpm and filtering through a 0.22 μm filter. Glycerol was added to a final concentration of 10% and the extract was frozen at -80°C in small aliqouts.

Binding assay

For the assay of Pho4^{WT}-zz and Pho4$_{\Delta 157\text{-}164}$-zz binding to Pse1-GFP, Sxm1-GFP, and Kap123-GFP, saturating amounts of bacterial lysate from either Pho4^{WT}-zz or Pho4$_{\Delta 157\text{-}164}$-zz were bound to 25 μL of IgG Sepharose by incubating for 90 min at 4°C. The resin with bound Pho4-zz (~4mg/ml) was washed with IgG buffer plus protease inhibitors (1 mM PMSF, 2 mM benzamidine, 1 μg/ml pepstatin A, 1 mg/ml leupeptin), followed by IgG buffer containing 1 M NaCl, and then re-equilibrated with IgG buffer. Immobilized Pho4-zz proteins were incubated for 3 hr at 4°C with 1.65 mg of total protein from a Y57 extract expressing one of the importin β family members tagged with GFP. The resin was washed extensively with IgG buffer and proteins bound to Pho4-zz proteins were eluted with IgG buffer containing 1 M $MgCl_2$ and concentrated using methanol-chloroform precipitation. Pho4^{WT}-zz proteins were eluted with 0.5 M HOAc pH 3.4. For binding Pho4^{WT}-zz to purified Pse1-6His, Pho4^{WT}-zz was purified from bacterial lysate as described above and incubated with 50 μg of purified Pse1-6His in the presence of either 1.65 mg of total protein from a Y57 extract or 1.65 mg of BSA for 3 hr at 4°C. Samples were processed as described above. For binding of phosphorylated and unphosphorylated Pho4^{WT}-zz to Pse1-GFP in a yeast extract, 30 μg of immobilized phosphorylated or unphosphorylated Pho4^{WT}-zz were incubated with 1.65 mg of protein from a Y57 extract containing Pse1-GFP and samples were processed as described above. For binding of

Pho4^{WT}-zz to purified Pse1, 30 μg of immobilized phosphorylated or unphosphorylated Pho4^{WT}-zz was purified away from the yeast extract by incubating with 1 M $MgCl_2$ for 10 min at room temperature, re-equilibrating with IgG buffer and incubating further with 10 nM purified Pse1-6His in the presence of 10% glycerol and 2 mg/ml BSA for 1 hr at room temperature. The resin was washed extensively with IgG buffer, eluted with 1 M $MgCl_2$, and concentrated using methanol-chloroform precipitation.

In vitro phosphorylation of Pho4-zz proteins

30 μg of purified Pho4^{WT}-zz or Pho4^{SA}-zz were incubated with approximately 1 mg of yeast whole cell extract containing HA-Pho80 expressed from the *GPD* promoter for 1 hr at room temperature in the presence of phosphatase inhibitors (80 mM β-glycerophosphate, 10 mM NaF, 10 nM calyculin A), and protease inhibitors (1 mM PMSF, 2 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A), either in the presence of an ATP regenerating system (1 mM ATP, 5 mM creatine phosphate, 50 μg/ml creatine kinase), or in the absence of an ATP regenerating system (mock-phosphorylation). The reaction was placed on ice, diluted with IgG buffer to approximately 3 mg/ml, and incubated with 25 μl of IgG Sepharose beads for 2 hr at 4°C. Bound Pho4-zz proteins were purified by extensive washes with IgG buffer followed by a wash with IgG buffer containing 1 M NaCl and then was re-equilibrated in IgG buffer for binding studies.

Dissociation of the Pho4-Pse1 complex by Gsp1-GTP

60 μg of purified Pse1-6His and 60 μg of Pho4^{WT}-zz were bound in a 240 μl reaction for 1 hr at 4°C. Complex containing Pse1-6His bound to Pho4^{WT}-zz was purified by incubating the reaction with 35 μl of IgG Sepharose beads for 2 hr at 4°C. The resin containing immobilized Pho4^{WT}-zz bound to Pse1 was washed extensively with IgG buffer and split into three equal parts. One third of the immobilized Pho4^{WT}-zz bound to Pse1 was incubated with 10 μg of purified Myc-Gsp1 loaded with GTP, one third with 10

μg of purified Myc-Gsp1 loaded with GDP, and one third with buffer alone for 20 min at room temperature. Proteins released during this incubation period were collected (eluate), the resin was washed once with 0.5 ml of IgG buffer, bound proteins were eluted with 1 M $MgCl_2$, and concentrated using methanol-chloroform precipitation. Proteins were separated on 10% SDS-PAGE and visualized by staining with Coomassie blue.

Nucleotide loading of Myc-Gsp1 was performed as follows. Purified Myc-Gsp1 (0.67 mg/ml) was incubated in the presence of 20 mM EDTA, 2 mM GTP or GDP, and 2 mM DTT for 1 hr at room temperature. $MgCl_2$ was added to a final concentration of 50 mM and the reaction was further incubated on ice for 20 min. Unbound nucleotides were removed by loading the sample over an Bio-Spin P-6 spin column (Bio-Rad) equilibrated in F-50 and the Myc-Gsp1 was frozen in small aliquots.

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Figure Legends

Figure 1. Pho4-GFP does not accumulate in the nucleus in *pse1-1* yeast. Pho4-GFP localization was monitored in *PSE1*+ (PSY580) or *pse1-1* yeast grown at 25°C in either high or no phosphate medium.

Figure 2. Nuclear import of $Pho4^{SA}$-GFP is defective in *pse1-1* yeast. The steady-state localization of $Pho4^{SA}$-GFP and Pho4-GFP-NLS was monitored in *PSE1+ KAP123+* (PSY580), *pse1-1*, or in *kap123Δ* yeast grown in high phosphate medium at 25°C. $Pho4^{SA}$-GFP is a mutant derivative of Pho4 that contains serine to alanine substitutions at all of the sites of phosphorylation by Pho80-Pho85 (O'Neill et al., 1996). Pho4-GFP-NLS contains the SV40 NLS fused to the C-terminus of Pho4-GFP.

Figure 3. Pho4 amino acids 140-166 are necessary and sufficient for nuclear localization. (*A*) Structure and function of Pho4. A domain important for Pho4 transactivation is contained within Pho4 amino acids 1-109 (hatched) (Ogawa and Oshima 1990). The Pho4 DNA binding domain has been localized to amino acids 248-312 (grey shaded) (Ogawa and Oshima 1990). The five sites on Pho4 phosphorylated by the kinase Pho80-Pho85 are at amino acids 100, 114, 128, 152, and 223 (O'Neill et al., 1996) and are shown with an asterisk (*). The Pho4 NLS lies within amino acids 140-166 (black). (*B*) Localization of Pho4-GFP derivatives. Pho4-GFP derivatives expressed in *pho4Δ pho80Δ* yeast were grown at 30°C in high phosphate medium. Pho4 deletion mutants smaller than 27 kDa were fused to three copies of GFP (GFP_3), and those larger were fused to a single GFP. Localization of the Pho4-GFP derivatives is indicated (N, nuclear; C, cytoplasmic). (*C*) Pho4 amino acids 140-166 are sufficient to target GFP_3 to the nucleus in a *PSE1*-dependent manner. Localization of $Pho4_{140\text{-}166}$-GFP_3 was monitored in *PSE1+ (pho4Δ pho80Δ)* and in *pse1-1* strains grown at 25°C in high phosphate medium.

(D) The Pho4 NLS resides within amino acids 140-166. The fourth phosphorylation site at Pho4 serine 152 is boxed.

Figure 4. Pho4 binds to Pse1. (*A*) Amino acids 157-164 are required for nuclear localization of Pho4 in vivo. Pho4 lacking amino acids 157-164 fused to GFP ($Pho4_{\Delta 157-164}$-GFP) was expressed in *pho4Δ* yeast grown in high or no phosphate medium at 30°C. (*B*) Pho4 associates with Pse1, but a mutant Pho4 lacking residues required for nuclear localization does not. Wild-type Pho4 and Pho4 lacking amino acids 157-164 were expressed as zz fusion proteins in *E. coli.* zz-containing proteins were purified using IgG Sepharose beads and incubated with yeast extract containing Pse1-GFP. The resin was washed extensively and proteins bound to Pho4-zz were eluted with 1 M $MgCl_2$. Eluted proteins were precipitated, separated on 7.8% SDS-PAGE, transferred to a PVDF membrane, and visualized by immunoblotting with anti-GFP monoclonal antibodies. Lane 1, 3% of the Pse1-GFP extract loaded (L); lane 2, Pse1-GFP bound to $Pho4^{WT}$-zz (WT); lane 3, Pse1-GFP bound to $Pho4_{\Delta 157-164}$-zz (Δ); lane 4, Pse1-GFP bound to IgG Sepharose beads (-). Half of the 1 M $MgCl_2$ eluate was loaded onto the gel. (*C*) Pho4 associates with Pse1, but not Kap123 or Sxm1. $Pho4^{WT}$-zz and $Pho4_{\Delta 157-164}$-zz immobilized on IgG Sepharose beads and IgG Sepharose beads alone were incubated with yeast extract containing expressing either Pse1-GFP, Kap123-GFP, or Sxm1-GFP. The resin was washed extensively and proteins bound to Pho4-zz were eluted with 1 M $MgCl_2$. Eluted proteins were precipitated, separated on 7.8% SDS-PAGE, transferred to PVDF membrane, and visualized by immunoblotting using anti-GFP monoclonal antibodies. For each panel: lane 1, ~3% of the importin-GFP loaded; lane 2, importin-GFP bound to $Pho4^{WT}$-zz; lane 3, importin-GFP bound to $Pho4_{\Delta 157-164}$-zz; lane 4, importin-GFP bound to the IgG Sepharose control.

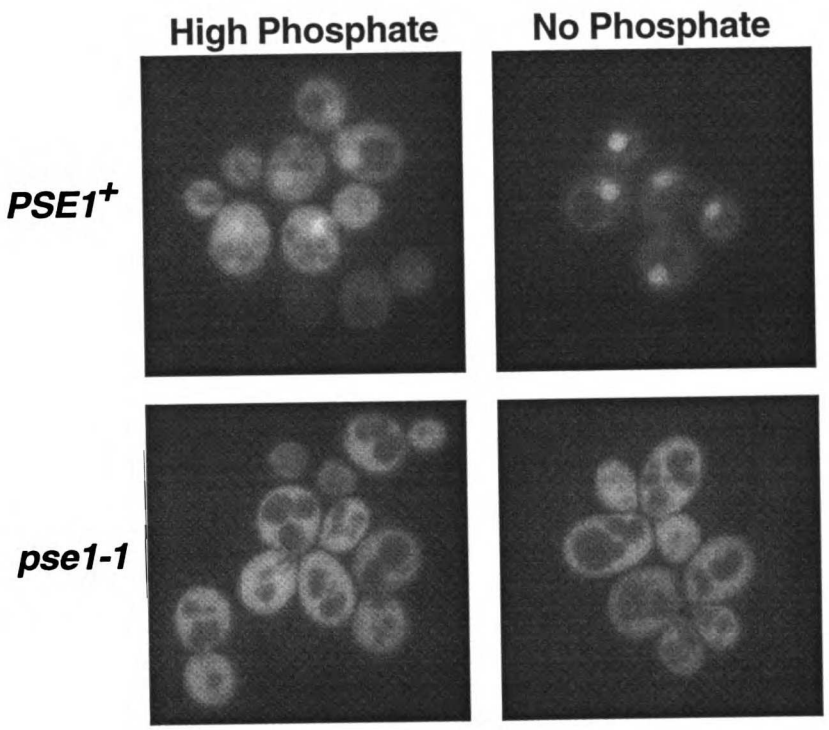
Figure 5. Pho4 interacts directly with Pse1. Pho4^{WT}-zz bound to IgG Sepharose beads was incubated with Pse1-6His purified from *E. coli* in the presence of either yeast extract or bovine serum albumin (BSA). The resin was washed extensively and proteins bound to Pho4^{WT}-zz were eluted with 1 M $MgCl_2$. Eluted proteins were precipitated, separated on 7.8% SDS-PAGE, and visualized by staining with Coomassie blue. Approximately 50% of the Pse1-6His preparation consists of an N-terminally truncated form of Pse1 (denoted by *). Lane 1, Pse1-6His bound to Pho4^{WT}-zz in the presence of whole cell extract (WCE); lane 2, Pse1-6His bound to Pho4^{WT}-zz in the presence of BSA; lane 3, Pse1-6His bound to IgG Sepharose beads alone in the presence of WCE; lane 4, 4% of the load.

Figure 6. Gsp1-GTP dissociates the Pho4-Pse1 complex. Purified Pse1-6His and Pho4^{WT}-zz were incubated and the Pse1-Pho4^{WT}-zz complex was purified using IgG Sepharose beads. The resin was washed extensively, split into three equal parts, and incubated with Gsp1-GTP, Gsp1-GDP, or buffer alone. Proteins released during this treatment were collected as the eluate. The N-terminally truncated form of Pse1-6His (denoted by *), which cannot bind Ran-GTP (data not shown), is not dissociated from Pho4^{WT}-zz by incubation with Gsp1-GTP. The Pho4^{WT}-zz resin was washed once with IgG buffer, and proteins bound to Pho4^{WT}-zz were eluted with 1 M $MgCl_2$ and precipitated (bound fraction). Proteins were separated on 10% SDS-PAGE and visualized by staining with Coomassie blue. Lanes 1 and 4, Gsp1-GTP; lanes 2 and 5, Gsp1-GDP; lanes 3 and 6, buffer alone. One-third of the eluate and one-half of the bound proteins were loaded onto the gel.

Figure 7. Phosphorylated Pho4 has reduced affinity for Pse1. (*A*) Phosphorylation affects binding of Pho4 to Pse1 in an extract. Purified Pho4^{WT}-zz and Pho4^{SA}-zz, a mutant containing serine to alanine substitutions at the sites of phosphorylation, were phosphorylated or mock-phosphorylated in vitro in an extract containing the Pho80-Pho85

cyclin-CDK complex. zz-containing proteins were purified from the extract with IgG Sepharose beads, washed extensively, and incubated with extract containing Pse1-GFP. The resin was washed and proteins bound to $Pho4^{WT}$-zz and $Pho4^{SA}$-zz were eluted with 1 M $MgCl_2$. Eluted proteins were precipitated, separated on 7.8% SDS-PAGE, transferred to PVDF membrane, and visualized by immunoblotting with anti-GFP monoclonal antibodies (top panel). To ensure that the same amount of Pho4 was immobilized on IgG Sepharose, Pho4-zz proteins were eluted from IgG Sepharose with acetic acid, separated on 7.8% SDS-PAGE, and visualized by staining with Coomassie blue (bottom panel). Lane 1, mock-phosphorylated $Pho4^{WT}$-zz; lane 2, phosphorylated $Pho4^{WT}$-zz; lane 3, mock-phosphorylated $Pho4^{SA}$-zz; lane 4, phosphorylated $Pho4^{SA}$-zz. Phosphorylation by Pho80-Pho85 causes $Pho4^{WT}$-zz to migrate slower than unphosphorylated $Pho4^{WT}$-zz in SDS-PAGE. (*B*) Phosphorylation affects Pho4 binding to purified Pse1. $Pho4^{WT}$-zz was phosphorylated or mock-phosphorylated and the immobilized proteins were incubated with Pse1-6His purified from *E. coli*. The resin was washed and proteins bound to $Pho4^{WT}$-zz were eluted with 1 M $MgCl_2$. Eluted proteins were precipitated, separated on 7.8% SDS-PAGE, and visualized by staining with silver (top panel). To ensure that the same amount of Pho4 was immobilized on IgG Sepharose, $Pho4^{WT}$-zz proteins were eluted from IgG Sepharose with acetic acid, separated on 7.8% SDS-PAGE, and visualized by staining with Coomassie blue (bottom panel). The truncated form of Pse1 is indicated (*).

Figure 1



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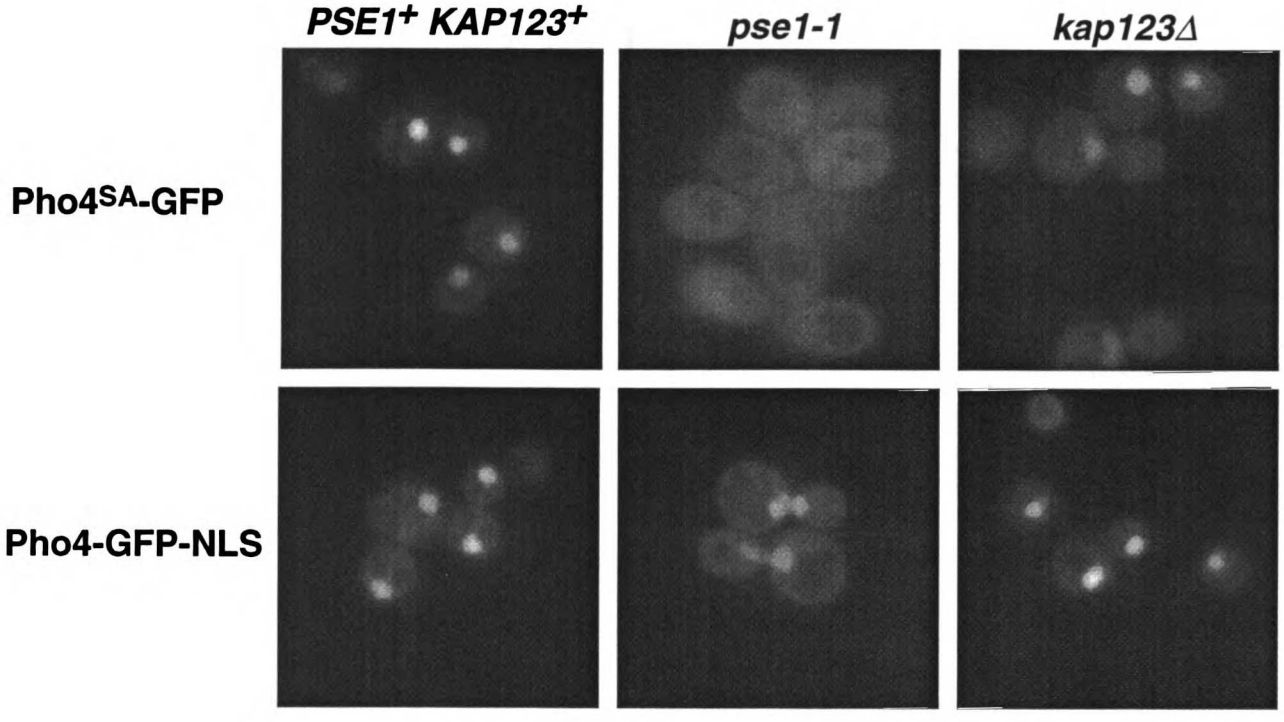

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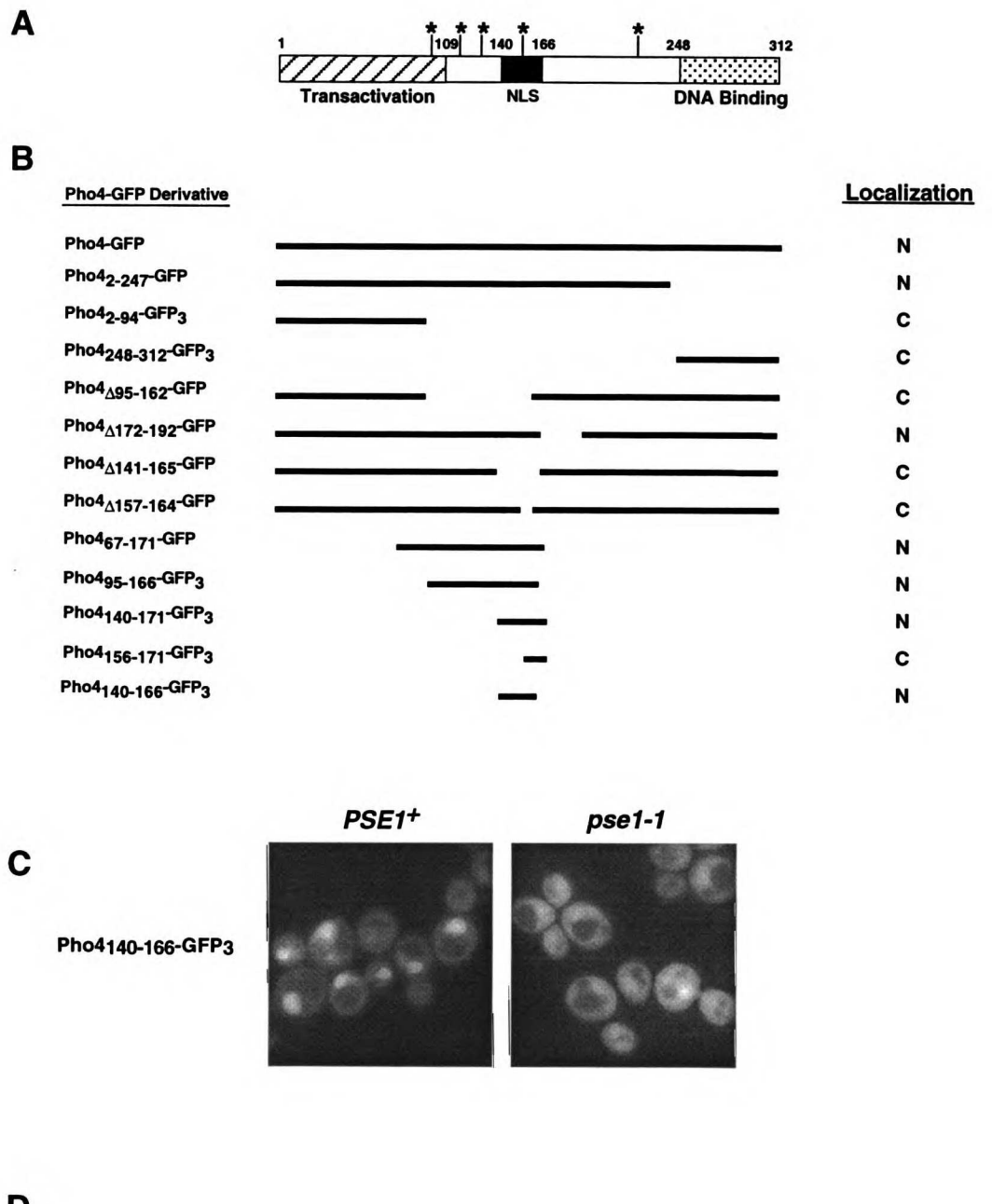
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Figure 4

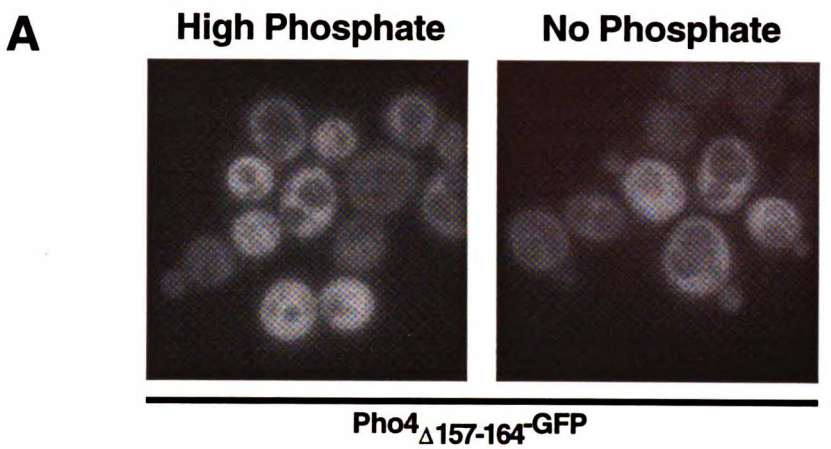
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Figure 5

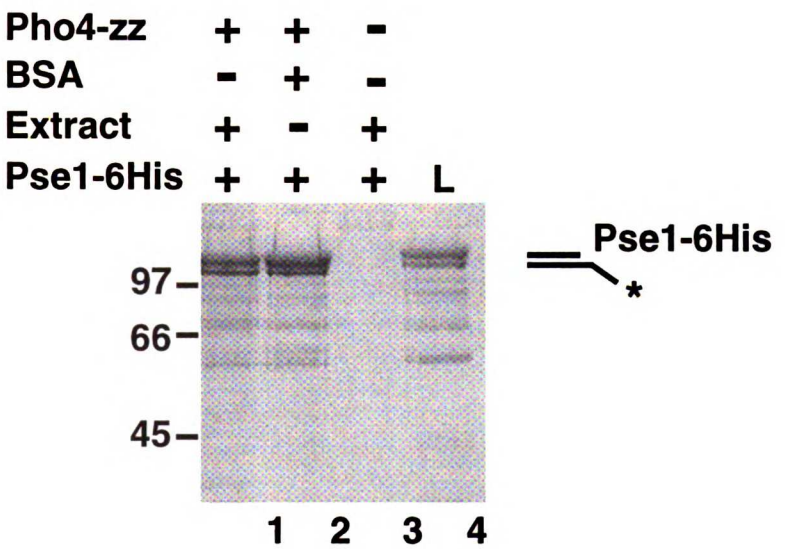

Figure 6

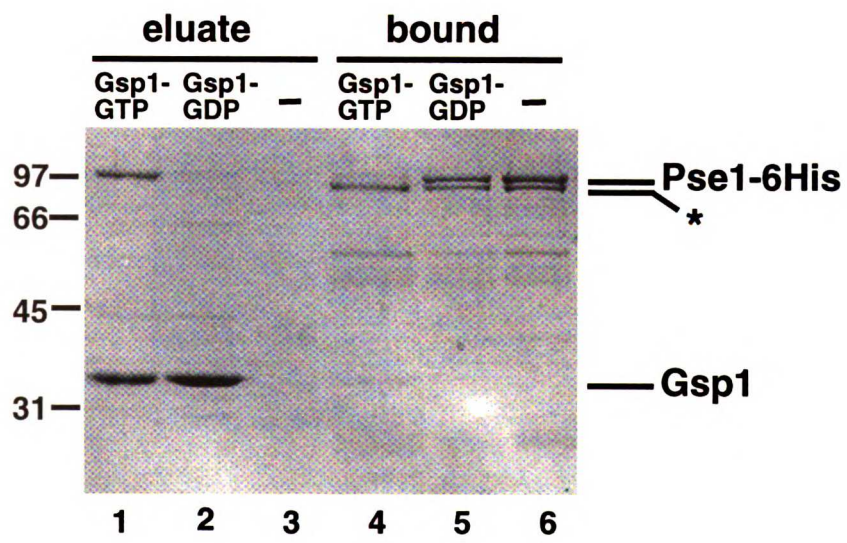
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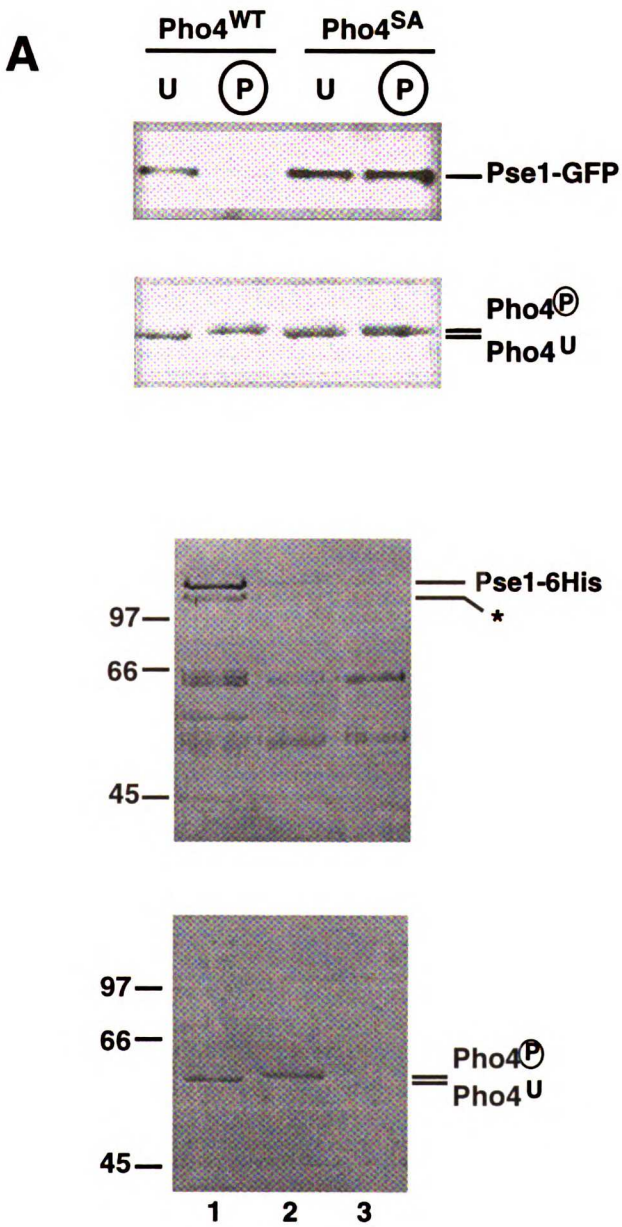
Figure 7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Chapter 3

The Receptor Msn5 Exports the Phosphorylated Transcription Factor Pho4 Out of the Nucleus

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Credits: Nicole Rank helped with microscopy, making the figures, and reproduced Elizabeth O'Neill's data shown in figure 1. Elizabeth O'Neill is responsible for figure 2. Linda Huang made the Msn5Δ strain and the Myc-Msn5 construct and I made the initial observation that Msn5 is required for export of Pho4 in vivo and that Msn5 binds preferentially to phosphorylated Msn5 (Fig 3 and 4). Reprinted by permission from Nature 396: 482-86 copyright (1998) Macmillan Magazines Ltd.

Movement of many transcription factors, kinases, and replication factors between the nucleus and cytoplasm plays an important role in regulating their activity[1]. In some cases, phosphorylation of a protein regulates its entry into the nucleus[2]. In other cases, phosphorylation of a protein leads to its export to the cytoplasm[3-6]. The mechanism by which phosphorylation promotes nuclear export is poorly understood. Here we examine how export of the yeast transcription factor Pho4 is regulated in response to changes in phosphate availability. We show that phosphorylation of Pho4 by a nuclear cyclin-CDK complex, Pho80-Pho85, triggers its export from the nucleus. We demonstrate that the importin β family member Msn5[7,8] is the nuclear export receptor for Pho4. Msn5 is required for nuclear export of Pho4 *in vivo* and binds directly and exclusively to phosphorylated Pho4 in the presence of the GTP-bound form of yeast Ran *in vitro*. These results reveal a simple mechanism by which phosphorylation enhances nuclear export of a protein and serve as a paradigm for understanding other examples of phosphorylation-induced export.

When yeast are grown in phosphate-rich medium, the transcription factor Pho4 is phosphorylated by the Pho80-Pho85 cyclin-CDK complex[9] and is localized to the cytoplasm[10], thereby turning off transcription of genes specific to phosphate starvation[11]. When yeast are starved for phosphate, the CDK inhibitor Pho81 inactivates Pho80-Pho85[12,13], and Pho4 is not phosphorylated. Unphosphorylated Pho4, but not phosphorylated Pho4, is recognized by the non-classical import receptor Pse1/Kap121, suggesting that import of Pho4 is regulated in response to phosphate availability[14].

For several reasons, the regulation of Pho4 import alone is unlikely to be sufficient to account for the change in Pho4 localization induced by phosphorylation. First, we observed that the Pho80-Pho85 cyclin-CDK complex was localized to the nucleus (Fig. 1a), while previously, it was shown that phosphorylated Pho4 is cytoplasmic[10]. These observations suggest that Pho4 is phosphorylated in the nucleus and exported to the cytoplasm.

Second, we observed that phosphorylated Pho4 was rapidly exported from the nucleus. To monitor export of Pho4, a yeast strain expressing Pho4-GFP was first grown in medium lacking phosphate, leading to accumulation of unphosphorylated Pho4-GFP in the nucleus (Fig. 1b, top). Cycloheximide was added to prevent further protein synthesis. Phosphate was then added to activate the Pho80-Pho85 kinase, and localization of Pho4-GFP was monitored. After three to six minutes the localization of Pho4-GFP was

indistinguishable from that observed in cells grown in phosphate-rich medium (Fig. 1b, top). If no phosphate was added to the culture, Pho4-GFP remained in the nucleus for several hours (data not shown). An alternative model, where phosphorylation of Pho4-GFP leads to degradation of nuclear Pho4-GFP followed by cytoplasmic accumulation of newly synthesized protein, is unlikely because protein synthesis was inhibited. To test the role of phosphorylation in the rapid export of Pho4 we examined export of $Pho4^{SA}$, a mutant Pho4 containing five serine to alanine substitutions at the sites of phosphorylation by Pho80-Pho85[10]. $Pho4^{SA}$ did not leave the nucleus upon addition of phosphate to a starved culture of yeast (Fig. 1b, bottom). In addition, in strains lacking either the cyclin Pho80 or the CDK Pho85, Pho4-GFP did not leave the nucleus upon addition of phosphate (data not shown). These data indicate that phosphorylation of Pho4 is required for its cytoplasmic accumulation.

To test directly whether nuclear export of Pho4 is also regulated by phosphorylation, it is necessary to examine export in the absence of import[15]. If export of Pho4 is enhanced by phosphorylation, phosphorylated Pho4 will accumulate in the cytoplasm more rapidly than unphosphorylated Pho4 when import is blocked. By contrast, if export of Pho4 is not regulated by phosphorylation, both forms of Pho4 will accumulate in the cytoplasm at the same rate when import is blocked (Fig. 2a).

We first demonstrated that two yeast mutants with a conditional defect in protein import, *nsp1*^{ts}[16,17] and *nup49-313*[16,18], were defective for import of Pho4 when grown at the restrictive temperature (data not shown). An experiment using the *nsp1*^{ts} strain expressing Pho4-GFP was performed as follows (Fig. 2b, top). First, the yeast were starved for phosphate at 23°C, allowing unphosphorylated Pho4-GFP to accumulate in the nucleus. The culture was then shifted to 38.5°C to impair protein import, treated with cycloheximide, and split in half. Phosphate was added to half of the culture to activate the Pho80-Pho85 kinase and trigger phosphorylation of Pho4, and was not added to the other half. Pho4-GFP was rapidly transported to the cytoplasm in the culture to which phosphate had been added, while it remained nuclear in the untreated culture for several more hours (Fig. 2b, bottom). In addition, when phosphate was added, Pho4^{SA} remained in the nucleus for several hours when import was blocked (data not shown). These data suggest that phosphorylation regulates nuclear export of Pho4.

To understand the molecular mechanism by which phosphorylation regulates Pho4 export, we wished to identify the Pho4 export receptor. We therefore examined the localization of Pho4-GFP in yeast strains containing mutations in putative nuclear transport receptors. Thirteen proteins in yeast have homology to the nuclear localization signal (NLS) import receptor importin β[7,8]. Some family members are transport receptors, while others have not yet been characterized. No defect in Pho4 export was

observed in yeast strains containing mutations in the importin β family members Kap104[19], Cse1[20], Xpo1[21], Sxm1[22], Kap123[22,23], and Mtr10[24]. However, in a strain lacking the importin β family member Msn5/Ste21, Pho4-GFP could not be exported and was localized constitutively to the nucleus (Fig. 3a). The defect in Pho4 localization in the *msn5Δ* strain is not an indirect result of a defect in the phosphate signal transduction pathway, since Pho4 is still phosphorylated normally in this strain (Fig. 3b). Although Pho4-GFP was localized to the nucleus in the *msn5Δ* strain, expression of the secreted acid phosphatase Pho5 was not induced due to additional mechanisms for regulating the transcriptional activity of Pho4 (manuscript in preparation).

One model to explain regulated export of Pho4 is that Msn5 preferentially binds the phosphorylated form of Pho4. We tested this hypothesis by performing an *in vitro* binding experiment. Since all protein export pathways characterized to date require the small GTPase Ran in the GTP-bound state[25-28], we tested if Ran-GTP was required for Msn5 to bind Pho4. We observed that phosphorylated Pho4 bound to Msn5 in yeast extract when a Ran mutant was added that is locked in the GTP-bound state (human RanQ69L-GTP)[29] (Fig. 4a, top, lane 1). In the absence of RanQ69L-GTP, no interaction was observed between phosphorylated Pho4 and Msn5 (Fig. 4a, top, lane 2). Pho4 must be phosphorylated to interact with Msn5, since even in the presence of RanQ69L-GTP no

interaction was seen between Msn5 and the mutant Pho4^{SA}, which cannot be phosphorylated (Fig. 4a, top, lane 3).

There are several models that could explain the preferential binding of Msn5 to phosphorylated Pho4. Preferential binding: (1) might require an adapter protein to facilitate the interaction with phosphorylated Pho4; (2) might require a protein that masks the Msn5 binding site on unphosphorylated Pho4; or (3) may be a direct consequence of the ability of Msn5 to distinguish between the two forms of Pho4. To determine whether Msn5 is sufficient to bind preferentially to the phosphorylated form of Pho4, we used purified recombinant proteins to test binding of Pho4 to Msn5 and the yeast Ran homologue Gsp1. Purified Msn5 bound preferentially to phosphorylated Pho4 in the presence of Gsp1 loaded with GTP (Fig. 4b, top, lane 1), but not when Gsp1 was loaded with GDP (Fig. 4b, top, lane 3). Purified Msn5 did not interact with unphosphorylated Pho4, even in the presence of Gsp1-GTP (Fig. 4b, top, lane 2). These data indicate that no additional proteins are required for Msn5 to bind preferentially to phosphorylated Pho4 and Gsp1, and that the interaction between Pho4 and Msn5 requires Gsp1-GTP. The observation that Gsp1-GTP bound to Pho4 in the presence of Msn5 (Fig. 4b, middle panel), but not in its absence (data not shown) suggests that phosphorylated Pho4, Gsp1-GTP, and Msn5 form a ternary complex. These results are consistent with the properties

of other characterized export receptor-cargo complexes, which have been shown to require the export receptor, cargo, and Ran-GTP for stable complex formation[25-28].

Our data suggest a model to explain how localization of Pho4 is regulated by phosphorylation (Fig. 4c). In high phosphate medium the activated Pho80-Pho85 cyclin-CDK complex phosphorylates Pho4 in the nucleus, triggering association between phosphorylated Pho4, the export receptor Msn5, and Gsp1-GTP. This trimeric complex is then rapidly exported from the nucleus. By analogy to what has been shown for the other export receptors[25-28], we propose that the Pho4-Msn5-Gsp1-GTP complex is disassembled in the cytoplasm by hydrolysis of GTP by Gsp1, stimulated by Rna1 (yeast RanGAP) and Yrb1 (yeast RanBP1). Phosphorylated cytoplasmic Pho4 is then prevented from re-entering the nucleus because phosphorylation of Pho4 inhibits interaction with the import receptor Pse1[14]. We have determined that the phosphorylation sites necessary and sufficient for regulating export are distinct from the site that regulates Pho4 import (manuscript in preparation). The dual role for phosphorylation in promoting export and preventing reimport is likely to be a general mechanism, as it allows cells to rapidly and efficiently regulate gene expression in response to environmental signals.

Two distinct models can explain the ability of Msn5 to preferentially recognize phosphorylated Pho4. The Msn5 binding site present on phosphorylated Pho4 could be revealed by a phosphorylation-induced conformational change. Alternatively, Msn5 may

recognize a phosphopeptide within Pho4. In this case, Msn5 may also function as an export receptor for other phosphorylated proteins. The existence of an export receptor dedicated to phosphorylated cargo would provide a simple mechanism for selectively exporting proteins that are phosphorylated in response to cellular signals.

While many proteins are regulated by phosphorylation-induced export from the nucleus, the molecular mechanism by which phosphorylation induces nuclear export has not previously been understood. Our studies reveal a novel pathway for nuclear export which utilizes an export receptor that requires only Ran-GTP to bind exclusively to the phosphorylated form of its cargo. These results provide a paradigm for understanding how phosphorylated proteins are exported from the nucleus.

Methods

Plasmids and strains. *PHO4*, *PHO80*, and *PHO85* were deleted in strain K699 (*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*) and *MSN5* and *PHO4* were deleted in strain IH3195 (*MATa HMLa HMRa ura3-52 ade2-101 met1 leu2Δ1 trp1Δ99*), which is isogenic to JC2-1B[30], by standard gene replacement. *nup49-313*, the isogenic *NUP49*$^{+}$ strain[16,18], and *nsp1*^{ts} and its isogenic strain[16,17] have been previously described. High phosphate cultures were grown in standard synthetic dropout medium (SD), low phosphate cultures were grown in phosphate-depleted SD dropout medium, and no

phosphate cultures were grown in SD dropout medium with KCl replacing KH_2PO_4[14]. The construction of plasmids expressing $Pho4^{WT}$ and $Pho4^{SA}$ fused to either GFP or to the zz tag from Protein A, and the plasmid expressing Myc-Gsp1 have been described[14]. Pho80-GFP and Pho85-GFP were expressed from their own promoter on a high-copy or low-copy plasmid, respectively. Both plasmids complement the phenotype of the respective deletion strain. Detailed descriptions of plasmids used in this study are available upon request.

Detection of phosphorylation of Pho4-zz. Isogenic *pho4Δ* and *pho4Δ msn5Δ* strains expressing $Pho4^{WT}$-zz from a plasmid were inoculated at an OD_{600} of 0.1-0.3 and grown in low phosphate medium for 8 h. Cells were harvested 10 min after the addition of KH_2PO_4 or KCl to a final concentration of 20 mM and frozen in liquid nitrogen. Samples were lysed by boiling for 5 min in the presence of SDS-PAGE loading buffer followed by bead-beating with acid-washed 0.5 mm glass beads for 5 min. Samples were reboiled for 3 min and 10-15 μg of total protein was separated using 7.8% SDS-PAGE and immunoblotted to detect the zz tag.

Phosphate starvation and microscopy. Phosphate starvation and microscopy were performed as described[14], except that photographs for Fig. 2 were taken using Olympus PM-30 camera.

Examination of Pho4^{WT}-GFP in *nsp1^{ts}*. The *nsp1^{ts}* strain harboring a plasmid expressing Pho4^{WT}-GFP was starved for phosphate for 4-8 hours in low phosphate medium at 23°C, resulting in the accumulation of Pho4^{WT}-GFP in the nucleus. Cells were then shifted to 38.5°C for 4-6 hours to impair protein import. Cycloheximide was added to a final concentration of 0.1 mg/ml and the culture was incubated for 10 min. For the + phosphate samples, 4.5 µl of culture plus 0.5 µl of 15mg/ml KH_2PO_4 were mixed on a slide and Pho4-GFP localization was examined by direct fluorescence. Similar results were obtained with *nup49-313* (data not shown).

Recombinant proteins expression and purification. Msn5 was expressed in *Escherichia coli* with a 6His tag at the C-terminus essentially as described for Pse1-6His[14], with the following modifications: Cells were lysed in lysis buffer containing 10 mM imidazole and Msn5-6His was eluted with a 10 mM to 1000 mM imidazole gradient. Myc-Gsp1, Pho4^{WT}-zz and Pho4^{SA}-zz were expressed and purified as described[14]. RanQ69L was expressed in BL21 grown at 37°C in LB + 100µg/ml carbenicillin to OD_{600} of 0.4 and induced with 0.4 mM IPTG at 30°C for 1.5 h and was purified as reported for Myc-Gsp1[14].

Binding assay. *In vitro* phosphorylation of Pho4-zz and loading of Myc-Gsp1 with GTP or GDP were performed as described[14]. Yeast whole cell extract was prepared essentially as described[14] except that phosphatase inhibitors were included (80 mM β-

glycerophosphate, 10 mM NaF, 10 nM calyculin A). Binding of Myc_3-Msn5 to Pho4-zz was essentially as described for Pse1[14], with the following modifications: (1) Purified RanQ69L was added to a final concentration of 5 μM; (2) An ATP regnerating system was added (1 mM ATP, 1 mM GTP, 50 μg/ml creatine kinase, 5 mM creatine phosphate); (3) Phosphatase inhibitors were reduced to 40 mM β-glycerophosphate, 5 mM NaF, and 5 nM calyculin A. Binding with purified components was done by incubating 25 μg of purified Pho4-zz immobilized on IgG-Sepharose beads with a final concentration of 1 μM Myc-Gsp1 and 1 μM Msn5-6His in the presence of 0.5 mg/ml bovine serum albumin for 1.5 h at 4°C.

Figure Legends

Figure 1 Phosphorylation of Pho4 by nuclear Pho80-Pho85 promotes its rapid export from the nucleus. a, Direct fluorescence microscopy of cells harboring a plasmid expressing Pho80-GFP or Pho85-GFP grown in high or no phosphate media. Since both Pho80 and Pho85 are required to phosphorylate Pho4[9], the localization of Pho80 restricts the kinase activity to the nucleus. b, Fluorescence microscopy of cells harboring a plasmid expressing $Pho4^{WT}$-GFP or $Pho4^{SA}$-GFP under the control of the *PHO4* promoter. Cells were starved for phosphate (no phosphate) and incubated with cycloheximide for 10 min. At t = 0 min phosphate was added to the culture and localization of Pho4-GFP was monitored as a function of time.

Figure 2 The rate of Pho4 nuclear export is regulated in response to phosphorylation. a, Predicted outcomes of blocking import when export is either regulated or is constant. b, Top: Outline of the experiment used to determine if the rate of export is regulated in response to phosphorylation. Bottom: Localization of $Pho4^{WT}$-GFP in an *nsp1*^{ts} strain[16,17] that has been starved for phosphate and incubated in cycloheximide at 38°C. The culture was split and phosphate was added to half of the culture (+ phosphate) and

not to the other half (- phosphate). The addition of phosphate to *nsp1*^{ts} resulted in export of Pho4-GFP with the same kinetics as was observed in the wild-type strain.

Figure 3 Msn5 is required for export of Pho4. a, Localization of Pho4^{WT}-GFP in wild-type (*MSN5*$^{+}$) and *msn5Δ* strains grown in high phosphate medium, no phosphate medium, or 10 min after the addition of phosphate to phosphate-starved cells (+ phosphate). b, Immunoblot detecting phosphorylation of Pho4^{WT}-zz in extracts made from *MSN5*$^{+}$ and *msn5Δ* strains. Yeast strains were grown in low phosphate medium, and phosphate was either added (+ P_i) or not added (- P_i) to the culture. Addition of phosphate to *MSN5*$^{+}$ and *msn5Δ* strains expressing Pho4^{WT}-zz grown in phosphate-depleted medium resulted in a shift in the electrophoretic mobility of Pho4, indicating that Pho4 was phosphorylated[9].

Figure 4 Msn5 binds directly to phosphorylated Pho4 in the presence of Ran-GTP. a, *In vitro* phosphorylated Pho4^{WT}-zz (P), Pho4^{SA}-zz (SA), or a no Pho4 control (-) were purified using IgG-Sepharose beads, and incubated with extract containing Myc_3-tagged Msn5 in the presence or absence of RanQ69L[29]. Top: Bound proteins were eluted with 1 M $MgCl_2$, concentrated using methanol-chloroform precipitation, separated on 7.8% SDS-PAGE and immunoblotted with anti-Myc antibodies. Phosphorylated Pho4^{WT}-zz

with RanQ69L (lane 1); phosphorylated Pho4^{WT} without RanQ69L (lane 2); phosphorylated Pho4^{SA}-zz with RanQ69L (lane 3); beads alone with RanQ69L (lane 4). Bottom: To ensure that the same amount of Pho4-zz was immobilized on IgG Sepharose, Pho4-zz was eluted with 0.5 M acetic acid pH 3.4, separated on 7.8% SDS-PAGE, and visualized with Coomassie blue. Lanes are as described above. b, Pho4^{WT}-zz was phosphorylated or mock phosphorylated *in vitro*, and purified using IgG-Sepharose beads. Immobilized proteins were incubated with 1 μM purified Msn5-His in the presence of 1 μM purified Myc-Gsp1 loaded with either GTP or GDP. Top: Bound proteins were eluted with 1M $MgCl_2$, concentrated using methanol-chloroform precipitation, separated on 7.8% SDS-PAGE and visualized with silver staining. Phosphorylated Pho4^{WT}-zz (P; lanes 1, 3); mock phosphorylated Pho4^{WT}-zz (U; lanes 2, 4); Myc-Gsp1 loaded with GTP (lanes 1, 2); Myc-Gsp1 loaded with GDP (lanes 3, 4). Approximately 20% of the Msn5-6His bound to phosphorylated Pho4 in the presence of Gsp1-GTP. Bottom: To ensure that the same amount of Pho4-zz was immobilized on IgG Sepharose, Pho4^{WT}-zz was eluted with 0.5 M acetic acid pH 3.4, separated on 7.8% SDS-PAGE, and visualized with Coomassie blue. Lanes are as described above. c, Model for the regulated nucleocytoplasmic localization of Pho4 in response to extracellular phosphate.

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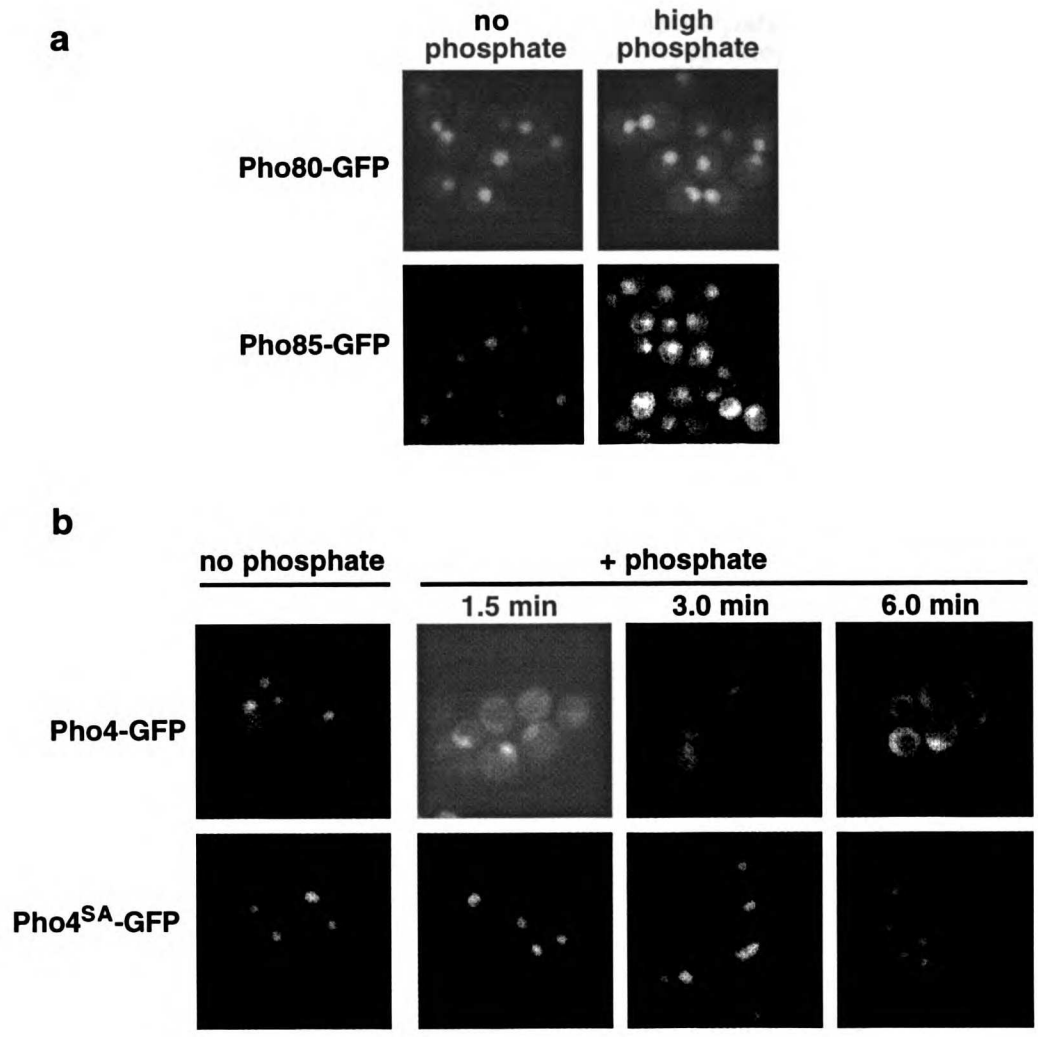
Figure 1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no phosphate
high phosphate
Pho80-GFP
Pho85-GFP
b
no phosphate
+ phosphate
1.5 min
3.0 min
6.0 min
Pho4-GFP
Pho4SA-GFP

Figure 2

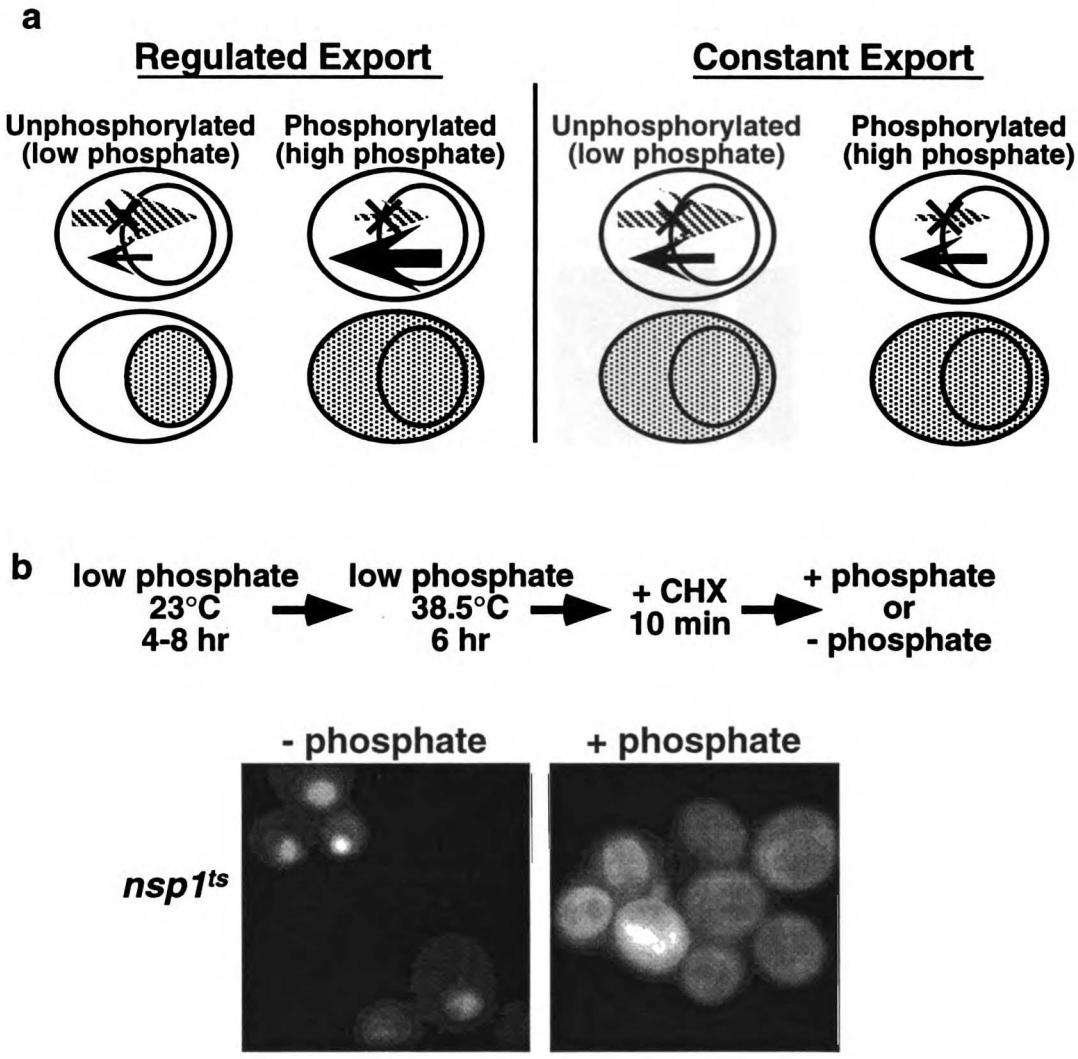

a
Regulated Export
Constant Export
Unphosphorylated (low phosphate)
Phosphorylated (high phosphate)
Unphosphorylated (low phosphate)
Phosphorylated (high phosphate)
b
low phosphate 23°C 4-8 hr
low phosphate 38.5°C 6 hr
+ CHX 10 min
+ phosphate or - phosphate
- phosphate
+ phosphate
nsp1ts

Figure 3

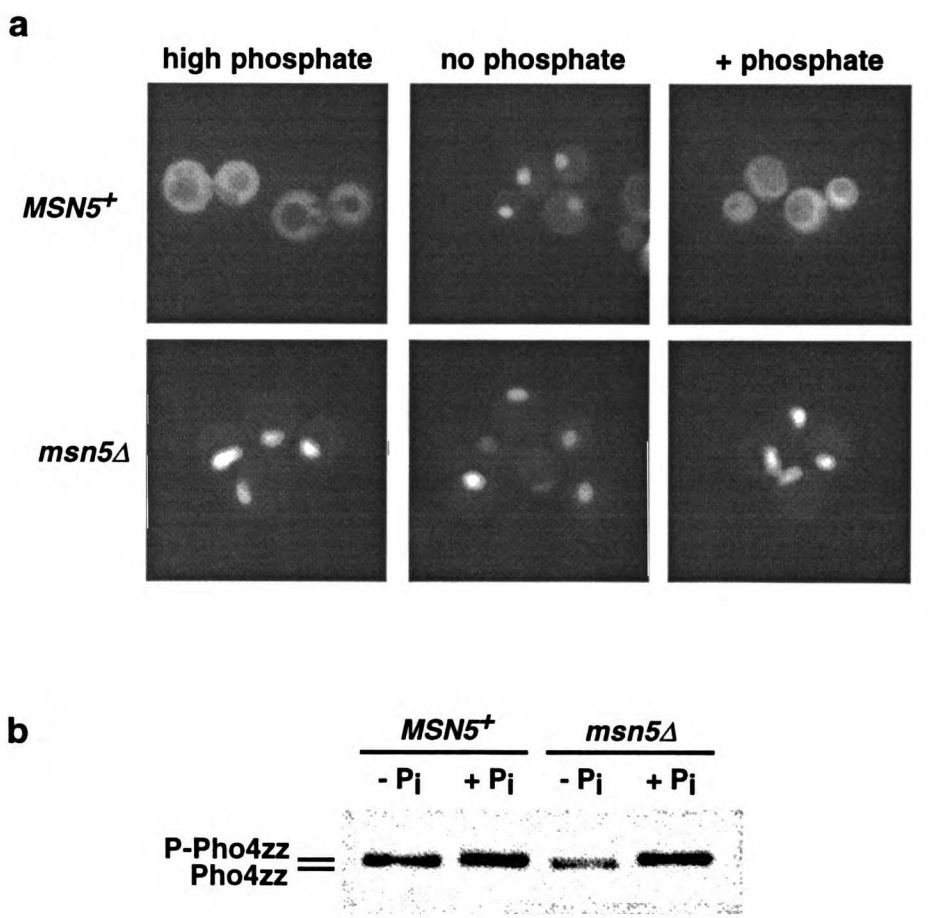
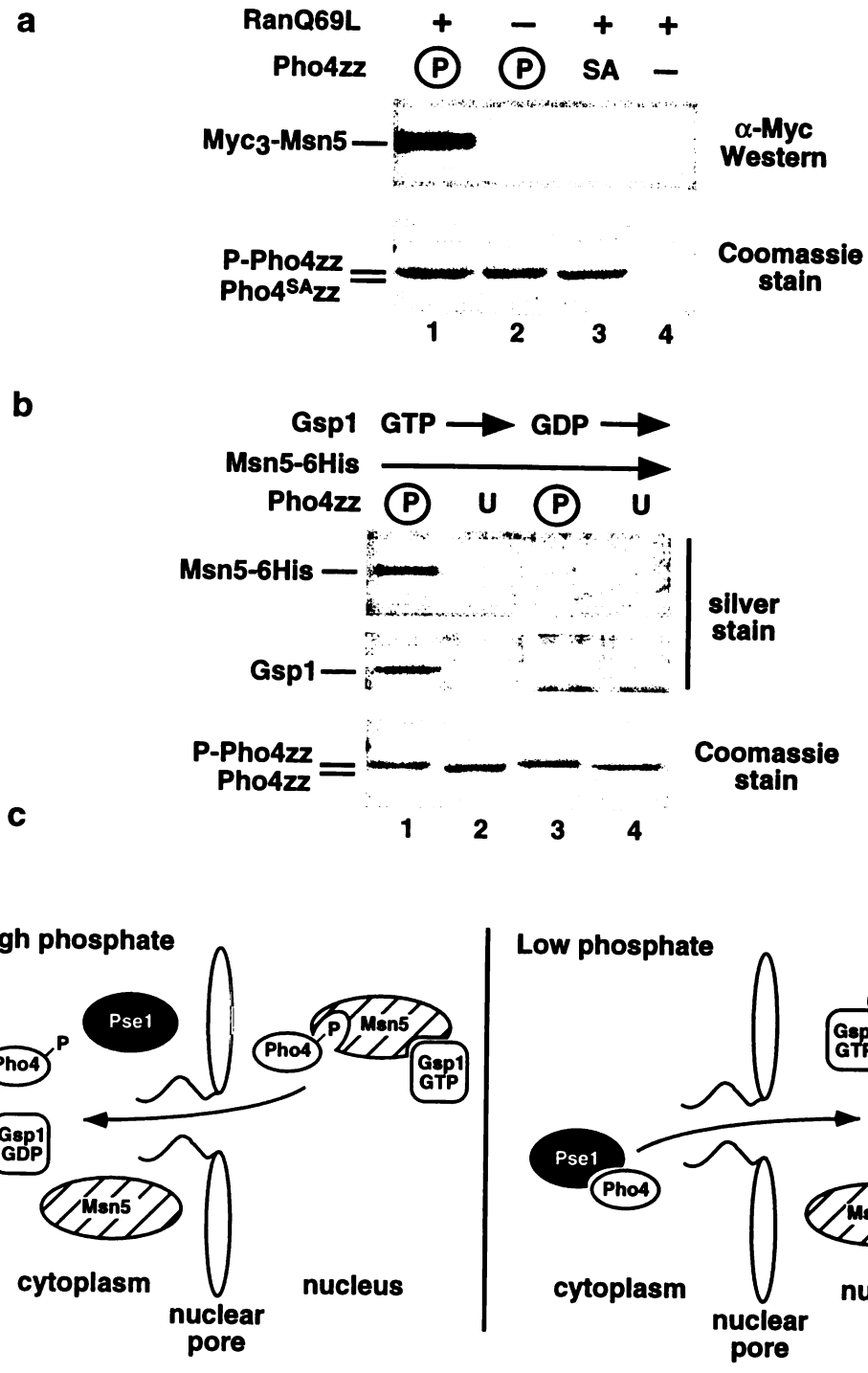

a
high phosphate
no phosphate
+ phosphate
MSN5+
msn5Δ
b
MSN5+
msn5Δ
- Pi
+ Pi
- Pi
+ Pi
P-Pho4zz
Pho4zz

Figure 4


a
RanQ69L + − + +
Pho4zz P P SA −
Myc3-Msn5
α-Myc Western
P-Pho4zz
Pho4SAzz
Coomassie stain
1 2 3 4
b
Gsp1 GTP → GDP →
Msn5-6His →
Pho4zz P U P U
Msn5-6His
Gsp1
silver stain
P-Pho4zz
Pho4zz
Coomassie stain
1 2 3 4
c
High phosphate
Pse1
Pho4 P
Pho4 P
Msn5
Gsp1 GTP
Gsp1 GDP
Msn5
cytoplasm
nuclear pore
nucleus
Low phosphate
Pse1
Gsp1 GTP
Pho4
Pse1
Pho4
Msn5
cytoplasm
nuclear pore
nucleus

Appendix

In Vitro Nuclear Import Assay in Budding Yeast

Introduction: Studies using the in vitro nuclear import assay in mammalian cells have proved to be instrumental in understanding the mechanism by which proteins enter the nucleus (Mattaj & Englmeier 1998). Two attempts to establish an in vitro import assay in *Saccharomyces cerevisiae* were published in the past ten years (Kalinich & Douglas 1989; Schlenstedt et al 1993). However, neither approach required the addition of exogenous extract to reconstitute import. In the case of Schlenstedt et. al. this is probably due to the difficulty of depleting endogenous import factors from yeast spheroplasts, and in the system used by Kalinich et. al. the reticulocyte lysate they used to radiolabel the import cargo probably allowed import into their purified nuclei even in the absence of additional extract. This extract-independent import makes it difficult to use these approaches to identify soluble factors required for nuclear import [for an interesting exception see (Corbett et al 1995)]. A large number of import receptors (importins) have been identified in yeast in the past two years using reverse genetics and biochemical binding assays (see Table 1, page 73); in none of these cases was the ability of these importins to import their putative cargoes demonstrated using the available import assays. This observation further suggests that the available import assays in yeast are not very useful experimental tools. In contrast, newly identified import receptors in mammalian cells are routinely shown to support import of their cargoes using the mammalian in vitro import assay (Jackal & Girlish 1998; Polaroid et al 1996).

Even though a robust import assay is not yet available in yeast, the genetic tools and the ease with which movement of proteins can be monitored in live yeast using the green fluorescent protein make *Saccharomyces cerevisiae* a very useful model organism to study the movement of proteins in and out of the nucleus. Three goals guided and motivated my attempt to develop an alternative in vitro import assay in yeast. (1) To identify the minimum set of proteins required for the import of Pho4; (2) To determine the effect that phosphorylation has on the rate of Pho4 import; (3) To establish a robust assay that may prove to be a useful tool in further understanding the process of nuclear import.

The assay: Nuclear import in vitro relies on monitoring the nuclear accumulation of fluorescently tagged protein carrying a nuclear localization signal (NLS) into semi-permeabilized yeast spheroplasts (spheroplasts with a perforated plasma membrane). This assay is a modification of a protocol reported by Schlenstedt et. al. (Schlenstedt et al 1993). In short, semi-permeabilized spheroplasts are incubated with a fluorescently tagged protein, ATP, and extract at room temperature for twenty minutes. The reaction is stopped by fixation with formaldehyde and import is scored by calculating the percentage of cells that accumulated fluorescently tagged protein in their nuclei (see Materials and Methods for details). The fluorescently tagged proteins we used are rhodamine-labeled human serum albumin (HSA) conjugated to either the wild type or a mutant form of the SV40 large-T antigen NLS (HSA-NLS), or recombinant Pho4 labeled with Texas Red. Two changes that we made to the original procedure allowed import to occur only upon addition of exogenous ATP and extract. First, we noticed that if spheroplasts were made from cells that were depleted of ATP (see Materials and Methods for details) import was now dependent on the addition of exogenous ATP (see also experiment 105 in my notebook). Second, washing the spheroplasts with 0.03% Tween-20 before adding them to the import assay allowed the reconstitution of import only when extract was added to the reaction (Exp 117).

Results: We used either a wild type peptide corresponding to the SV40 large-T antigen NLS or a mutant NLS peptide (Kalderon et al 1984) and conjugated these peptides to rhodamine labeled HSA (HSA-NLS^{SV40}). We then used the HSA-NLS^{SV40} as an import cargo for developing the assay. The SV40 NLS is imported efficiently into yeast in vivo in a manner that requires its import receptor Srp1 (the yeast homologue of importin α) (Shulga et al 1996) and the HSA-NLS^{SV40} is routinely used in the mammalian-import assay (Adam et al 1990). Since import in vivo requires physiological temperature, an

energy source, soluble factors, and a NLS we wished to demonstrate that import in our assay has the same requirements. Figure 1a shows import of HSA-NLS^{SV40} into yeast spheroplasts under different conditions. Nuclear import requires physiological temperature, the addition of ATP, and extract and a functional NLS sequence; a mutant form of the SV40 NLS does not target HSA into the nucleus. A quantitative summary of a typical import assay with HSA-NLS^{SV40} is shown in Fig 1b. We routinely see 10-20% import in the absence of ATP, or extract, or when import is carried out at 4°C. In contrast 60-80% of the spheroplasts show import when cells are incubated at room temperature in the presence of ATP and extract. It is important to note that the background import seen in the absence of ATP and/or extract is much fainter compared to the bright nuclear signal seen in the presence of both ATP and extract (compare No ATP and Complete Reaction in Figure 1a), and therefore the 3-8 fold stimulation seen in the presence of extract and ATP (Fig 1b) is probably an underestimate of the real difference in import between these conditions.

We next wanted to test whether import of Pho4 also requires incubation at physiological temperature, addition of ATP and extract, and a functional NLS. Recombinant Pho4 was fluorescently labeled with maleimide Texas Red (Pho4-TR) and was used as a cargo in the import assay. We chose to fluorescently label Pho4 this way because maleimide Texas Red specifically modifies a unique cysteine in the C-terminus of Pho4 that is dispensable for its proper localization in vivo (our unpublished observation), and therefore should not interfere with its ability to enter the nucleus in vitro. Here we demonstrate that import of Pho4-TR requires extract, ATP, and physiological temperature (Figure 2a). We also made use of a mutant Pho4 lacking amino acids 157-164 (Pho4ΔNLS), deletion of these amino acids prevents Pho4 from entering the nucleus in vivo and binding to Pse1 in vitro (Kaffman et al 1998b). The observation that Pho4ΔNLS-TR is not imported efficiently suggests that import of Pho4 in vitro requires a

functional NLS (Fig 2a). Imported Pho4-TR seems to localize, for unknown reasons, to a subnuclear structure that looks like the nucleolus.

Since Pse1/Kap121 is required to import Pho4 in vivo (Kaffman et al 1998b) we wished to determine whether it is also required for import of Pho4 in vitro. However, we were unable to show a defect in Pho4-TR import using spheroplasts and extracts made from a strain carrying the *pse1-1* allele (See Exp167, Exp168). In vivo, *pse1-1* shows a defect in Pho4 import even at the permissive temperature (Kaffman et al 1998b), but no difference in import was observed in vitro when extract and spheroplasts were made at the permissive or the restrictive temperature. We reasoned that the ability of the *pse1-1* allele to import Pho4 in vitro is due to the fact that it is only a partial loss of function and that the residual activity in the import reaction is sufficient to import Pho4. Lower levels of Pse1 activity are probably required in this assay, compared to in vivo, because the Pho4 concentration in the assay (~ 400nM) is probably much higher than its concentration in vivo, allowing import of Pho4 even with low levels of Pse1 activity. We next tried to biochemically deplete Pse1 from the extract and to compare import of Pho4 of extract that was either depleted or not depleted of Pse1. To do this we constructed a strain that is deleted for *PSE1* and is maintained by expressing Pse1-zz from the *MET3* promoter. This strain grows slower in the presence of 4mM methionine (see 5/7/98) suggesting that: (1) full expression from the *MET3* promoter is essential for normal growth; and (2) the presence of 4mM methionine does not completely block Pse1 expression. Pse1-zz can be depleted completely from an extract made from this strain by incubating it with IgG-Sepharose beads (see 5/7-12/98). However, no difference in import of Pho4 was observed with extracts depleted or not depleted of Pse1 (Exp 170 & Exp 174). These results suggested that either Pho4 can be imported into spheroplasts in the absence of Pse1 or that the residual Pse1 activity in the spheroplasts is sufficient to import Pho4 in vitro. Three reasons lead us to believe that the latter explanation is responsible for the observed import of Pho4 with the Pse1-depleted extract. (1) Pse1 binds specifically to Pho4 in vitro,

whereas related importins such as Kap123 and Sxm1 do not bind Pho4 in vitro (Kaffman et al 1998b). (2) Import of Pho4 in vitro requires amino acids 157-164 necessary for binding to Pse1 (Figure 2a). (3) Addition of recombinant Pse1 enhances import of Pho4 when added to import reactions containing small amounts of extract that support very little import on their own (Figure 2b), suggesting that Pse1 activity is limiting under these conditions. Showing that Pse1 activity is required for the import of Pho4 in vitro is essential to demonstrate the usefulness of this assay and therefore more work is needed to establish this point (see Future Directions).

Since phosphorylation of Pho4 maintains it in the cytoplasm by enhancing its rate of export and at the same time inhibiting its rate of import, we wanted to test whether phosphorylation of Pho4 also correlates with its inability to accumulate in the nucleus in vitro. Pho4-TR was phosphorylated or mock phosphorylated in vitro and the phosphorylated and unphosphorylated Pho4-TR were used in the import assay. Preliminary results show that unphosphorylated Pho4-TR accumulates in the nucleus more efficiently than phosphorylated Pho4-TR (Fig 2c). However, because phosphorylated Pho4 is efficiently exported from the nucleus in vivo we cannot determine from this data whether the inability of phosphorylated Pho4-TR to accumulate in the nucleus is due to a block in import and/or efficient export (see Future Directions for further experiments).

We next wanted to identify the minimal set of proteins required for Pho4 import. Since no one has been able to reconstitute import in yeast with purified components we hoped that this would further demonstrate that our assay represents an improvement over existing import assays in yeast. In addition, we wanted to know whether the same proteins that are sufficient to reconstitute import into mammalian cells are also sufficient to reconstitute nuclear import in yeast. A requirement for a different set of proteins to reconstitute import in yeast compared to mammalian cells may reflect either a difference in import strategies between the two species and/or a difference in the ability to deplete soluble factors in the two experimental systems. We have purified as recombinant proteins from

bacteria all the yeast homologues of the mammalian proteins that are required to reconstitute import in mammalian cells. These include: Gsp1 (Ran), Ntf2, Rna1 (Ran-GAP), Pse1 and tested the ability of these purified proteins to support import of Pho4. Dramatically less import is observed when purified components are used compared to levels of import seen with yeast extract (Fig 2d; see also experiments 156, 159, 160, 173). Note that the observation that the import signal seen with purified components is much weaker compared to the signal seen with crude extract is not reflected in Fig 2d. However, some increase in nuclear import over background (nothing added) is seen upon addition of purified components. The recombinant material does not seem to inhibit import because it enhances import when added to yeast extract (compare lanes 2 and 3 in Fig 2d). These results indicate, that under the conditions used, purified components are unable to support import of Pho4 and that further work (see below) is needed to establish import of Pho4 using purified components.

Future Directions: Three requirements need to be met in order to demonstrate that this import assay represents an improvement over existing import assays in yeast. (1) Demonstrate that Pse1 is required to import Pho4 in vitro. (2) Reconstitute import of HSA-NLS^{SV40} and Pho4 using purified components. (3) Develop a more objective and quantitative way to score import.

The first step to accomplish these goals should be to make HSA-NLS^{Pho4}. This can be done by conjugating a peptide containing the NLS of Pho4 to rhodamine labeled HSA (HSA-NLS^{Pho4}) and use it as an import substrate instead of the Pho4-TR. In vivo, this 31 amino acid peptide (140-171) is sufficient to target GFP into the nucleus in a Pse1-dependent manner (Kaffman et al 1998b). Based on the difference in the import signals seen between HSA-NLS^{SV40} and Pho4-TR (i.e. the localization of Pho4-TR to a subnuclear structure) the HSA-NLS^{Pho4} should give brighter and cleaner import signal that can also be more directly compared to import seen with HSA-NLS^{SV40}.

Two experimental approaches can be used to demonstrate that Pse1 is required for Pho4 import in vitro. First, spheroplasts and extract from a strain carrying Pse1-zz as its only copy of Pse1 can be incubated with antibodies (or IgG-Sepharose beads) in the presence of ATP prior to the addition of HSA-NLS^{Pho4} to the import reaction. This step should inactivate all the Pse1 in the reaction, and should inhibit import of HSA-NLS^{Pho4}, but not of HSA-NLS^{SV40}. Addition of recombinant Pse1-6His can now be used to reconstitute import. Second, find conditions that further deplete endogenous pools of Pse1 from spheroplasts. This may be difficult, but if achieved will solve what seems to be a fundamental problem of this assay. Finding the right conditions that deplete Pse1 pools should be done using spheroplasts made from a strain that expresses Pse1 tagged with GFP or zz as its only source of Pse1. Depletion can be assessed by monitoring levels of Pse1 (by immunoblot) and import of HSA-NLS^{Pho4} in spheroplasts before and after different wash conditions. Pse1 depletion may be enhanced by preincubating the spheroplasts with Ran-GTP and an ATP regenerating system at room temperature before using them in the import assay. This treatment has been shown to deplete mammalian cells of Crm1 (Kehlenbach et al 1998), and Christelle Sabatier observed a reduction in the import background (i.e. import in the absence of adding extract) when she tried this. In addition, washes with higher salt, or experimenting with different detergents may help. It may also be worth exploring the option of using purified nuclei instead of spheroplasts in the import reaction. This would require some work but may provide a much cleaner system to perform the import assay, and may reduce the level of endogenous Pse1 substantially.

It is imperative to determine whether the low level of import seen with purified components is due to low level of activity of one of the components. I have titrated all the components up to very high levels (Exp 160) except for Pse1 in which the highest concentration used was ~150nM (Exp 156); much lower compared to the ~2μM (Jakel & Gorlich 1998; Truant et al 1998) concentration of import receptor used in the mammalian

import assay. In addition, the Pse1-6His used contained some zz-RanQ69L that was used to purify the full length Pse1 away from some breakdown products (see 3/29/98 in the note book labeled Proteins associated with Pho4 #2). The presence of zz-RanQ69L may be responsible for the inhibition of import seen when higher concentrations of Pse1 (~150nM, Exp156) were used. I did not use the concentrated stock of Pse1-6His since it contains an N-terminal truncation of Pse1 that may inhibit import. It is therefore worth testing whether increasing the Pse1 concentration in the reaction will enhance import with the purified system. One can try and use the more concentrated prep of Pse1 and test whether the N-terminal truncation of Pse1 does block import. Alternatively, the full length Pse1 can be separated from the truncated form either by using zz-RanWT loaded with GTP (instead of the Q69L) or by conventional chromatography (Mono Q and hydroxyapatite are good resins to try). It might be useful to test the activities of the purified components in the mammalian system (see Truant et. al. 1998 for an example). However, if all attempts to reconstitute import with the available purified components fail, additional proteins in the extract may be required. This can be tested by fractionating the extract and identifying activities that are required to enhance import in the presence of the purified components. Partial fractionation of the extract might be a useful thing to do anyway as it will probably remove inhibitory activities from the extract, providing a more robust import signal upon addition of extract. A more quantitative measurement of import can be achieved either by using the CCD camera (Palacios et al 1996) or by using FACS (Paschal & Gerace 1995) .

Demonstrating that import of Pho4 requires Pse1, that it can be reconstituted with purified components, and be quantitatively measured will allow us to use this tool to address many interesting problems; three are mentioned briefly below. The effect that phosphorylation has on the rate of Pho4 import can be determined. This can be done by conjugating either a phosphorylated peptide containing the NLS of Pho4, or a NLS carrying a serine to aspartic acid substitution, to rhodamine labeled HSA, and comparing the import rates of phosphorylated and unphosphorylated HSA-NLS^{Pho4}. Alternatively,

one can study the inhibitory effect of adding excess unlabeled phosphorylated and unphosphorylated NLS peptides on the import of HSA-NLS^{Pho4}. Since the Pho4 NLS is not required for Pho4 export in vivo (observation made by EKO) or binding to Msn5 in vitro (the first 140 amino acids of Pho4 are sufficient to bind Msn5 AK) we can assume that no export takes place with HSA-NLS^{Pho4}, and therefore the difference in nuclear accumulation observed between phosphorylated and unphosphorylated HSA-NLS^{Pho4} reflects different rates of import and not a difference in the steady state localization (i.e. import and export) of the two constructs.

We can also use this assay to reconstitute Pho4 export and determine what soluble factors are required to trigger export of Pho4 in vitro. The simplest way to do this would be to induce export of Pho4^{wt}-GFP from spheroplasts expressing this construct in a strain deleted for *MSN5*. Pho4^{wt}-GFP is trapped in the nucleus in this strain (Kaffman et al 1998a) and the addition of purified Msn5 and Ran-GTP to spheroplasts made from this strain should trigger export if these components are sufficient for export. If Msn5 and Ran-GTP are not sufficient to export Pho4^{wt}-GFP one can identify other activities in the extract that are also required to trigger export. Pho4^{SA}-GFP can be used as a control and should not be exported. This experiment was tried once by Erin and did not work but should be tried more systematically.

It will also be interesting to use this assay in combination with some in vivo work to determine how Prp20 (Ran-GEF) enters the nucleus. Since the nuclear localization of Prp20 is essential to establish the Ran-GTP gradient, understanding the mechanism that ensures its proper nuclear localization is of great importance. Assuming that (1) nuclear entry is not accomplished by diffusion (Prp20 is 53 kDa and diffusion is not commonly used to transport molecules into and out of the nucleus), and (2) the exchange activity of Prp20 does not require binding to chromatin, it will be interesting to identify the NLS and the import receptor that target Prp20 into the nucleus. In addition, it will be interesting to determine how the interaction between this import receptor and Prp20 is maintained in the

presence of Prp20-exchange activity, because in almost all cases binding of Ran-GTP to importin causes the cargo to dissociate from its import receptor.

More work is clearly required before the import assay can be used as a productive experimental tool, but I strongly believe that this can be achieved with a reasonable amount of effort, and once established will prove to be an important contribution to the field of nuclear transport.

Materials and Methods

Preparing Extract

1. Grow 500ml of Y57 cells to OD~0.5 (~250 OD total)

Note: Extract from Y57 works better than K699, and I routinely harvested at this OD.

2. Harvest in GSA bottles by spinning 10 min at 4Krpm. To minimize proteolysis work fast on ice from here.

3. Wash cells in each GSA bottle with 10 ml of buffer I, transfer to a 50 ml conical tube, and spin at 3.5Krpm in the RC3B for 5 min.

4. Resuspend pellet (~250 OD) in 2 ml buffer I, and add cell suspension to a 2 ml screw-cap tube filled with about 1ml acid-washed beads. Remove all air bubbles.

5. Bead beat 5x1 min, with 1 min on ice in between beating (check lysis under the microscope should get ~85-95% cell lysis)

6. Poke a hole at the bottom of the 2 ml screw-cup and transfer supernatant to a new eppendorf.

7. Spin 20 min in microfuge.

8. Transfer supernatant to TLA 120.1 tubes and spin 55Krpm (100,000g) for 1 hr in a table-top ultracentrifuge.

Note: High speed spin is important to obtain good import.

9. Record concentration of lysate (should be ~20-30 mg/ml) and freeze in 50λ aliquots.

Solutions:

Buffer I: 0.25M sorbitol, 20mM Pipes-KOH (pH 6.8), 150mM KOAc, 5mM MgOAc, 3μg/ml leupeptin, 3μg/ml pepstatin, 0.5mM PMSF, 1mM DTT

Making Spheroplasts

1. Grow 100 ml of Y57 strain to OD~0.4

Note: (a) Spheroplasts from Y57 import very nicely, I can get import with other strains but the import signal is not as nice as with Y57.

(b) Harvesting cells in early log phase is important.

(c) This prep gives spheroplasts for about 100 import reactions and you can scale up by growing 500ml culture, but then spheroplasts are left in the -80°C for a very long time and I think they lose some activity. Use 500ml culture if you plan to titrate the lyticase (see step 6).

2. Harvest cells in 50 ml conical tubes 5 min 3.5Krpm in the RC3B

3. Resuspend cells in 20 ml of minimal media +10mM sodium azide and 10mM 2-deoxyglucose (DG). Transfer to a 100ml flask and shake at 30°C for 1 hr.

Note: this step depletes cells of ATP and makes the import reaction more ATP dependent and also more robust (for some unknown reason).

4. Spin as above (step 2) and resuspend cells in 10ml buffer T + azide and DG in a 50 ml conical tube. Incubate 10 min at 30°C with gentle shaking (~100 rpm).

Note: this step reduces the cell wall and facilities digestion with lyticase.

5. Spin cells as above.

6. Resuspend cells in 2.3 ml SS media + azide and DG and place in a 15 ml conical tube. Add ~30λ of lyticase and incubate for 30 min at 30°C with gentle agitation (100 rpm).

Note: (a) Good lyticase digestion is key and you don't want to over digest or to under digest. The best way to determine this is to do a titration of the lyticase, but the guidelines

above should work. One can also get a measurement of the degree of digestion by measuring the ratio of OD600 of the spheroplasts in water and A1.2 which has sorbitol.

(b) The lyticase prep that I have been using was stored at 4°C and accumulated precipitation over time. I routinely mixed the contents of the tube before adding the lyticase in an attempt to add similar amounts of lyticase for different preps of spheroplasts. Probably the best way to accomplish this is to freeze the lyticase in small aliquots that are then thawed and used only once.

7. Stop lyticase activity by adding 5 ml cold 1X Pre-buffer A1.2 and spin 5min in the clinical centrifuge at setting 5.

8. Remove the cloudy supernatant and wash again with 5 ml of 1XPre-buffer A1.2. Spin as above (Step 7).

9. Resuspend pellet in 0.5 ml of A1.2 + protease inhibitors. Freeze in 50λ aliquots in 0.5ml eppendorf tubes in liquid nitrogen and store at -80C. Use 0.5ml eppendorfs because they do not pop open so easily compared to 1.5 ml eppendorfs and also they are more convenient for thawing (see step 1 in the in vitro assay section)

Solutions:

Buffer T: 100mM Tris-Cl (pH 7.5), 10mM DTT (Fresh)

minimal media: Synthetic media + amino acids, no glucose!

1M Azide; 1M 2DG: I usually make fresh 1ml solutions for each prep

SS media: 20mM potassium phosphate (pH 7.5), 1.2M sorbitol, 10mM Azide, 10mM DG, in 1X minimal media (I usually make only 10 ml)

1XPre-A1.2 buffer: 1.2M sorbitol, 20mM Pipes-KOH (pH 6.8), 150mM KOAc, 5mM MgOAc (Filter and keep at 4°C). I usually make 100ml which lasts a long time.

1X A1.2 + protease inhibitors: To the 1X Pre-A1.2 buffer add: 3μg/ml leupeptin, 1μg/ml pepstatin, 0.5mM PMSF, 1mM DTT, 1mM EDTA (I usually make 5 ml)

In Vitro import assay

Preparing spheroplasts:

1. Thaw spheroplasts rapidly by placing the frozen 0.5 ml eppendorf tube in a 1.5ml tube containing water from the 30°C bath and incubate the 1.5 ml tube in the 30°C bath for 3 min.

Note: freeze/thawing spheroplasts causes rupture of the plasma membrane which is essential for the assay to work. Rumors from the Schekman Lab say that fast thawing is important for appropriate rupture of the plasma membrane. I have not checked this but I routinely thawed spheroplasts as describe above.

2. Place 25λ of thawed spheroplasts in a new 0.5 ml eppendorf and add 200λ of A1.2 plus 0.03% Tween-20, and incubate on a rotator for 20 min at 4°C.

Note: (a) 25λ of thawed spheroplasts provides spheroplasts for 5 import reactions, and I usually try not to run more than 8-10 import reactions per experiment because it is difficult to count so many samples. I also recommend to use the volumes as described here and not to scale up at least until you get it to work.

(b) Use high quality Tween-20 (Fluka #93773). 0.03% of Tween-20 is ~5X the critical micelle concentration (CMC) and this is important for removing residual activity in the spheroplasts that cause import even in the absence of exogenous added extract. In addition, Tween-20 allows more efficient import probably due to increase in permeability of the plasma membrane. It is important to note that at this concentration, and up to 50X the CMC of Tween-20, the reaction is still energy and temperature dependent so when there is a problem with extract dependency titrating Tween-20 might be a good idea.

3. Wash spheroplasts twice with 200λ of A1.2 (no detergent). Spin spheroplasts gently at 3Krpm for 2 min as they rupture easily.

Note: this step removes the detergent from the reaction and presumably depletes endogenous import activities from spheroplasts.

4. Resuspend pellet (from the original 25λ of spheroplasts - see step 2) in 40λ of A1.2 and place on ice.

Import reaction

1. Add the following to pre-chilled 0.5 ml eppendorf tubes, and incubate for 5 min at room temperature:

    a. Buffer I to bring the total volume of the reaction to 20λ (including b,c, & d)

    b. 8λ washed spheroplasts

    c. 7λ extract (or purified components), or buffer I - for no extract control

    d. 1λ of 20X ATP^{reg} mix for the +ATP reaction and nothing or 2 units of apyrase (Sigma #A-6410) for no ATP. Adding apyrase seems to make the reaction a little bit more ATP dependent.

Note: (a) I usually add these things in the above order but I don't know how important that is. The idea here is to allow things to equilibrate before adding the import substrate and also for more efficient ATP depletion.

(b) You can probably use less than 7λ of extract if more volume is needed.

2. Add labeled substrate and incubate 20-30 min at room temperature. I use 5μg/ml final conc. of HSA-NLS, and ~15μg/ml final conc. of Pho4-Texas Red.

Note: Time of incubation is something that is worth checking. My impression is that 30 min is definitely long enough, and 15- 20 min may be sufficient (See exp #150).

3. Terminate reaction by adding 2λ of 37% formaldehyde and incubate 15 min at room temperature.

4. Add 1λ of 2.5μg/ml DAPI to each reaction followed by 100λ of A1.2 to wash the DAPI and excess of substrate.

5. Spin 3min 4krpm in the microfuge.

6. Resuspend pellet in 20λ of A1.2 and use 4λ to score import under the microscope.

7. Count 3 sets of 100 cells each. Import is scored when the signal in the nucleus is brighter than the cytoplasmic signal. This scoring method is very subjective and difficult to assess at times, it is therefore important to count a large number of cells.

Solutions:

1X Pre-Buffer I: 0.25M sorbitol, 20mM Pipes-KOH (pH 6.8), 150mM KOAc, 5mM MgOAc (Filter and keep at 4°C). I usually make 100ml that lasts for a long time.

1X A1.2 or buffer I: To the 1X Pre-buffer add: 3μg/ml leupeptin, 1μg/ml pepstatin, 0.5mM PMSF, 1mM DTT, 1mM EDTA

Note: (a) Benzamidine seems to have some inhibitory effect on import.

(b) I usually make 5ml of A1.2 plus proteases and only 1ml of Buffer I plus proteases for each experiment using fresh protease inhibitors.

20X ATP^{reg}: 300mM creatine phosphate (Boeringer Mannheim #621-714), 1mg/ml creatine kinase (Boeringer Mannheim #126-969), 20mM ATP (Pharmacia 27-2056-01), 20mM GTP (Pharmacia 27-2076-01)

Preparing HSA-NLS conjugated with rhodamine

Note: Before starting the procedure prepare two G-25 columns, see steps 5 and 11

Coupling HSA to rhodamine

1. Dissolve 8mg of HSA (CalBiochem #126658) in 8ml of 50mM Na_2Co_3 pH 9.1
2. Add 800λ of 1mg/ml rhodamine (Molecular Probes #L-20) in DMF
3. Incubate 1.5 hrs at room temperature covered with aluminum foil.
4. Concentrate to 1 ml in Centriprep 10 by spinning 3.6 Krpm in the GH 3.8 rotor (GS-6 centrifuge) for ~30 min.

Note: (a) Rhodamine works by covalently attaching to lysines so it is important to carry the reaction at high pH to deprotonate lysines

(b) This reaction gives a 1:10 molar ratio of HSA and rhodamine.

(c) Aluminum foil prevents bleaching of rhodamine.

Remove unconjugated rhodamine and change buffer

5. Load labeled HSA onto a10 ml G-25 column equilibrated with PBS pH 7.5

6. Collect fractions containing HSA (These are the first fractions to elute that are bright red, unconjugated rhodamine elutes much later and is seen as a second peak of red material).
7. Record volume (should be around 2.7 ml with ~ 10-100mM HSA concentration).

Cross linking HSA conjugated to rhodamine (HSA^R) to MBS

8. Add 250λ of 20mg/ml of MBS (Pierce #22311) in DMF to the 2.7 ml HSA^R. This should give about 1:100 molar ratio between HSA and MBS
9. Incubate 30 min at room temperature covered with aluminum foil.
10. Concentrate to 1 ml in Centriprep 10 by spinning 3.6 Krpm in the GH-3.8 for ~10 min

Note: MBS works by crosslinking lysines in HSA^R to succinimide in MBS and the cysteine in the NLS to the maleimide group in MBS.

Removing unbound MBS and conjugating NLS to HSA^R

11. Load HSA^R-MBS onto a10 ml G-25 column equilibrated with 100mM sodium phosphate (pH 7).
12. Collect fractions containing proteins (bright red).
13. Keep 10λ of unconjugated sample.
14. Split HSA^R-MBS pool into two fractions (each should be ~1.1 ml) and add 40λ of 38mg/ml of either wild type or mutant SV40 large T-antigen peptide NLS.

Note: The NLS peptides were obtained from the BRC and their masses determined by mass spectrometry. WT NLS is: CTPPKKKRKV and mutant NLS is: CTPPK**T**KRKV.

15. Incubate the two samples overnight at room temperature covered with aluminum foil.
16. Stop the reaction by adding βME to a final concentration of 14mM and incubate for 20 min at room temperature.
17. Dialyze each sample against 500ml of buffer I for 4 hrs. Replace buffer with another 500ml buffer I and dialyze for an additional 4 hrs.

18. Check conjugation by comparing the mobility shift of the unconjugated sample (step 13) and the conjugated samples. From the change in the mobility shift I estimated ~5-15 peptides per HSA.

19. Measure concentration (should be ~2mg/ml), freeze in liquid nitrogen in 30λ aliquots.

20. To use the HSA-NLS, thaw an aliquot and dilute it to 0.5mg/ml with buffer I and store wrapped in aluminum foil at 4°C. I use the same stock for about a month before I thaw a new frozen aliquot.

Conjugating Pho4 with Texas Red (Pho4-TR)

1. Purify Pho4 and dialyze it against 500 ml of K0.1 buffer overnight.

2. Spin protein 10 min in microfuge.

3. Measure protein concentration and dilute Pho4 to 1.2 mg/ml (~35μM). Add 3λ of 15mM stock solution of Texas Red (TR)(Molecular Probes #T-6008) dissolved in DMSO to 1ml of the 1.2 mg/ml stock of Pho4 (the color of the Texas Red changes when you mix the two).

Note: this should give a 1:1 molar ratio of Pho4 and TR. TR specifically modifies cysteines at the pH used here. The concentration and amount of Pho4 used here provides a convenient amount of material to work with and should not be difficult to obtain.

4. Incubate 5 hrs at 4°C covered with aluminum foil.

5. Stop reaction by adding 1λ of the βME stock solution (14.3M) and incubate an additional 10 min at 4°C (color changes).

6. Spin 5 min in microfuge.

7. To remove free TR, load the reaction at 1ml/min onto a 5 ml HiTrap G-25 column equilibrated with K0.1.

Note: in this labeling procedure the color of Pho4 is very light-purplish color, different from the bright color seen with the HSA conjugated to rhodamine.

8. Record volume and concentration of Pho4-TR and add sorbitol to a final concentration of 0.25M. I usually get ~1ml of 0.16 mg/ml, which is a 10X stock for the import assay. Note: sorbitol is used as a cryoprotectant instead of glycerol because glycerol inhibits import in mammalian cells.

9. Freeze in 30λ aliquots in liquid nitrogen and store at –80°C.

10. Use Pho4-TR at a final concentration of about 15μg/ml in the import reaction and use thawed tube only once.

Solutions:

Buffer K0.1: 30mM Hepes (pH 7), 100mM NaCl

Figure Legends

Figure 1. Import of HSA-NLS^{SV40} requires a functional NLS, physiological temperature, and the addition of ATP and extract. **a**. Nuclear import of rhodamine labeled HSA-NLS^{SV40} into semi-permeabilized spheroplasts. Complete reaction includes ATP, extract, wild type SV40 NLS and incubation at room temperature. Mutant NLS refer to HSA-NLS^{SV40} carrying a mutant form of the SV40 NLS. Pictures on the left are of rhodamine labeled HSA-NLS^{SV40} with DAPI staining of the same cells shown to the right. **b**. Percentage of cells showing import of HSA-NLS^{SV40} under different import conditions. No extract or ATP added (lane 1); no extract added (lane 2); no ATP added (lane 3); complete reaction (lane 4); complete reaction at 4°C (lane 5); complete reaction of HSA-NLS^{SV40} carrying a mutant form of the SV40 NLS (lane 6).

Figure 2. In vitro nuclear import of Pho4-TR. **a**. Nuclear import of Pho4-TR requires a functional NLS, physiological temperature, and the addition of ATP and extract. Complete reaction with wild type Pho4 (lane 1); complete reaction with ΔNLSPho4 (lane 2); no ATP added (lane 3); no extract added (lane 4); complete reaction at 4°C (lane 5). WT, wild type

Pho4 labeled with Texas Red; Δ, Pho4 deleted for amino acids 157-164 labeled with Texas red. **b**. Addition of recombinant Pse1-6His enhances import of Pho4-TR in vitro. No recombinant Pse1(lane 3), 30nM final concentration of Pse1-6His (lane 4), or 75nM final concentration of Pse1-6His (lane 5) were added to an import reaction containing 42μg total proteins made from a *Pse1-1* strain grown at the permissive temperature. Import with the same amount of proteins made from a Y57 wild type strain is shown in lane 2 (note that the two extracts are not made from isogenic strains). **c**. Unphosphorylated Pho4 accumulates more efficiently in the nucleus of semi-permeabilized spheroplasts compared to phosphorylated Pho4. Pho4-TR was phosphorylated or mock phosphorylated in vitro and import of the unphosphorylated and the phosphorylated forms were compared in vitro. **d**. Import of Pho4-TR can be partially reconstituted with purified components. No extract added (lane 1); crude extract (lane 2); crude extract with purified components (lane 3); Gsp1, Rna1, Ntf2 (lane 4); Gsp1, Rna1, Ntf2, and Pse1 (lane 5). Note that even though import looks impressive in terms of percentage of cells with purified components (lane 5) the import signal is much weaker compared to import with crude extract (lane 2). Final concentrations used: 3μM Gsp1, 300nM Rna1, 300nM Ntf2, 120nM Pse1.

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Figure 1

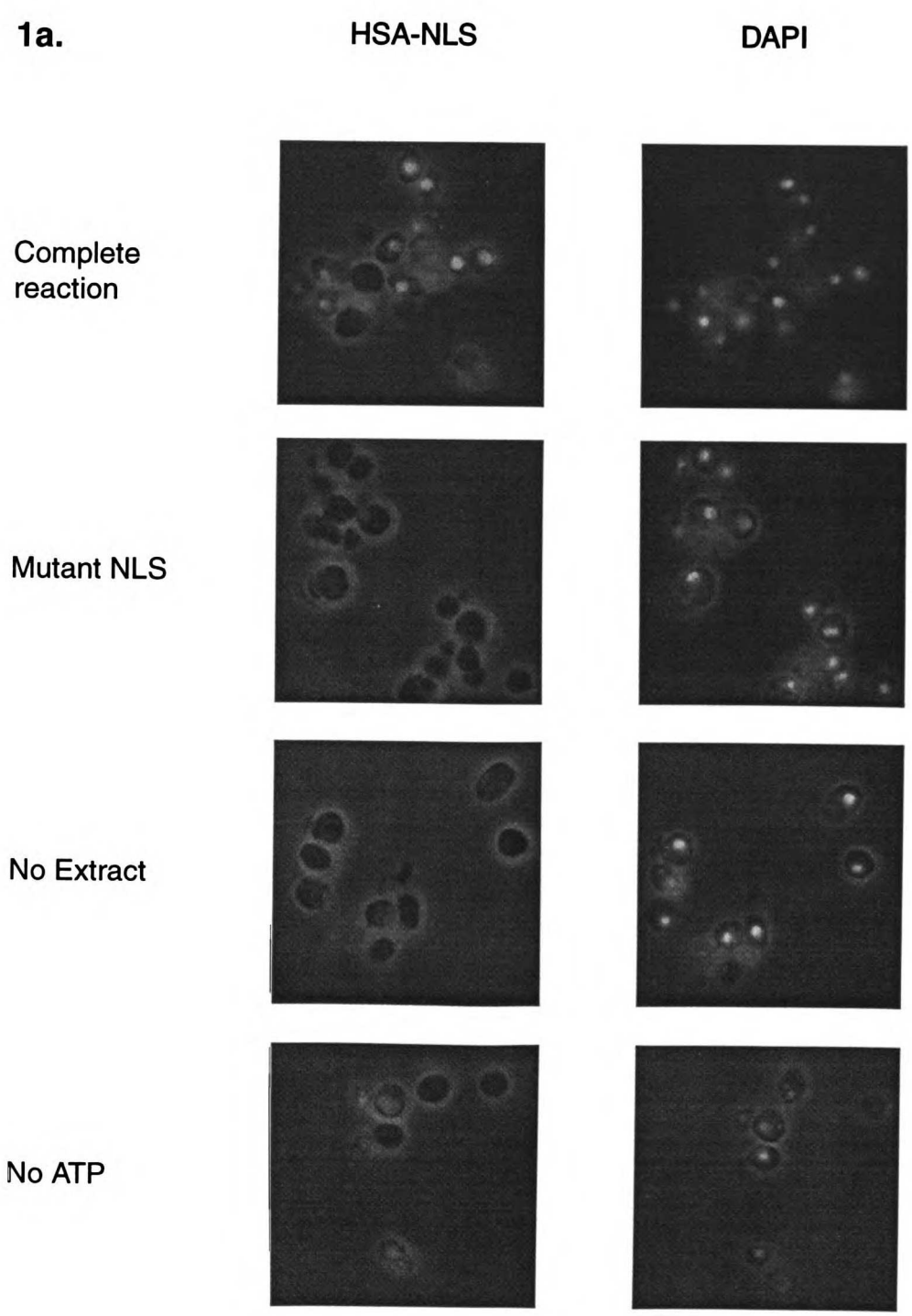
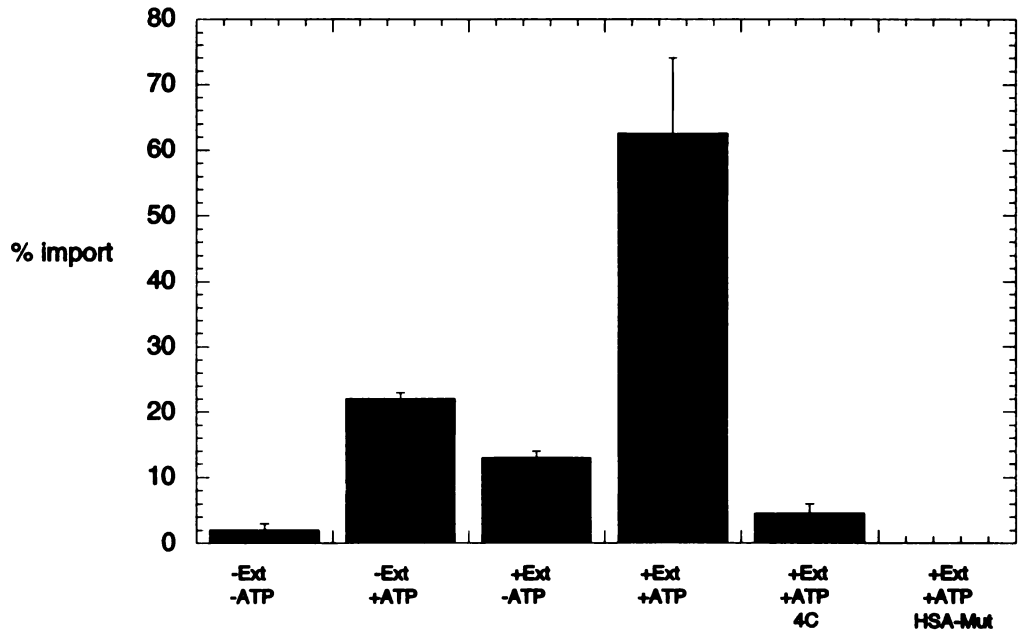

1a.
HSA-NLS
DAPI
Complete reaction
Mutant NLS
No Extract
No ATP

Figure 1

1b.


% import
80
70
60
50
40
30
20
10
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-Ext -ATP
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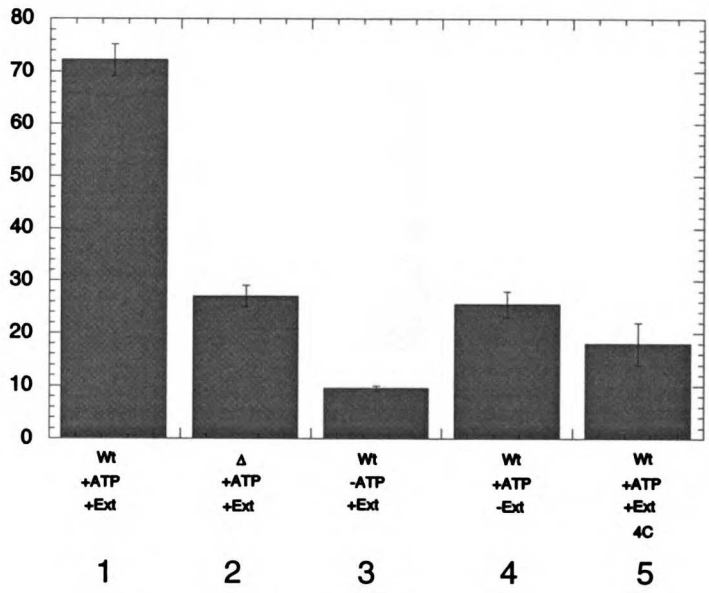
+Ext +ATP HSA-Mut

Figure 2

2a.



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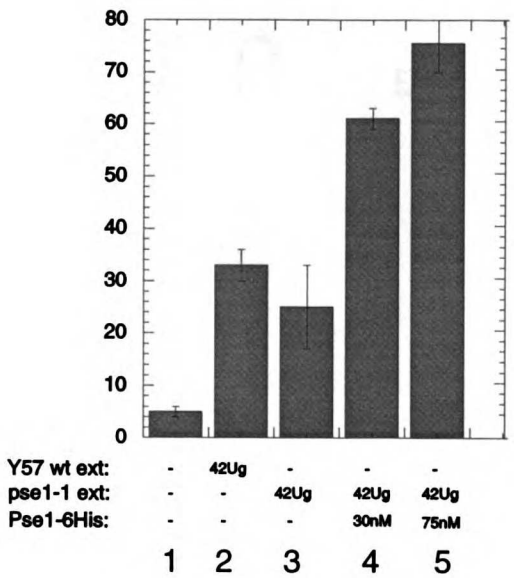
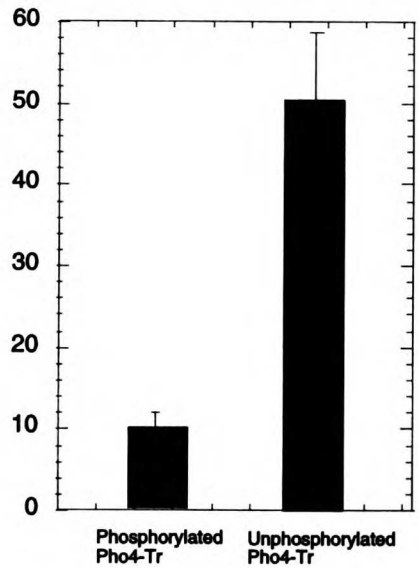
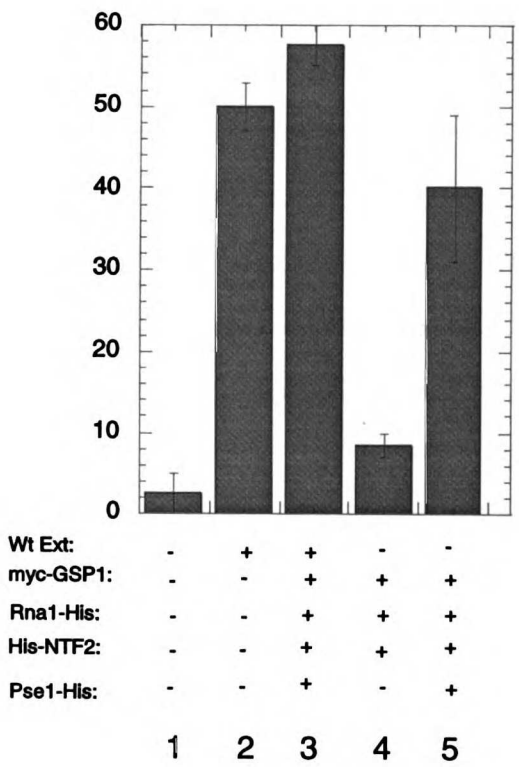


Figure 2

2c.



2d.



Conclusions

In the last year we made substantial progress in understanding how phosphorylation of Pho4 regulates its activity. We demonstrated that phosphorylation controls the subcellular localization of Pho4 by enhancing its affinity for its export receptor and preventing its association with its import receptor, making the localization of Pho4 one of the best understood example of regulated subcellular localization. We propose that such concomitant regulation of the rates of nuclear import and export will be a common way to control the subcellular localization of proteins, because it provides a rapid way to remove a protein from the nucleus (i.e. trigger its export), and also maintains the protein in the cytoplasm by blocking its reimport.

We also demonstrated that phosphorylation regulates the transcriptional activity of Pho4 by controlling its ability to associate with Pho2. Different phosphorylation sites regulate different activities of Pho4. In addition, regulating the subcellular localization of Pho4 and its ability to bind Pho2 are both required to fully inactivate Pho4 in high phosphate. These observations suggest that multiple phosphorylation sites cooperate to inactivate the transcriptional activity of Pho4 by regulating the abilities of distinct functional domains within Pho4 to execute their function. We propose that such cooperativity between multiple phosphorylation sites represents a more general mechanism that allows reversible modification of a protein to be used to construct an efficient molecular transcriptional switch.

Many questions still remain unanswered with regard to Pho4 localization and I will summarize below the ones that I believe have broader implications for the field of nuclear transport. First, we need to identify the NES in Pho4 and explain how phosphorylation allows Pho4 to bind to Msn5. This issue is important because many proteins are phosphorylated prior to their export, but very little is known about this process. The observation that all known cargoes of Msn5 seem to require phosphorylation prior to their

export suggests that Msn5 might be an export receptor dedicated to phosphorylated cargoes. Such an export receptor may be able to bind specifically to a phosphorylated NES, providing a simple explanation for how Msn5 is able to export several phosphorylated cargoes that do not share any obvious sequence similarity. If Msn5 is an export receptor dedicated to phosphorylated proteins it will be interesting to determine whether similar export receptors exist in other model organisms. Alternatively, if the phosphorylation sites are not part of the NES, but rather serve to induce a conformational change that exposes a hidden NES in Pho4, it will be interesting to understand how such a mechanism is conserved among a group of proteins with such different primary sequences.

Second, more work should be directed towards further characterizing the import and export pathways of Pho4. This is because, so far, Pho4 is the only characterized export cargo for Msn5 and the only bone fide import cargo for Pse1. This line of work should address issues such as: (1) What is the minimal set of proteins required for import and export of Pho4? (2) What allows Pho4 to be specifically imported into the nucleus by Pse1? In contrast, the ribosomal protein L25 that shares a similar NLS with Pho4 seems to be imported by both the Kap123- and the Pse1-import pathways. In addition, the NLS in Pho4 conforms to the classical NLS sequence but is not imported by importin α. These observations provide us with a unique opportunity to address an important issue in the nuclear transport field - what dictates which import pathway will be used by a particular cargo? (3) Does Msn5 have low affinity for Ran-GTP (as was shown for all other exportins) in the absence of Pho4 that is enhanced in the presence of Pho4? Low affinity between exportin and Ran-GTP presumably allows the export receptor to exit the nucleus only in the presence of its cargo, thereby making nuclear export more efficient.