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Calcineurin-dependent nuclear import of the transcription factor Crz1p requires Nmd5p

Renée S. Polizotto and Martha S. Cyert

Department of Biological Sciences, Stanford University, Stanford, CA 94305

Calcineurin is a conserved Ca^{2+}/calmodulin-specific serine-threonine protein phosphatase that mediates many Ca^{2+}-dependent signaling events. In yeast, calcineurin dephosphorylates Crz1p, a transcription factor that binds to the calcineurin-dependent response element, a 24-bp promoter element. Calcineurin-dependent dephosphorylation of Crz1p alters Crz1p nuclear localization. This study examines the mechanism by which calcineurin regulates the nuclear localization of Crz1p in more detail. We describe the identification and characterization of a novel nuclear localization sequence (NLS) in Crz1p, which requires both basic and hydrophobic residues for activity, and show that the karyopherin Nmd5p is required for Crz1p nuclear import. We also demonstrate that the binding of Crz1p to Nmd5p is dependent upon its phosphorylation state, indicating that nuclear import of Crz1p is regulated by calcineurin. Finally, we demonstrate that residues in both the NH_2- and COOH-terminal portions of Crz1p are required for regulated Crz1p binding to Nmd5p, supporting a model of NLS masking for regulating Crz1p nuclear import.

Introduction

Signal transduction pathways play a critical role in altering and controlling cell proliferation and differentiation. Transitory rises in cytosolic-free Ca^{2+}, due to either the opening of plasma membrane Ca^{2+} channels and/or release from internal stores, are used to activate Ca^{2+}-binding proteins such as calmodulin, which in turn activate downstream target enzymes (Klee et al., 1988). One such Ca^{2+}/calmodulin target is calcineurin, a serine-threonine protein phosphatase. In mammalian cells, calcineurin regulates many processes including cation homeostasis (Lieberman and Mody, 1994; Cameron et al., 1995), neutrophil chemotaxis (Lawson and Maxfield, 1995), heart development (Molkentin et al., 1998), and T cell activation (Clipstone and Crabtree, 1992).

In mammalian systems, calcineurin plays an essential role in T cell activation by regulating the translocation of the nuclear factor of activated T cells (NFAT)* family of transcription factors via dephosphorylation (for review see Cyert, 2001). In turn, NFAT transcriptionally activates many genes such as interleukin-2 (Jain et al., 1995). The immunosuppressant drugs FK506 and cyclosporin A inhibit calcineurin activity and block nuclear localization of NFAT thereby inhibiting NFAT-dependent gene expression (Shaw et al., 1995).

A similar calcineurin-dependent signal transduction pathway has been described in the yeast *Saccharomyces cerevisiae*. Yeast calcineurin is encoded by three genes: *CNA1* and *CNA2* are functionally redundant genes, which encode the catalytic subunit (Cyert et al., 1991; Liu et al., 1991b), and *CNB1* encodes the regulatory subunit (Kuno et al., 1991; Cyert and Thorner, 1992). Yeast calcineurin can be functionally inactivated in vivo by mutating either both the *CNA1* and *CNA2* genes or the *CNB1* gene or by the addition of FK506 (Liu et al., 1991a). In yeast, calcineurin-dependent signaling is activated by a rise in intracellular Ca^{2+}, resulting from a variety of stress conditions such as exposure to high temperature, prolonged exposure to mating pheromone, and changes in extracellular ions such as Na^{+} and Ca^{2+} (for review see Aramburu et al., 2000). Calcineurin is required for viability under most of these conditions and exerts its effect via activation of its target protein Crz1p, a zinc-finger transcription factor (Matheos et al., 1997; Stathopoulos and Cyert, 1997). When Crz1p is activated, it binds to a promoter element called the calcineurin-dependent response element (CDRE) and turns on a variety of genes (Stathopoulos and Cyert, 1997), including *PMC1*, *PMR1*, and *PMR2*, which encode P-type ATPases that regulate different aspects of ion homeostasis (Cunningham and Fink, 1996), and *FKS2*, which encodes a major cell wall biosynthetic enzyme (Mazur et al., 1995).

Address correspondence to Martha Cyert, Dept. of Biological Sciences, 371 Serra Mall, Stanford University, Stanford, CA 94305-5020. Tel.: (650) 723-9970. Fax: (650) 725-8309. E-mail: mcyert@stanford.edu

*Abbreviations used in this paper: CDRE, calcineurin-dependent response element; GFP, green fluorescent protein; GST, glutathione *S*-transferase; NFAT, nuclear factor of activated T cells; NLS, nuclear localization sequence; nt, nucleotide; SRR, serine-rich region.

Key words: calcineurin; karyopherin; nuclear transport; transcription factor; yeast

Calcineurin regulates the phosphorylation state and subcellular localization of Crz1p but not its ability to bind DNA (Stathopoulos-Gerontides et al., 1999). Crz1p isolated from *cnb1Δ* cells displays a slower electrophoretic mobility than Crz1p isolated from wild-type cells, demonstrating that Crz1p is hyperphosphorylated in the absence of calcineurin (Stathopoulos-Gerontides et al., 1999). Calcineurin has also been shown to dephosphorylate Crz1p in vitro, demonstrating that Crz1p is a calcineurin substrate (Stathopoulos-Gerontides et al., 1999). Upon addition of Ca^{2+} to wild-type cells, Crz1p rapidly translocates from the cytosol to the nucleus, whereas in *cnb1Δ* cells Crz1p remains cytosolic, indicating that calcineurin regulates Crz1p localization (Stathopoulos-Gerontides et al., 1999). Calcineurin-independent nuclear localization is observed when a region of Crz1p that is serine-rich and homologous to the NFAT serine-rich region (SRR) is deleted, suggesting that this domain plays a role in regulating Crz1p localization (Stathopoulos-Gerontides et al., 1999).

The rapid translocation of Crz1p from the cytosol to the nucleus may be mediated by calcineurin-dependent regulation of its rate of nuclear import, nuclear export, or both. In this study, we examine the regulation of Crz1p localization by investigating the mechanism of nuclear import. We describe the identification and characterization of the Crz1p nuclear localization sequence (NLS) and the import factor, Nmd5p, that is responsible for shuttling Crz1p into the nucleus. Finally, we demonstrate that the binding of Crz1p to Nmd5p is dependent on its phosphorylation state, establishing that calcineurin regulates the nuclear import of Crz1p.

Results

Identification of the Crz1p NLS

To identify the location of the Crz1p NLS, a series of green fluorescent protein (GFP)–Crz1p fusions were analyzed for calcineurin-dependent regulation of localization (Fig. 1 A). In wild-type cells, full-length GFP–Crz1p localizes to the cytoplasm until the addition of Ca^{2+}, after which it rapidly localizes to the nucleus. This localization is dependent on calcineurin because GFP–Crz1p remains cytoplasmic in *cnb1Δ* cells even in the presence of Ca^{2+} (Fig. 1 B; Stathopoulos-Gerontides et al., 1999). A fusion of the NH_2-terminal 425 amino acids of Crz1p to GFP exhibits a localization pattern identical to that of full-length GFP–Crz1p (Fig. 1, N-term-425); however, a fusion including only the first 388 residues displays constitutively cytoplasmic localization, suggesting that residues 388–425 contain an NLS (Fig. 1 A, N-term-388). Furthermore, full-length Crz1p lacking amino acids 394–425 is constitutively localized to the cytoplasm, demonstrating that this region is necessary for nuclear localization (Fig. 1 A, NLS#1Δ).

If this region functions as an NLS, it should be capable of targeting a nonnuclear protein to the nucleus. Construct GFP-NLS#1, consisting of Crz1p residues 394–422 fused to three tandem copies of GFP, displays calcineurin-independent nuclear localization, indicating that this region ($_{394}$IINGRKLKLKKSRRRSSQTSNNSFTSRRS$_{422}$) is sufficient to target a protein to the nucleus but does not contain all the residues required to impart calcineurin regulation of localization (Fig. 1). Since NLSs are enriched in basic residues with one or more of these basic residues required for activity, several of the lysines and arginines in this region were changed to alanine, resulting in a loss of nuclear localization (Fig. 1, mutNLS#1), demonstrating that residues 394–422 contain the Crz1p NLS (see below for further analysis).

Additional fusion proteins were analyzed to determine if Crz1p contains more than one NLS. The region NH_2-terminal to NLS#1 (N-term-388) and the region between residues 437 and 607 (btwNLS#1&2) are both constitutively cytoplasmic (Fig. 1 A); however, a GFP fusion containing the COOH-terminal portion of Crz1p downstream of NLS#1 (amino acids 469–679) localizes to the nucleus, indicating the presence of a second NLS in the COOH terminus of Crz1p (Fig. 1 A, C-term-469). Sequence analysis identified residues 612–615 as a potential second NLS. A protein containing these residues fused to three tandem copies of GFP displays calcineurin-independent nuclear localization, confirming that these residues are also sufficient to target proteins to the nucleus (Fig. 1, GFP-NLS#2). Thus, Crz1p contains two NLSs, but it is likely that only NLS#1 is relevant for in vivo calcineurin-dependent Crz1p nuclear localization for several reasons. First, constructs that contain NLS#2 without NLS#1 never display calcineurin-regulated localization. Second, deletion of NLS#2 has no effect on calcineurin-regulated localization. Third, nuclear localization of Crz1p is disrupted by the deletion of NLS#1 even when NLS#2 is present. Finally, NLS#2 functions independently of Nmd5p (see below).

Analysis of the Crz1p NLS#1

Clearly, residues 394–422 are sufficient to target GFP to the nucleus, but it is unclear which residues are necessary for nuclear localization; therefore, a variety of deletions and mutations were made in this region and analyzed for their ability to target GFP to the nucleus. NLSs can be of two types, either monopartite, consisting of a single cluster of basic residues, or bipartite, consisting of two basic clusters separated by a spacer region (for review see Jans and Hubner, 1996). The Crz1p NLS could be of either type, thus the Crz1p NLS was initially divided into two fragments to separate the two basic clusters and fused to GFP to test their competence for nuclear targeting. The COOH-terminal half (NLS#1-1, amino acids 410–422) was not able to target GFP to the nucleus, whereas the NH_2-terminal half (NLS#1-2, amino acids 394–410) displayed partial nuclear localization, suggesting that this region was sufficient to drive nuclear import (Fig. 2). Deletion of the first two isoleucines (NLS#1-3, amino acids 396–410) completely abolished nuclear localization, indicating that these hydrophobic residues play a critical role in Crz1p NLS function (Fig. 2). Significant inhibition of nuclear localization was also observed by mutagenesis of the lysine (mutNLS#1-6) or arginine (mutNLS#1-5) clusters to alanine (Fig. 2 A), suggesting that the basic residues along with the hydrophobic residues are crucial for Crz1p NLS function. Finally, to determine if complete nuclear localization could be restored, clusters of residues were added back to NLS#1-2 until the complete NLS sequence was obtained (Fig. 2 A, NLS#1-4, NLS#1-5,

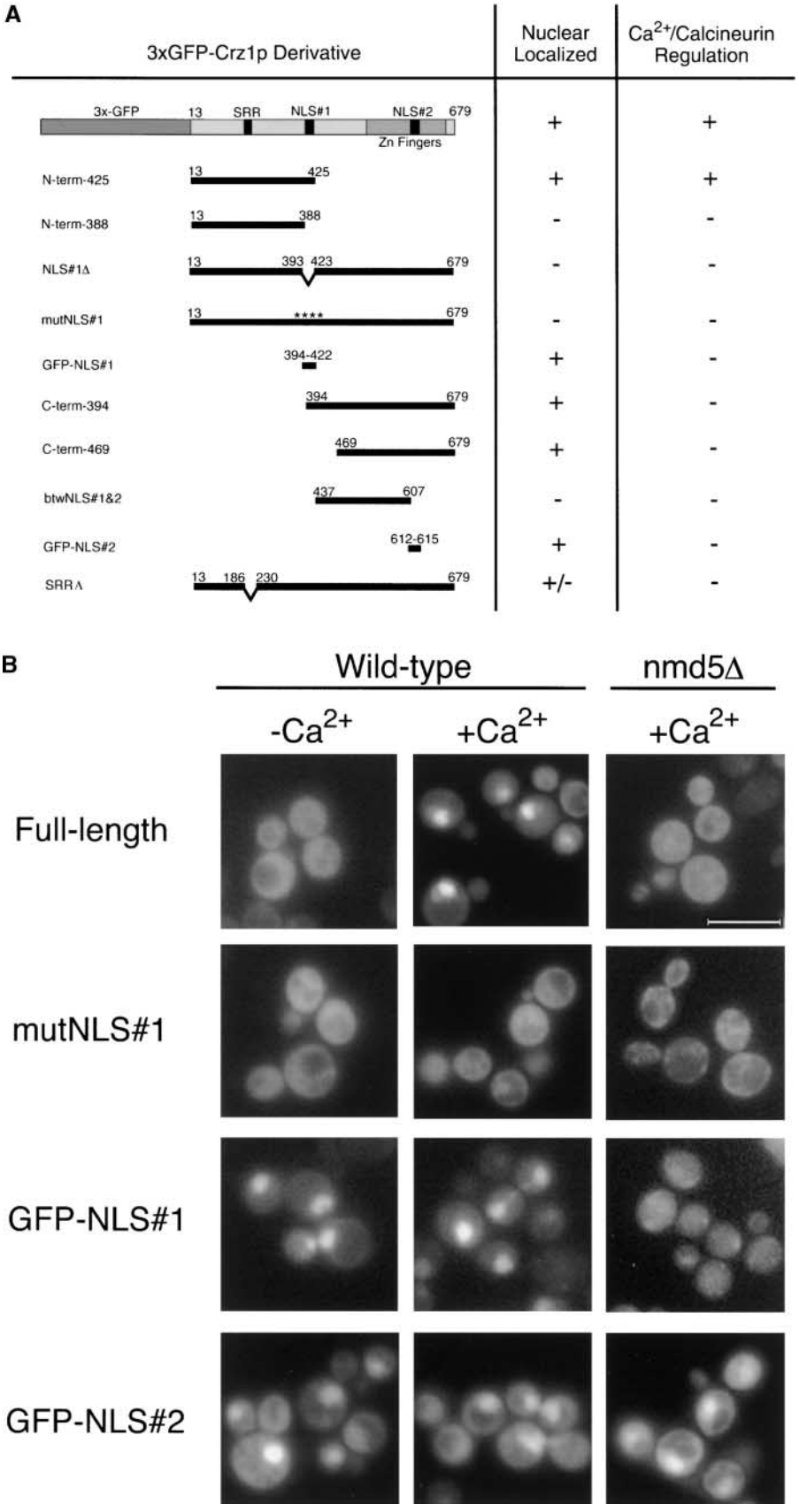

Figure 1. **Identification of the Crz1p NLS and requirement of Nmd5p activity for Crz1p nuclear localization.** (A) Localization of GFP–CRZ1 fusions in strains ASY472 (*crz1Δ*) and ASY475 (*crz1Δcnb1Δ*) as determined by fluorescence microscopy. Localization was observed in cells incubated at 21°C for 10 min with or without 200 mM $CaCl_2$ and scored for both nuclear localization and Ca^{2+}/calcineurin-dependent regulation. A protein was scored as positive for Ca^{2+}/calcineurin-dependent regulation if its nuclear localization was dependent on the addition of Ca^{2+} and failed to localize in a *cnb1Δ* strain after Ca^{2+} addition. (B) Residues 394–422 are required for Ca^{2+}-dependent nuclear localization of Crz1p, and this sequence is recognized by Nmd5p. Living cells of strain ASY472 (WT) or RPY176 (nmd5Δ) containing pRSP97 (full-length), pRSP114 (mutNLS#1), pRSP40 (GFP-NLS#1), or pRSP92 (GFP-NLS#2) were grown at 21°C and incubated with ($+Ca^{2+}$) or without ($-Ca^{2+}$) 200 mM $CaCl_2$ at 21°C for 10 min. GFP–Crz1p localization was observed using fluorescence microscopy. Bar, 20 μm.

NLS#1). NLS#1-4 and NLS#1-5 also exhibited partial nuclear localization, and thus the last two arginines are required for full activity of the NLS. Mutagenesis of the basic clusters in the full-length protein showed similar results (unpublished data). Substitution of either the NH_2-terminal lysine (mutNLS#1-6) or arginine (mutNLS#1-5) clusters to

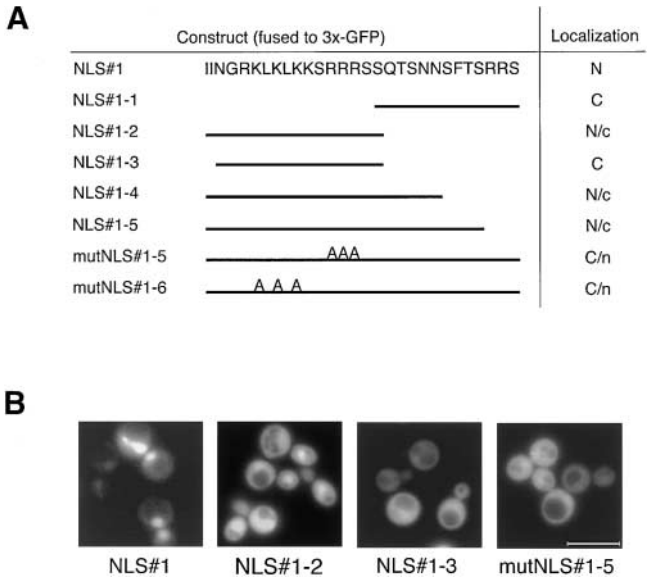

Figure 2. **Analysis of the Crz1p NLS#1.** (A) Localization of fragments of the Crz1p NLS#1 (residues 394–422) fused to three tandem copies of GFP in strain ASY472 as determined by fluorescence microscopy. Localization was observed in untreated log-phase cells growing at 21°C and scored for nuclear localization. (B) Hydrophobic and basic residues are required for NLS function. Untreated living cells of strain ASY472 containing pRSP40 (NLS#1), pOM9-1 (NLS#1-3), pRSP136 (NLS#1-2), or pRSP153 (mutNLS#1-5) were grown at 21°C. GFP localization was observed using fluorescence microscopy. Bar, 20 μm.

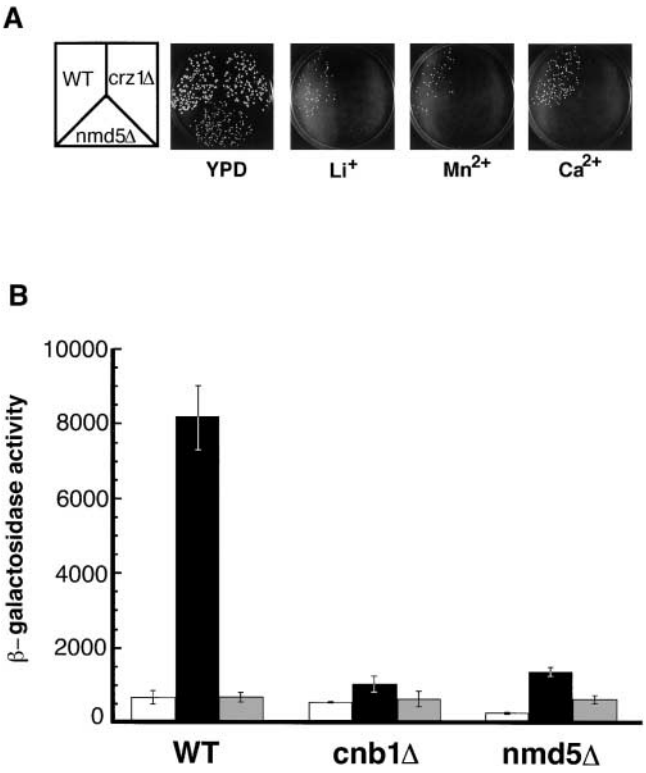

Figure 3. **Nmd5p is required for cation resistance and CDRE::lacZ activity.** (A) *nmd5Δ* and *crz1Δ* strains share similar cation sensitivities. Yeast strains were plated onto YPD alone or containing 150 mM LiCl, 400 mM $CaCl_2$, or 6 mM $MnCl_2$ and grown at 30°C for 3 d. WT, strain YPH499; *crz1Δ*, strain ASY472; *nmd5Δ*, strain RPY176. (B) Nmd5p is required for CDRE::lacZ activity. Strains YPH499 (WT), ASY472 (crz1Δ), and RPY176 (nmd5Δ) containing the CDRE::lacZ reporter (pAMS366) were grown in synthetic medium at 21°C for 6 h as follows: untreated (white bars), with 200 mM $CaCl_2$ (black bars), with 200 mM $CaCl_2$ and 2 μg/ml FK520 (gray bars), and β-galactosidase activity was assayed and done in triplicate. The SD is representative of the error between the samples.

alanine significantly inhibited Crz1p nuclear localization (Fig. 2 A), whereas mutagenesis of both clusters together completely eliminated nuclear localization (Fig. 1 A, mutNLS#1). However, mutagenesis of the COOH-terminal arginines to alanine had no effect on localization (unpublished data), indicating that these residues enhance nuclear localization but are not absolutely required.

Nmd5p is required for Crz1p nuclear localization

To understand the mechanism by which Crz1p is localized to the nucleus, we sought to identify the import receptor, or karyopherin, required to transport Crz1p to the nucleus. This was accomplished by screening a variety of yeast strains that were either conditional or null mutants disrupted for the function of a particular karyopherin. There are 14 known members of the karyopherin-β family (Gorlich et al., 1997; Wozniak et al., 1998). Full-length GFP–*CRZ1* was transformed into 11 of these strains, each defective for one karyopherin (*kap114*, *kap120*, and *kap122* were not available at the time of this study) and assayed for loss of GFP–Crz1p localization to the nucleus. Only two mutants had an effect on GFP–Crz1p localization. First, in *msn5Δ* cells GFP–Crz1p localizes to the nucleus of untreated cells, indicating a defect in nuclear export (L. Boustany, personal communication). Second, in *nmd5Δ* cells GFP–Crz1p fails to localize to the nucleus in the presence of Ca^{2+}, indicating that Nmd5p is required for Crz1p nuclear import (Fig. 1 B, full-length). Karyopherins involved in nuclear import recognize their cargo via the NLS of the cargo. We found that the constitutive nuclear localization of GFP-NLS#1 is disrupted in the *nmd5Δ* strain (Fig. 1 B), suggesting that Nmd5p recognizes NLS#1 for import. In contrast, nuclear localization of GFP-NLS#2 is unaffected by the loss of Nmd5p (Fig. 1 B).

Next, we examined the physiological consequences of disrupting Crz1p nuclear localization in the *nmd5Δ* strain. Crz1p-dependent transcriptional activation occurs through binding to the CDRE promoter element (Stathopoulos and Cyert, 1997); thus, to determine if Nmd5p affects Crz1p-dependent transcription an *nmd5Δ* strain was transformed with a plasmid containing four tandem copies of the CDRE fused to *lacZ*. Cells were treated with Ca^{2+} or Ca^{2+} plus the calcineurin inhibitor FK520, an isomer of FK506, and assayed for β-galactosidase activity (Fig. 3 B). Wild-type cells treated with Ca^{2+} displayed a 15-fold stimulation of β-galactosidase activity, which was abrogated by the calcineurin inhibitor FK520. This activation was absent in cells lacking calcineurin (*cnb1Δ*) and significantly reduced in *nmd5Δ* cells. Phenotypic analysis of *nmd5Δ* cells also reveals a defect in Crz1p-dependent transcriptional activation. *crz1Δ* and *nmd5Δ* cells were plated on YPD containing high concentrations of Li^+, Mn^{2+}, or Ca^{2+}, ions to which *crz1Δ* cells are sensitive (Stathopoulos and Cyert, 1997). Both *nmd5Δ* and

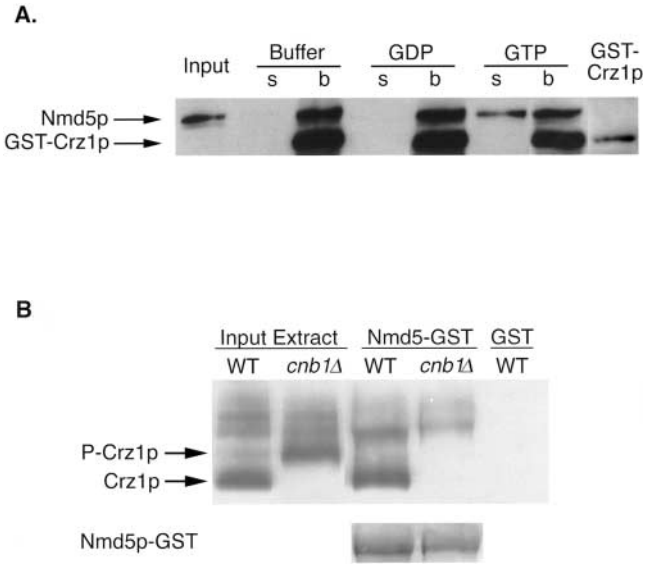

Figure 4. **Crz1p and Nmd5p form an import complex.** (A) Nmd5p binding to Crz1p is disrupted by Gsp1p–GTP. 5 μg GST–Crz1p was bound to glutathione resin and incubated with 5 μg thrombin-cleaved Nmd5p. After extensive washing, the resin was incubated with 10 μM $Gsp1p^{(Q71L)}$–GTP, $Gsp1p^{(Q71L)}$–GDP, or buffer alone. The supernatant was collected, and the washed resin was resuspended in Laemmli loading buffer. Equivalent amounts of the bound and unbound fractions were analyzed on a 6% SDS-PAGE followed by Western blotting using an anti-Nmd5p antibody that also recognizes GST (GST–Crz1p is shown as an internal control). (B) Nmd5p binds Crz1p in a calcineurin-dependent manner. 200 μg yeast cytosol from *crz1Δ* strains ASY472 (WT) and ASY475 (cnb1Δ) expressing HA-tagged Crz1p (pAMS451) were incubated with 50 μl Nmd5p–GST bound to glutathione resin. Equal amounts of the bound fractions were analyzed by Western blotting using an anti-HA antibody. The mobility of HA-Crz1p was compared with untreated yeast cytosol (Input Extract). As a control, WT yeast cytosol was incubated with GST-bound resin. The bottom displays a Ponceau stain analysis of the Western blot to demonstrate equivalent amounts of Nmd5p–GST.

crz1Δ strains display similar phenotypes (Fig. 3 A) consistent with Nmd5p being required for calcineurin/Crz1p-dependent transcriptional activation in vivo.

Nmd5p binds Crz1p, and the binding is disrupted by Gsp1p-GTP

A Crz1p–Nmd5p complex was demonstrated biochemically using a glutathione *S*-transferase (GST) pull-down assay consisting of immobilized GST–Crz1p incubated with Nmd5p, both of which were produced in *Escherichia coli*. Analysis of the bound fractions by Western blotting revealed that Crz1p binds to Nmd5p (Fig. 4 A). Several labs have demonstrated that RanGTP dissociates the interaction between most import karyopherins and their target cargo proteins (Rexach and Blobel, 1995; Albertini et al., 1998; Kaffman et al., 1998). Incubation of immobilized Nmd5p–Crz1p complex with a mutant version of Gsp1p, the yeast homologue of Ran, that is locked in the GTP-bound state ($Gsp1p^{(Q71L)}$–GTP), significantly disrupted the Nmd5p–Crz1p complex (Fig. 4 A), whereas addition of Gsp1p–GDP or buffer alone failed to disrupt Crz1p–Nmd5p binding.

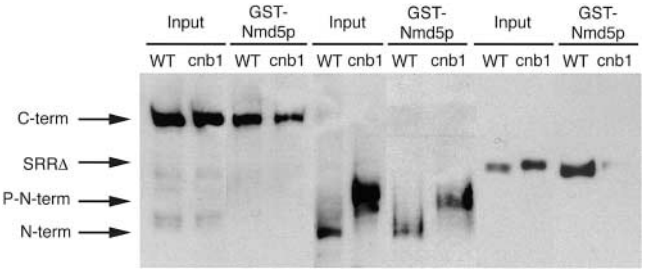

Figure 5. **Calcineurin regulation of Crz1p–Nmd5p binding requires resides on either side of the Crz1p NLS.** 200 μg yeast cytosol from *crz1Δ* strains ASY472 (WT) and ASY475 (cnb1Δ) expressing pAMS478 (N-term), pRSP37 (C-term), and pAMS490 (SRRΔ) were incubated with 50 μl GST–Nmd5p bound to glutathione resin. Equal amounts of the bound fractions were analyzed on an 8.5% SDS-PAGE followed by Western blotting using an anti-GFP antibody. For each of the GFP–Crz1p fusion proteins, its electrophoretic mobility in WT and *cnb1Δ* cells is shown (Input).

Nmd5p–Crz1p binding is calcineurin-dependent

To determine if the phosphorylation state of Crz1p affects its ability to bind Nmd5p, immobilized GST–Nmd5p was incubated with yeast extracts made from wild-type and *cnb1Δ* cells expressing HA-Crz1p. Crz1p isolated from *cnb1Δ* cells is hyperphosphorylated and characterized as having a slower electrophoretic mobility than Crz1p isolated from wild-type cells (Stathopoulos-Gerontides et al., 1999). We observed that the dephosphorylated form of Crz1p, equivalent to the GST–Crz1p isolated from *E. coli* (non-phosphorylated), binds to Nmd5p (Fig. 4 B). In contrast, the hyperphosphorylated form of Crz1p isolated from *cnb1Δ* cells fails to bind Nmd5p (Fig. 4 B). Ponceau S staining established that both samples contained equivalent amounts of Nmd5p; therefore, binding of Crz1p to Nmd5p is dependent on its phosphorylation state, establishing that calcineurin regulates Crz1p nuclear import.

Regulation of Crz1p import requires residues in the NH_2 and COOH termini

The Crz1p NLS#1 itself is not regulated by calcineurin; therefore, using GFP–Crz1p fusion proteins we analyzed the involvement of the NH_2-terminal, COOH-terminal, and the SRR regions in regulating Crz1p binding to Nmd5p. The COOH-terminal half of Crz1p (amino acids 394–679) displays neither an electrophoretic mobility shift nor regulated localization (Fig. 1 A, C-term-394, and Fig. 5, C-term). Accordingly, Nmd5p binds the COOH-terminal fragment isolated from both wild-type and *cnb1Δ* cells equally well (Fig. 5, C-term). Surprisingly, the NH_2-terminal fragment of Crz1p (amino acids 13–425), which displays a calcineurin-dependent electrophoretic mobility shift and regulated localization also binds to Nmd5p in both its phosphorylated and dephosphorylated forms (Fig. 5, N-term), suggesting that sequences on both the NH_2- and COOH-terminal sides of the Crz1p NLS#1 are required to regulate binding to Nmd5p. Deletion of the SRR region (amino acids 186–230) in full-length Crz1p results in calcineurin-independent nuclear localization and reduces the calcineurin-dependent mobility shift, implicating this region in calcineurin regulation (Sta-

thopoulos-Gerontides et al., 1999). Analysis of the binding of GFP–Crz1p–SRRΔ to Nmd5p revealed that only the dephosphorylated form shows efficient binding to Nmd5p (Fig. 5, SRRΔ), suggesting that the SRR region is not required for regulation of Crz1p nuclear import.

Discussion

Regulation of subcellular localization via phosphorylation

In yeast, calcineurin is part of a stress response pathway which elicits an immediate response. Within minutes after a rise in intracellular Ca^{2+}, calcineurin dephosphorylates Crz1p, resulting in Crz1p translocation from the cytosol to the nucleus. Regulation by nuclear localization is a common theme among transcription factors and allows rapid regulation of transcription factor activity. The subcellular localization of many transcription factors is regulated by their phosphorylation state (for review see Cyert, 2001). For example, Pho4p and NFAT are both localized to the nucleus in their dephosphorylated form (Beals et al., 1997; Kaffman et al., 1998; Zhu et al., 1998). Proteins can rapidly translocate from the cytosol to the nucleus in response to either an increase in nuclear import, a decrease in nuclear export, or both. The studies presented here elucidate the mechanism of Crz1p nuclear import, whereas independent studies in the lab have analyzed the mechanism of its nuclear export (L. Boustany, personal communication).

Identification of the Crz1p NLS

Deletion analysis of GFP–Crz1p identified two independent NLSs; however, only one is required for calcineurin-dependent nuclear import. There are two types of NLS, either monopartite, a single cluster of basic amino acids, or bipartite, two clusters of basic amino acids separated by a spacer region (Jans and Hubner, 1996). There are also two types of karyopherin-mediated nuclear import. Classical nuclear import occurs via the interaction of the cargo with the karyopherin-α/karyopherin-β complex (Kap60p/Kap95p in *S. cerevisiae*) through the binding of the cargo NLS to Kap95p (for review see Jans and Hubner, 1996). The other members of the karyopherin-β family mediate nuclear transport via the direct interaction of the cargo and karyopherin-β homologue via the NLS of the cargo (Gorlich et al., 1997; Wozniak et al., 1998), and each of these karyopherins may have unique requirements for NLS recognition.

In this report, we have characterized the interaction of Nmd5p with its cargo protein Crz1p. The Crz1p NLS contains two hydrophobic isoleucine residues and two basic lysine and arginine clusters that are critical for Nmd5p-dependent nuclear import. In addition, two arginine residues at the COOH terminus of the NLS enhance Crz1p nuclear import but are not absolutely required. Thus, Crz1p contains a novel NLS in which both basic and hydrophobic residues are required for NLS activity.

Recently, another stress-response protein involved in starvation, Ssa4p, has been shown to contain an NH_2-terminal short hydrophobic sequence containing a single basic residue that is able to drive starvation-dependent nuclear localization and requires Nmd5p activity (Chughtai et al., 2001). This Ssa4p sequence differs from the Crz1p NLS, but a direct interaction between Nmd5p and Ssa4p has not been demonstrated, and thus the Ssa4p sequence may interact with a linker protein that in turn interacts with Nmd5p. Conversely, Nmd5p has been shown to bind directly to TFIIS, a transcription elongation factor, but the TFIIS NLS has been identified only by computer scanning rather than functional analysis (Albertini et al., 1998). Hence, Crz1p is the first Nmd5p-dependent cargo that had been demonstrated to bind directly to Nmd5p and whose NLS had been functionally characterized.

Identification of the Crz1p importin, Nmd5p

Proteins that contain NLSs also require an import karyopherin, or importin, to transport the protein into the nucleus. We have presented both cytological and biochemical evidence that Nmd5p is the importin for Crz1p. Not only is Nmd5p required for nuclear localization of Crz1p, but Nmd5p is also required for Crz1p-dependent CDRE transcriptional activation. In accordance with the localization and transcriptional data, *nmd5Δ* and *crz1Δ* strains also share similar phenotypes. Recent studies have shown that *nmd5Δ* cells have other phenotypes in addition to those described here (Entian et al., 1999). The pleiotropic nature of the mutation likely reflects its involvement in nuclear transport of many different substrates. Albertini et al. (1998) have shown biochemically that TFIIS, is a major substrate of Nmd5p, and Nmd5p has also been shown to be required for nuclear localization of Hog1p, a mitogen-activated protein kinase required for osmotic stress survival (Ferrigno et al., 1998), although whether Nmd5p interacts directly with Hog1p is not known.

Regulation of Crz1p nuclear localization

We have shown that Nmd5p and Crz1p form a complex that is dependent on the phosphorylation state of Crz1p, demonstrating that Crz1p nuclear import is regulated by calcineurin. Some transcription factors, such as Swi5p, are regulated by direct phosphorylation of the NLS, making the NLS nonfunctional (Moll et al., 1991); however, neither GFP-NLS#1 nor C-term-394 displays calcineurin-dependent nuclear localization, and thus it is unlikely that the NLS is acted upon directly by calcineurin. Our analyses of the Crz1p–Nmd5p interaction demonstrate that Crz1p fusion proteins containing sequences only NH_2-terminal or only COOH-terminal to the NLS bind Nmd5p regardless of their phosphorylation state. In contrast, the binding to Nmd5p of a Crz1p fusion protein lacking the SRR region is sensitive to phosphorylation state. Taken together, these results indicate that regions both upstream and downstream of the NLS, but excluding the SRR region, are required for calcineurin-regulated binding of Crz1p to Nmd5p.

These data suggest two possible mechanisms for regulating Crz1p nuclear import. In one scenario, there could be a cytosolic Crz1p-binding protein or tether that interacts with Crz1p in a phosphorylation-dependent manner and thereby regulates Crz1p binding to Nmd5p. We do not favor this model because genetic analyses have failed to identify candidates for such a tethering protein. Furthermore, this model predicts that overexpression of Crz1p would exceed the

binding capacity of the cytosolic tether and result in mislocalization. In contrast, we observe normal regulation of Crz1p localization even when it is highly overexpressed.

Instead, we favor a mechanism involving masking of the Crz1p NLS through intramolecular interactions between regions on either side of the NLS. Since the Crz1p NH_2-terminal fragment displays a calcineurin-dependent mobility shift, it may contain phosphorylated residues that interact with basic residues downstream of the NLS in the COOH-terminal portion. Thus, in the resting state the conformation of Crz1p would limit the access of the NLS to Nmd5p. Upon dephosphorylation by calcineurin, a Crz1p NLS would be exposed, favoring binding to Nmd5p and increasing the rate of Crz1p nuclear import.

Our findings also indicate that regulation of Crz1p nuclear import may explain only part of its mechanism of localization. We observed that regulated binding of Crz1p containing fusion proteins to Nmd5p in vitro did not correlate completely with their localization in vivo. The NH_2-terminal–Crz1p fusion protein (N-term-425) displays regulated nuclear localization even though its binding to Nmd5p is not sensitive to its phosphorylation state, and the SRRΔ fusion protein displays calcineurin-independent localization while retaining calcineurin-dependent binding to Nmd5p. These findings suggest that nuclear export also contributes to Crz1p localization in vivo, and independent studies from our laboratory have established that Crz1p nuclear export is also regulated by calcineurin (L. Boustany, personal communication).

The studies described here elucidate the mechanism by which Crz1p is imported into the nucleus and demonstrate that calcineurin regulates this process, but there are still many unanswered questions. The next step will be to identify the sites in Crz1p that are dephosphorylated by calcineurin and to characterize the role of these residues in Crz1p binding to Nmd5p. Furthermore, the kinase(s) responsible for phosphorylating Crz1p has yet to be identified. Once these questions have been answered, a much more complete understanding of the calcineurin pathway will be obtained.

Materials and methods

Plasmids

Standard procedures for the manipulation of plasmid DNA and transformation into *E. coli* (TOP10 cells, Invitrogen; BLR cells, Novagen) by electroporation were performed (Ausubel et al., 1994). The high fidelity Vent polymerase (New England Biolabs, Inc.) was used in PCR for cloning. The TOPO-TA cloning kit (Invitrogen) was used to ligate the PCR products into a bacterial vector before transfer to yeast vectors. The high fidelity *Pfu* Turbo polymerase (Stratagene) was used to mutagenize the *CRZ1* NLS#1 in pRSP97 using the ExSite PCR-based site-directed mutagenesis kit (Stratagene).

A GFP fusion vector with three tandem copies of GFP, pOM4, was constructed using PCR to modify the GFP fusion vector, pGFP-N-FUS (Niedenthal

Table I. **Plasmids**

Plasmid	Relevant information	Source
pAMS459	pGFP-N-FUS	Niedenthal et al., 1996
pAMS478	pGFP-N-FUS-CRZ1-N-term-425 (amino acids 13–425)	Stathopoulos-Gerontides et al., 1999
pAMS490	pGFP-N-FUS-CRZ1-SRRΔ (amino acids 13–679, Δ186–230)	Stathopoulos-Gerontides et al., 1999
pOM4	pGFP-N-FUS with two tandem copies of M3-GFP	This study
pOM9-1	pOM4-CRZ1-NLS#1-3 (amino acids 396–410)	This study
pRSP37	pOM4-CRZ1-C-term-394 (amino acids 394–679)	This study
pRSP40	pOM4-CRZ1-NLS#1 (amino acids 394–422)	This study
pRSP49	pOM4-CRZ1-C-term-469 (amino acids 469–679)	This study
pRSP50	pOM4-CRZ1-NLS#1-1 (amino acids 410–425)	This study
pRSP70	pOM4-CRZ1-btwNLS#1&2 (amino acids 437–607)	This study
pRSP84	pOM4-CRZ1-N-term-388 (amino acids 13–388)	This study
pRSP92	pOM4-CRZ1-NLS#2 (amino acids 612–615)	This study
pRSP93	pOM4-CRZ1-mutNLS#1-3 (amino acids 13–679 with arginines 406–408 mutated to alanine)	This study
pRSP97	pOM4-CRZ1 (amino acids 13–679)	This study
pRSP102	pOM4-CRZ1-NLSΔ (amino acids 13–679, Δ394–422)	This study
pRSP112	pOM4-CRZ1-mutNLS#1-2 (amino acids 13–679 with lysines 399,401,403 mutated to alanine)	This study
pRSP114	pOM4-CRZ1-mutNLS#1 (amino acids 13–679 with lysines 399,401,402 and arginines 406–408 mutated to alanine)	This study
pRSP134	pOM4-CRZ1-mutNLS#1-4 (amino acids 13–679 with arginines 420–421 mutated to alanine)	This study
pRSP136	pOM4-CRZ1-NLS#1-2 (amino acids 394–410)	This study
pRSP139	pOM4-CRZ1-NLS#1-4 (amino acids 394–415)	This study
pRSP146	pOM4-CRZ1-NLS#1-5 (amino acids 394–418)	This study
pRSP153	pOM4-CRZ1-mutNLS#1-5 (amino acids 394–422 with arginines 406–408 mutated to alanine)	This study
pRSP156	pOM4–CRZ1-mutNLS#1-6 (amino acids 394–422 with lysines 399,401,402 mutated to alanine)	This study
pAMS435	YEp351-CRZ1	Stathopoulos and Cyert, 1997
pAMS451	pRS315-HA-CRZ1	Stathopoulos and Cyert, 1997
pAMS366	pLG178-4X-CDRE::cyc1::lacZ	Stathopoulos and Cyert, 1997
pRD56	pRS316-GAL1-GST	R. Deshaies
pRSP121	pGEX-4T-3-GST-CRZ1	This study
pGST-NMD5	pGEX-2TK-GST-NMD5	M. Rexach
pKW581	pQE-GSP1-Q71L	K. Weis

Table II. **Yeast strains**

Strain	Relevant genotype	Source
YPH499	MATa ura3-52 lys2-801 ade2-101 trip-Δ63 his3Δ200 leu2-Δ1	Sikorski and Hieter, 1989
YPH500	same as YPH499, except MATα	Sikorski and Hieter, 1989
DD12	same as YPH499, except cnb1::hisG	Cyert and Thorner, 1992
ASY472	same as YPH499, except crz1::loxP-kanMX-loxP	Stathopoulos and Cyert, 1997
ASY475	same as DD12, except crz1::loxP-kanMX-loxP	Stathopoulos and Cyert, 1997
RPY197	same as YPH499, except nmd5::HIS3	This study
HFY135	ade2-1 his3-11,15 leu2-3,11 trp1-1 ura3-1 can1-100 NMD5	A. Jacobson
HFY133	same as HFY135, except nmd5::HIS3	A. Jacobson
cse1-1	MATα ura3-52 ade2-101 his3-11,15 trp1-Δ901 cse1-1	Xiao et al., 1993
PSY967	MATα ura3-52 leu2Δ1 kap123::HIS3	Seedorf and Silver, 1997
PSY1200	MATa ura3-52 leu2Δ1 trp1Δ63 SXM::HIS3	Seedorf and Silver, 1997
PSY1201	MATa ura3-52 leu2Δ2 trp1Δ63 pse1-1	Seedorf and Silver, 1997
KWY121	MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 XPO1::LEU2 pKW456 (LEU2, xpo1-1)	Stade et al., 1997
X2316-3C	MATα LOS1 SUP4 ade2-1 can1-100 lys1-1 his5-2 trp5-48 ura3-1	Hopper and Schultz, 1980
201-1-5	same as X2316-3C, except los1-1	Hopper and Schultz, 1980
SWY1313	MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 kap95::HIS3 pSW509 (LEU2, kap95-L63A)	Iovine and Wente, 1997
kap104ts	MATa ura3-52 his3Δ200 trp1-1 leu2-3,112 lys2-801 KAP104::ura3::HIS3 p104(URA3, kap104-16)	Aitchison et al., 1996
T255	same as YPH500, except mtr10ts	Kadowaki et al., 1994

et al., 1996). An ~0.7-kb NheI-SpeI fragment of M3GFP ("enhanced GFP") (Valdivia et al., 1996) was made by PCR and cloned into the XbaI and SpeI sites of pGFP-N-FUS to create two tandem copies of GFP. Another copy of amplified M3GFP was ligated into the new vector digested with SpeI to create three tandem copies of GFP, two of which are the M3GFP version.

A series of GFP–*CRZ1* fusions (Table I) were constructed by PCR and cloning using the GFP fusion vector pOM4 and portions of the *CRZ1* gene from pAMS435 (Stathopoulos and Cyert, 1997). Full-length GFP–*CRZ1* (pRSP97) was constructed by inserting an ~2-kb SpeI-HindIII fragment containing the *CRZ1* ORF into the SpeI-HindIII sites of pOM4. CRZ1-C-term-394 (pRSP37) was constructed by inserting an ~0.9-kb EcoRV-HindIII fragment from pAMS435 into the SmaI-HindIII sites of pOM4. pRSP40 (GFP-NLS#1), pRSP153 (GFP-mutNLS#1-5), and pRSP156 (GFP-mutNLS#1-6) were created by digesting pRSP37, pRSP93, and pRSP112, respectively, with XhoI and re-ligating the ~7.7-kb fragment. pOM9-1 (GFP-NLS#1-3) containing the NH_2-terminal half of NLS#1 (amino acids 396–410) and pRSP50 (GFP-NLS#1-1) containing the COOH-terminal half of NLS#1 (amino acids 410–422) were created by oligo hybridization (Ausubel et al., 1994) to generate 42- and 39-bp SpeI-HindIII fragments, respectively, and ligated into the SpeI-HindIII sites of pOM4. GFP-NLS#2 (pRSP92) was created by oligo hybridization to generate a 21-bp SpeI-HindIII fragment, which was ligated into the SpeI and HindIII sites of pOM4. pRSP136 (GFP-NLS#1-2) and pRSP139 (GFP-NLS#1-4) were generated by oligo hybridization to create 57- and 72-bp SpeI-HindIII fragments, respectively, which were ligated into the SpeI and HindIII sites of pOM4. pRSP49 (CRZ1-C-term-469), was constructed using PCR to introduce a SpeI site at nucleotide (nt) 1411 and extended to the end of the *CRZ1* ORF to create an ~0.6-kb SpeI-HindIII fragment, which was inserted into the SpeI-HindIII sites of pOM4. pRSP70 (CRZ1-btwNLS#1&2) was created using PCR to introduce a SpeI site at nt 1312 and a stop codon followed by a HindIII site after nt 1820 to create an ~0.5-kb SpeI-HindIII fragment which was inserted into the SpeI-HindIII sites of pOM4. pRSP84 (CRZ1-N-term-388) was constructed using PCR to create a stop codon and ClaI site after nt 1161 to create an ~1.1-kb SpeI-ClaI fragment, which was inserted into the SpeI-ClaI sites of pOM4. GFP-NLS#1-5 (pRSP146) was created using PCR to introduce an XhoI site at nt 1257 in full-length *CRZ1*. An ~2-kb SpeI-HindIII fragment of *CRZ1* containing the XhoI site was ligated into the SpeI-HindIII sites of pOM4 and then was further digested with XhoI, and the remaining ~7.7-kb fragment was religated. The *CRZ1* NLS#1 (nucleotides 1260–1800) was deleted using the ExSite PCR-based site-directed mutagenesis kit and pRSP97 as the template to create pRSP102 (GFP–CRZ1-NLSΔ). pRSP93 (CRZ1-mutNLS#1-3), pRSP112 (CRZ1-mutNLS#1-2), pRSP114 (CRZ1-mutNLS#1), and pRSP134 (CRZ1-mutNLS#1-4) were generated using the ExSite mutagenesis kit and pRSP97 as template to replace either arginines 406–408, lysines 399, 401, 403, both sets of arginines and lysines, or arginines 420–421 to alanine, respectively.

An HA-tagged version of *CRZ1* (pAMS451) contains a triple HA-epitope tag inserted between codons 12 and 13 of the *CRZ1* ORF (Stathopoulos and Cyert, 1997). pRD56, provided by R. Deshaies (California Institute of Technology, Pasadena, CA), contains the GST ORF under the control of the *GAL1* promoter. GST-tagged *CRZ1* (pRSP121) was made by inserting an ~3.2-kb BamHI-SalI fragment containing the *GAL1* promoter fused to the *CRZ1* ORF with a 5′-GST tag (from pRD56) into the BamHI-XhoI sites of pGEX-4T3 (Amersham Pharmacia Biotech). GST-*NMD5*, provided by M. Rexach (Stanford University, Stanford, CA), consists of the *NMD5* ORF in pGEX-2TK. Both GST fusions were expressed in the protease-deficient bacterial strain BLR. 6×-His-*GSP1*, provided by K. Weis (University of California, San Francisco, CA), contains a Q71L mutation in the *GSP1* ORF that locks Gsp1p in the GTP-bound state and was expressed in the SG10034 bacterial strain.

Yeast strains and media

Yeast media and culture conditions have been described previously (Stathopoulos and Cyert, 1997; Stathopoulos-Gerontides et al., 1999). Yeast strains used in this study are derived from YPH499 (Table II). All other strains have been described previously.

Plasmid DNA was introduced into yeast using lithium acetate transformation (Ausubel et al., 1994). Double-stranded DNA templates used for sequencing were prepared (Qiaprep Spin Miniprep kit; QIAGEN) and sequenced using BigDye (Perkin Elmer/ABI) according to the manufacturers' specifications. Sequence analysis was performed at the Stanford PAN Facility (Stanford University, Stanford, CA).

GFP analysis

GFP was visualized in living cells as described previously (Stathopoulos-Gerontides et al., 1999). Cells were grown at 21°C in synthetic medium containing 0.1 mg/ml methionine and 60 μg/ml adenine. Living log-phase cells (OD_{600} = 0.6–1) were treated with or without 200 mM $CaCl_2$ for 10 min and then analyzed at 21°C for GFP localization on an Eclipse E600 microscope (Nikon) equipped with fluorescence optics using a 100/1.4 objective lens, an HB100 mercury lamp, and fluorescein filter sets (Chroma). Images were recorded digitally using a CCD 4742-95 camera (Hamamatsu) and QED Imaging, Inc. software. Images were processed digitally using Adobe Photoshop® (Adobe Systems, Inc.).

β-Galactosidase assay

Exponentially growing cells were grown in synthetic medium containing ammonium chloride at 21°C for 6 h with either no treatment or 20 mM $CaCl_2$ with or without 2 μg/ml FK520 (Merck). Cell extracts were prepared as described previously (Stathopoulos and Cyert, 1997; Withee et al., 1997), and protein concentrations were determined using the Bio-Rad Laboratories Bradford assay kit using BSA for the standard curve. β-Galactosidase activity was determined as nmoles ONPG converted per min per mil-

ligram of protein. Values represent the average of triplicate readings, and the standard deviation is representative of the error between readings.

Protein purification

GST-tagged Nmd5p and GST–Crz1p were purified from log-phase *E. coli* cultures induced for 2 h with 0.3 mM IPTG at 21°C. Cultures were harvested and lysed in lysis buffer (50 mM Tris, pH 8, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 5 mM DTT, 5 μg/ml pepstatin A, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2.5 mM benzamidine, 1 mM PMSF) using a French press (900 psi) followed immediately by the addition of NaCl to 1.5 M. The cell lysate was clarified by centrifugation at 30,000 *g* for 15 min, and Tween-20 was added to 0.1% to the resulting supernatant. The extracts were incubated with 1.5 ml of packed washed glutathione sepharose 4B resin (Amersham Pharmacia Biotech) for 2 h at 4°C. The resin was sedimented and transferred to a disposable mini-column (Talon column; CLONTECH) and washed with 5 column volumes of wash buffer (50 mM Tris, pH 8, 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.1% Tween-20), 2 column volumes of wash buffer plus 1 mM ATP, and then 3 column volumes of wash buffer. GST fusion proteins were eluted using 6 column volumes of wash buffer plus 15 mM reduced glutathione.

6×-His-tagged Gsp1p$^{(Q71L)}$–GTP or –GDP was isolated from log-phase *E. coli* cultures that were induced with 0.3 mM IPTG for 4 h at 21°C. Cells were harvested and lysed in His lysis buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM imidazole, 5 μg/ml pepstatin A, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2.5 mM benzamidine, 1 mM PMSF) using the French press (900 psi). The cell lysate was clarified by centrifugation at 30,000 *g* for 15 min followed by addition of Tween-20 to 0.1%, 0.1 mM ATP, 0.1 mM GTP or GDP to the resulting supernatant. The extract was incubated with packed washed nickel resin (Ni-NTA agarose; QIAGEN) for 1 h at 4°C. The resin was sedimented, washed with His lysis buffer, and transferred into a Talon column and washed with 10 column volumes of His lysis buffer plus 0.1% Tween-20, 0.1 mM ATP, 10 column volumes of His wash buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 20 mM imidazole, 0.1% Tween) plus 0.1 mM ATP, 10 column volumes of His wash buffer plus 0.5% Tween, then 10 column volumes of wash buffer. HIS-Gsp1p$^{(Q71L)}$–GTP or –GDP was eluted with 6 column volumes of His elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.1% Tween-20) and stored in His elution buffer plus 0.1 mM ATP, 0.1 mM GTP or GDP, respectively. The monomeric form of Gsp1p was isolated using FPLC (S-75 column, no. ÄKTA FPLC; Amersham Pharmacia Biotech) and stored in binding buffer (20 mM Hepes, pH 6.8, 150 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT) plus 0.1% Tween-20, 0.1 mM GTP or GDP.

Solution binding assay

Yeast cytosol was prepared from exponentially growing *crz1Δ* and *crz1Δcnb1Δ* cells expressing HA-CRZ1 (pAMS451) or GFP–CRZ1 fusion proteins (pAMS478, pAMS490, pRSP37). Cells were harvested, resuspended in binding buffer plus 250 mM sorbitol, 5 μg/ml pepstatin A, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2.5 mM benzamidine, 1 mM PMSF, and a phosphatase inhibitor cocktail (50 mM NaF, 50 mM sodium molybdate, 50 mM EDTA, 50 mM EGTA), and lysed using glass bead shearing. Cell lysates were clarified by centrifugation at 20,000 *g* and desalted using a G-25 Sephadex column (PD10 column; Amersham Pharmacia Biotech).

200 μg yeast cytosol were incubated with 50 μl of immobilized GST–Nmd5p for 3 h at 4°C in binding buffer. The resin was sedimented and washed twice with binding buffer, twice with binding buffer plus 1 mM ATP, twice with binding buffer followed by spinning through binding buffer plus 1 mM sucrose and washing twice with binding buffer. The samples were analyzed by SDS-PAGE followed by Western blotting.

Gsp1p disruption assay

50 μl of GST–Nmd5p resin was incubated with 0.1 units of thrombin (Calbiochem) in cleavage buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$) for 10 min at 21°C followed by 4 U of hirudin (Calbiochem) for 4 min at 37°C to inhibit the cleavage reaction. 5 μg GST–Crz1p was bound to glutathione resin and incubated with 5 μg thrombin-cleaved Nmd5p for 2 h at 4°C. The resin was washed as described for the solution binding assay with the omission of the ATP wash and then incubated with 10 μM Gsp1$^{(Q71L)}$–GTP or –GDP for 1 h at 21°C. The supernatant was harvested, and the resin was washed twice with binding buffer. The samples were analyzed by SDS-PAGE followed by Western blotting.

Immunoblot analysis

Protein samples were resolved using either 6 or 8.5% reducing gels and transferred to nitrocellulose membranes. Immunoblot analysis was performed using anti–rabbit or anti–mouse IgG-coupled HRP (Amersham Pharmacia Biotech) as secondary antibody, and blots were developed using the ECL system (Amersham Pharmacia Biotech). HA-Crz1p was visualized using monoclonal anti-HA 12CA5 antiserum (Roche Molecular Biochemicals). Nmd5p was visualized using polyclonal anti-Nmd5p antiserum provided by M. Rexach. GFP–Crz1p was visualized using polyclonal anti-GFP antiserum provided by P. Silver (Harvard Medical School, Boston, MA). Images were digitally processed using Adobe Photoshop®.

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