The High Mobility Group Box Transcription Factor Nhp6Ap Enters the Nucleus by a Calmodulin-dependent, Ran-independent Pathway

Received for publication, July 17, 2007, and in revised form, September 5, 2007 Published, JBC Papers in Press, September 18, 2007 DOI 10.1074/jbc.M705875200

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A gradient of Ran-GTP typically regulates traffic through the nuclear pore by modulating association of receptors with cargo. However, here we demonstrate that the yeast high mobility group box transcription factor Nhp6Ap enters the nucleus via a novel nuclear localization signal recognized by calcium calmodulin in a process that does not require Ran. Calmodulin is strictly required for the non-diffusional nuclear entry of Nhp6Ap. Calmodulin and DNA exhibit mutually exclusive binding to NHP6A, indicating that the directionality of Nhp6Ap nuclear accumulation may be driven by DNA-dependent dissociation of calmodulin. Our findings demonstrate that calmodulin can serve as a molecular switch triggering nuclear entry with subsequent dissociation of calmodulin binding upon interaction of cargo with chromatin. This pathway appears to be evolutionarily conserved; mammalian high mobility group box transcription factors often have two nuclear localization signals: one a classical Ran-dependent signal and a second that binds calmodulin. The finding that Nhp6Ap nuclear entry requires calmodulin but not Ran indicates that Nhp6Ap is a good model for studying this poorly understood but evolutionarily conserved calmodulin-dependent nuclear import pathway.

Proteins enter the nucleus through nuclear pore complexes (NPCs). Those smaller than ~40–50 kDa can traverse these pores by passive diffusion (1–5). Many larger proteins require soluble receptors, termed importins or karyopherins, which bind cargo in the cytoplasm, accompany it through the NPC, and release it in the nucleus. Most cargo proteins contain short nuclear localization signals (NLS), motifs recognized by the various receptor proteins. The small GTPase Ran drives the accumulation of cargo proteins in the nucleus by regulating the association and dissociation of NLS-containing proteins with importin/karyopherins.

In the nucleus, most Ran is GTP-bound, whereas it is largely GDP-bound in the cytoplasm. Cargo proteins bind importins in the presence of Ran-GDP in the cytoplasm, and when an importin bound to cargo arrives in the nucleus, Ran-GTP induces cargo release (6). Thus, the Ran-GTP gradient across the NPC drives the accumulation of most NLS-containing cargo in the nucleus.

Some Ran-independent nuclear import pathways have been described, although most remain poorly understood (for review see Ref. 7). One of the most intriguing of these involves the movement of calmodulin across the nuclear pore (8–10). Calmodulin nuclear import was argued to be a facilitated mechanism that was blocked by the calmodulin antagonist peptide M13 and did not require cytosolic factors or an ATP-regenerating system (8, 10). In the presence of calcium, calmodulin was also shown to be able to facilitate movement of large molecules into the nucleus. This latter property suggested a Ca2+-inducible nuclear import function for calmodulin that might operate during cellular activation. The calmodulin–mediated pathway was proposed to be redundant with the canonical Ran-GTP-driven nuclear entry pathway (9).

The potential transport function of calmodulin has more recently been highlighted by studies on two architectural transcription factors involved in mammalian sex determination, SRY and SOX9 (11–13). Both are members of the large HMGB family of transcription factors, and each contains a calmodulin-binding NLS (14). Defective nuclear import of SRY, the primary trigger for male sex determination, is observed in SRY mutants with altered calmodulin binding; this import defect leads to autosomal sex reversal (13). Like SRY, SOX9 must be translocated into the nucleus of Sertoli cells within the developing gonad. Binding of calmodulin to SOX9 leads to a conformational change and is associated with nuclear import of the transcription factor. A missense mutation (A158T) in SOX9 was found in a patient with Campomelic dysplasia/autosomal sex reversal. This mutation altered the conformation of SOX9-calmodulin complexes, resulting in diminished nuclear transport and transactivation (11). Taken together, these findings are consistent with a role for calmodulin in the regulated nuclear import of SOX9 and SRY.

Many HMGB proteins contain sequences that are similar to the calmodulin-binding motifs in SOX9 and SRY. However, understanding the role of calmodulin in HMGB protein nuclear import is complicated by the fact that many of these proteins,
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TABLE 1

| Plasmid | Relevant characteristics | Source |
|---------|--------------------------|--------|
| pRI642  | PK-GFP under NHP6A promoter; TRP1 | Ref. 15 |
| pRI6010 | NHP6A(1–36)-PK-GFP under NHP6A promoter; TRP1 | Ref. 15 |
| pR6008  | NHP6A-(37–93)-PK-GFP under NHP6A promoter; TRP1 | this study |
| pWP1399 | NHP6A-(E34D)-PK-GFP under NHP6A promoter; TRP1 | this study |
| pWP1395 | NHP6A-(Y28C)-PK-GFP under NHP6A promoter; TRP1 | this study |
| pRI1522 | NHP6A-GFP under NHP6A promoter; TRP1 | Ref. 15 |
| pR6025  | NHP6A-PK-GFP under GAL1 promoter; TRP1 | Ref. 15 |
| pGEX-4T-3 | E. coli GST fusion expression vector | Amersham |
| pTS15  | NHP6A(1–36) in pGEX-4T-3 | this study |
| pTS16  | NHP6A(1–54) in pGEX-4T-3 | this study |
| pTS17  | NHP6A(37–93) in pGEX-4T-3 | this study |
| pTS22  | NHP6A in pGEX-4T-3 | this study |
| pTS23  | NHP6A(R23A,R36A) in pGEX-4T-3 | this study |
| pWP1383 | NHP6A-(E34D) in pGEX-4T-3 | this study |
| pWP1384 | NHP6A-(Y28C) in pGEX-4T-3 | this study |
| pPS72  | H2B1-GFP under GAL1 promoter; HIS3 | P. Silver |
| pPS832 | GSP1 under GAL1 promoter; URA3 | P. Silver |
| pPS833 | GSP1(G21V) under GAL1 promoter; URA3 | P. Silver |
| pADH1-GADGFP | SV40 NLS-GAL4AD-GFP under ADH1 promoter; URA3 | D. Goldfarb |
| pGAL1-GADGFP | SV40 NLS-GAL4AD-GFP under GAL1 promoter; URA3 | D. Goldfarb |
| pAC148 | GSP1; LEU2 | A. Corbett |
| pAC414 | gpl-2; LEU2 | A. Corbett |
| pAC1411 | NT2; LEU2 | A. Corbett |
| pAC1412 | nfg2-1; LEU2 | A. Corbett |

including SOX9 and SRY, also contain classical NLS motifs that can direct nuclear entry by importins/karyopherins. In this report, we have turned to an HMG protein in the yeast *Saccharomyces cerevisiae*, Nhp6Ap, and confirmed that it enters the nucleus in a Ran-independent fashion (15). We further demonstrate that it contains a novel, calmodulin-binding NLS that can direct the nuclear entry of a large cargo protein too big to enter the nucleus by passive diffusion. This import is strongly inhibited by specific calmodulin antagonists or by temperature-sensitive mutations in calmodulin, which do not affect Ran-dependent nuclear import. Binding of Nhp6Ap to calmodulin and DNA are mutually exclusive, suggesting that DNA binding drives the accumulation of Nhp6Ap in the nucleus. Our data support a model in which Ca\(^{2+}\)-calmodulin associates with Nhp6Ap in the cytoplasm, promoting assembly of a complex that facilitates its passage through nuclear pores. Ca\(^{2+}\)-calmodulin dissociates from Nhp6Ap upon binding to calmodulin in the nucleus. This may represent a general paradigm for how calmodulin triggers the nuclear entry of HMGB transcription factors that are critical for cellular differentiation and sex determination.

EXPERIMENTAL PROCEDURES

Strains and Plasmids Used in This Study—The strains and plasmids used in this study are listed in Tables 1 and 2. The cells were grown in synthetic complete medium (0.67% yeast nitrogen base and 2% glucose). For expression of proteins from the GAL1 promoter, the cells were grown in medium containing 2% raffinose in place of glucose, and expression was induced by the addition of 2% galactose. Ophiobolin A (Sigma-Aldrich) was added to the medium to a final concentration of 50 μM from a 5 mM stock in methanol.

Microscopy—The cells were imaged live in growth medium at room temperature using an Olympus BX61 microscope, UPlanApo 100X/1.35 lens, Qimaging Retiga EX camera. IPabs version 3.6.3 software was used for image acquisition and analysis.

Confocal images for fluorescence loss in photobleaching were captured with a Zeiss LSM 510 NLO laser scanning inverted microscope using a Plan-Neofluor 100X/1.3 oil objective with argon laser line 488 nm (optical slices less than 4.2 μm). LSM 510 software version 3.2 was used for image acquisition and analysis. Magnification, laser power, and detector gains were identical across samples. The images were captured at 2-s intervals, and the cells were photobleached every 4 s.

Calmodulin Binding Assay—The GST-Nhp6Ap fusions were expressed in *Escherichia coli*, bound to glutathione-agarose, and eluted with 10 mM glutathione. After purification, the fusion proteins were incubated in a buffer containing 1 mM CaCl\(_2\) for 10 min at room temperature to remove fragments of DNA bound to Nhp6Ap. The fusions were dialyzed overnight against 1 M NaCl and eluted with 10 mM glutathione. After purification, the fusion proteins were incubated in a buffer containing 1 M NaCl and eluted with 10 mM glutathione. The GST-Nhp6Ap fusions were expressed in a GAL1 promoter; URA3, and eluted with 10 mM glutathione. After purification, the fusion proteins were incubated in a buffer containing 1 mM CaCl\(_2\) for 10 min at room temperature to remove fragments of DNA bound to Nhp6Ap. The fusions were dialyzed overnight against 20 mM Tris, pH 7.4, 100 mM NaCl, 2 mM MgCl\(_2\). For the calmodulin binding assay, ~100 μg of each fusion protein was incubated for 1 h at 4 °C with glutathione-agarose (Sigma-Aldrich) that had been equilibrated with binding buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 2 mM MgCl\(_2\), 0.1% bovine serum albumin, 1 mM dithiothreitol, and either 2 mM CaCl\(_2\) or 10 mM EGTA). The agarose beads were then washed three times with binding buffer, and 1 mg of biotinylated bovine brain calmodulin (Calbiochem) was added. After incubation for 1 h at 4 °C, the agarose was washed three times with binding buffer, and the fusion proteins were eluted with 10 mM glutathione. Approximately 10% of the elution was separated by SDS-PAGE, and the gels were either stained with Coomassie to visualize the fusion proteins or blotted to nitrocellulose membranes for Western blotting using IRDye 800CW labeled streptavidin and the Odyssey Infrared Imaging System (Li-Cor Biosciences).

For DNA competition experiments, the GST fusion proteins were incubated with glutathione-agarose and biotinylated DNA that are critical for cellular differentiation and sex determination.
bovine brain calmodulin and washed as above. About 5 μg of plasmid DNA was added and incubated for 1 h at room temperature. The fusion proteins were then eluted and processed as above.

To determine whether Ophiobolin A treatment prevents calmodulin binding to Nhp6Ap, 1 mg of biotinylated bovine brain calmodulin was incubated for 1 h in 250 μl of binding buffer with 2 mM CaCl2 and 50 μM Ophiobolin A (from a 5 mM stock in methanol). In a control reaction, the equivalent amount of calmodulin was incubated with the same amount of binding buffer (methanol). In a control reaction, the equivalent amount of calmodulin was incubated with the same amount of binding buffer and methanol. The treated and mock treated calmodulin were used in a calmodulin binding assay with GST-Nhp6Ap as described above.

Immunoblotting—Immunoblots with anti-GFP antibody (Roche Applied Science) were performed with the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Screen for Mutations in Nhp6Ap That Block Nuclear Import—To mutagenize NHP6A, error-prone PCR was performed with GeneMorph II random mutagenesis kit (Stratagene). The product of this reaction was used to transform the strain SEY6210 together with plasmid pRJ6006 that had been cut with NdeI and XmaI. Transformants were selected on medium that lacked tryptophan. The transformants were visually screened for those containing plasmid DNA was added and incubated for 1 h at room temperature. The fusion proteins were then eluted and processed as above.

RESULTS

Identification of the Nhp6Ap NLS—Some proteins less than ~40–50 kDa can enter the nucleus efficiently by simple diffusion, unaided by karyopherins or other proteins (1, 4, 5). Because the yeast HMGB transcription factor Nhp6Ap is 10.8 kDa, it might diffuse into the nucleus. However, like many small nuclear proteins, it could be actively transported into the nucleus. When Nhp6Ap is fused to pyruvate kinase (PK) and GFP, the resulting protein (Nhp6A-PK-GFP) is too large (92 kDa) to enter the nucleus efficiently by diffusion but is still highly concentrated there (Fig. 1A and Ref. 15). In contrast, a PK-GFP fusion lacking Nhp6A is largely excluded from the nucleus (Fig. 1B). Accumulation of Nhp6A-PK-GFP in the nucleus does not require high affinity DNA binding. A mutant form of the Nhp6Ap fusion, in which two residues important for DNA binding were mutated, Nhp6A-(R23A,R36A)-PK-GFP, still concentrates in the nucleus, although not to the same extent as a fusion with native Nhp6Ap (Fig. 1C). We confirmed this by quantitation of the ratio of the fluorescence intensity in

the nucleus and cytoplasm (Fig. 1G). Thus, Nhp6Ap enters the nucleus in a process that is nondiffusional and does not require DNA binding.

Previous work has shown that nuclear import of the Nhp6A-PK-GFP fusion does not require Gsp1p (the yeast Ran homolog) or any of the known karyopherins (15). To investigate this unusual nuclear import pathway, we wanted to identify which residues in Nhp6Ap contain an NLS. A–F, cells constitutively expressing various GFP fusions are shown. The arrow indicates the location of the nuclei in cells in which the fusion proteins are largely excluded from the nucleus. G, the ratio of fluorescence intensity in the nucleus and cytoplasm of the cells shown in A–F was calculated as described under “Experimental Procedures.” The error bars indicate the S.E., n = 15–20 cells. H, cells expressing the fusions used in A–F were immunoblotted with anti-GFP antibodies. The numbers on the left indicate the position of molecular mass standards.
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**Nuclear Entry via the Nhp6Ap NLS Does Not Require Ran (Gsp1p)**—Having localized the NLS of Nhp6Ap, we verified that the nuclear import of Nhp6A-(1–36)-PK-GFP, like Nhp6A-PK-GFP (15), does not require Gsp1p (the yeast Ran homolog). A plasmid encoding an inducible dominant negative Gsp1p (G21V) mutant was introduced into cells expressing Nhp6A-(1–36)-PK-GFP. Induction of this Gsp1p mutant did not affect the nuclear localization of Nhp6A-(1–36)-PK-GFP (Fig. 2A). It also did not interfere with import of Nhp6A-PK-GFP (15). As a control, we determined the ability of Gsp1p (G21V) to inhibit the nuclear import of histone H2B fused to GFP (H2B-GFP). Both H2B-GFP and the dominant negative Ran were placed under the inducible GAL1 promoters. When both were induced simultaneously, the nuclear import of H2B-GFP was substantially inhibited. As a control, we induced wild-type Gsp1p instead of Gsp1p (G21V). We quantified the effect of Gsp1p (G21V) induction on H2B-GFP nuclear localization and found that the nuclear to cytoplasmic ratio changed from about 13:1 to 3:1. However, the nuclear to cytoplasmic ratio of Nhp6A-(1–36)-PK-GFP was not significantly affected by Gsp1p (G21V) under these conditions.

To verify that Nhp6Ap nuclear import does not require Ran/Gsp1p, we determined the ability of Nhp6A-PK-GFP to enter the nucleus in cells with conditional defects in Gsp1p or a protein needed for Gsp1p function. Cells containing gsp1-2 are not viable at nonpermissive temperature (37 °C) and have severe defects in Ran-dependent nuclear import (16). We induced expression of Nhp6A-PK-GFP in gsp1-2 cells at the same time that they were shifted to 37 °C. The ratio of Nhp6A-PK-GFP in the nucleus and cytoplasm was not affected (Fig. 2B). In contrast, gsp1-2 severely affected nuclear accumulation of a fusion protein containing the Ran-dependent NLS from SV40 T antigen (SV40-NLS-GFP); the nuclear/cytoplasmic ratio of SV40-NLS-GFP dropped significantly. Thus, gsp1-2, like the dominant negative Gsp1p (G21V), does not affect the nuclear import of Nhp6A-PK-GFP.

Nuclear localization factor 2 (Ntf2p) transports RanGDP into the nucleus and is required for Ran function (16, 17). Cells containing ntfl-1 are not viable at elevated temperatures and have defects in nuclear import (16). Like gsp1-2, ntfl-1 reduces the nuclear/cytoplasmic ratio of SV40-NLS-GFP at nonpermissive temperature (Fig. 2C). However, ntfl-2 had no effect on the nuclear import of Nhp6A-PK-GFP. Taken together, these results show that Nhp6Ap utilizes a Ran-independent nuclear entry mechanism dependent upon the first 36 residues of Nhp6Ap.

**Nhp6Ap Nuclear Import Requires NPC Phenylalanine-Glycine (FG) Repeats**—In addition to Ran, most proteins imported into the nucleus also require importins/karyopherins, proteins that facilitate the transfer of NLS-containing proteins across the nuclear pore. These nuclear transport receptors interact with the FG repeats found in numerous NPC proteins, although

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precisely how these interactions promote transfer across the pore is not understood. Previous work has shown that Nhp6Ap transport into the nucleus is not slowed in mutants lacking any of the known yeast karyopherins (15). Thus, either a number of these proteins may be able to redundantly import Nhp6Ap, or alternatively, novel proteins may be needed. To determine whether FG-interacting proteins are needed for Nhp6Ap import, we assessed Nhp6Ap localization in mutants lacking some FG repeats (ΔFG strains). In a systematic study, Wente and co-workers (18) found that large portions of the FG repeats in NPC proteins could be deleted without affecting cell viability. However, many of these ΔFG strains have significant defects in the nuclear import of cargo with some classes of NLS. We examined Nhp6A-ΔPK-GFP import in four ΔFG strains and found it was significantly slowed in all of them; one of these is shown in Fig. 3 (the other ΔFG strains used are listed in Table 1). Thus, one or more FG repeat-interacting proteins are likely needed for Nhp6A-ΔPK-GFP import.

We also examined the localization of Nhp6A-GFP in the ΔFG strains. Because this fusion protein is only 38 kDa, less than the diffusion limit of the nuclear pore, Nhp6A-GFP import likely requires an FG domain-interacting protein. This finding suggests that Nhp6A-GFP forms a complex in the cytoplasm that may be too large to diffuse rapidly into the nucleus but that interacts with one or more proteins, facilitating its movement through the nuclear pore.

Nhp6A-ΔPK-GFP Nuclear Import Requires Calmodulin—Nhp6Ap exhibits substantial homology to mammalian SRY and SOX9, two HMGB proteins that interact with calmodulin (11, 19). This similarity prompted us to ask whether calmodulin might play a role in the nuclear import of Nhp6Ap in yeast. To examine the role of calmodulin in Nhp6Ap import, we used the specific calmodulin antagonist Ophiobolin A, which covalently modifies a critical lysine residue in calmodulin (20–22). Expression of an Nhp6A-ΔPK-GFP fusion was induced in cells at the same time that Ophiobolin A was added to the growth medium. After 1 h, most of the newly synthesized Nhp6A-ΔPK-GFP remained in the cytoplasm (Fig. 4A). To rule out nonspecific toxic effects of the inhibitor, the same experiment was done using the cell constitutively expressing SV40-NLS-GFP, which contains a classical Ran-dependent NLS. As expected, Ophiobolin A had no effect on the nuclear accumulation of this fusion.

We confirmed that calmodulin is required for Nhp6Ap nuclear import by measuring import in cells with temperature-sensitive defects in calmodulin. Yeast expresses a single calmodulin (Cmd1p), which is essential for viability. We induced expression of Nhp6A-ΔPK-GFP in cmd1-1 cells at the same time that they were shifted to nonpermissive temperature. As shown in Fig. 4B, Nhp6A-ΔPK-GFP nuclear import was dramatically reduced in cmd1-1 cells compared with a wild-type CMD1 strain. In contrast, the accumulation of SV40-NLS-GFP in the nucleus was not affected by cmd1-1.

The dramatic effect of cmd1-1 and Ophiobolin A on the localization of Nhp6A-ΔPK-GFP could be explained if calmodulin were required for DNA binding by Nhp6Ap in vivo. This is a possibility because a DNA-binding mutant of Nhp6Ap is much less enriched in the nucleus than unaltered Nhp6Ap, which binds DNA with high affinity (Fig. 1 and Ref. 15). To rule out this possibility because a DNA-binding mutant of Nhp6Ap is much less enriched in the nucleus than unaltered Nhp6Ap, which binds DNA with high affinity (Fig. 1 and Ref. 15). To rule this out, we determined the effect of cmd1 mutants on the nuclear localization of Nhp6A-(1–36)-ΔPK-GFP, which like the DNA-binding mutant of Nhp6Ap is enriched in the nucleus but is not exclusively nuclear. When cmd1-1 and cmd1-7 cells expressing Nhp6A-(1–36)-ΔPK-GFP were shifted from 30 to 37 °C (nonpermissive temperature), this fusion was no longer enriched in the nucleus; the nuclear to cytoplasmic ratio shifted from slightly more than 2:1 to less than 1:1 (Fig. 4C).

Calmodulin Inhibition Does Not Cause Nhp6Ap Export—These findings suggest that calmodulin is required for the nuclear entry of Nhp6A-ΔPK-GFP. However, an alternative explanation could be that inhibiting calmodulin promotes the nuclear export of Nhp6Ap rather than preventing import. To address this possibility, we measured the rate at which GFP fusions exit the nucleus using fluorescence loss in photobleaching, shown in Fig. 5. In these experiments, a small portion of the cytoplasm (green boxes) of cells expressing GFP fusions was subjected to repeated rounds of photobleaching, and we determined the rates at which the fluorescent signals in the nucleus
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Mutations in Nhp6Ap That Block Its Nuclear Import—To learn more about how Nhp6Ap is imported into the nucleus, we screened for mutations in Nhp6Ap that would prevent the nuclear import of an Nhp6A-PK-GFP fusion. The NHP6A portion of an Nhp6A-PK-GFP fusion was mutated by error-prone PCR and gap repair by homologous recombination in cells (see “Experimental Procedures”). Approximately 5000 transformants were visually screened, and we isolated four independent mutants in which the fusion was excluded from the nucleus. DNA sequencing revealed that three of the mutant plasmids encode Nhp6A with an E34D mutation and the other a Y28C mutation (Fig. 6A). Remarkably, both mutations are within the first 36 residues of Nhp6Ap, consistent with the idea that these residues are within an NLS. Nhp6A-PK-GFP fusions containing these mutations were excluded from the nucleus as efficiently as the PK-GFP fusion (Fig. 6B; compare with Fig. 1, A and B), suggesting that they block nuclear import of the fusions. The locations of these residues in the structure of Nhp6Ap are shown in Fig. 6C. To rule out that the mutations in Nhp6Ap caused it to be proteolytically removed from the PK-GFP portion of the fusions in cells, we confirmed that the fusions stay largely intact by immunoblotting with anti-GFP antibodies (Fig. 6D). Therefore, these mutations in Nhp6Ap block the nuclear import of Nhp6A-PK-GFP fusions.

Nhp6Ap Nuclear Entry Requires Direct Calmodulin Binding to Its NLS—We determined whether Nhp6Ap interacts with calmodulin directly. GST fusions to various Nhp6Ap derivatives were bound to glutathione-agarose, and their ability to bind with biotinylated bovine brain calmodulin was evaluated (see “Experimental Procedures”). As shown in Fig. 7A, Nhp6Ap bound to calmodulin in a calcium-dependent manner. Similar binding was observed with other fusions containing the Nhp6Ap NLS including residues 1–36, residues 1–54, and the DNA-binding mutant R23A,R36A. In contrast, the fusion containing residues 37–93 of Nhp6Ap, which lacks the calmodulin-dependent NLS, exhibited little detectable calmodulin binding. In addition, the Nhp6A-GST fusions with mutations that blocks nuclear import (E34D or Y28C) also did not bind cal-

Nhp6Ap and H2B bind DNA with very high affinity. Therefore, we determined the rate at which Nhp6A fusions that cannot bind DNA exit the nucleus. In cells expressing Nhp6A-(1–54)-PK-GFP, the cytoplasmic pool bleaches rapidly (Fig. 5B). In contrast, the nuclear pool of the fusion is partially reduced but then remains unchanged for the remainder of the experiment. This small decrease could represent a pool of the fusion that can be exported from the nucleus. However, it is more likely that the large optical slices used in this experiment capture some of the cytosolic pool surrounding the nucleus. Thus, the Nhp6A-(1–54)-PK-GFP fusion does not rapidly leave the nucleus. Similar results were obtained with Nhp6A-(R23A,R36A)-PK-GFP, which is impaired for DNA binding (data not shown). If inhibiting calmodulin increased the rate at which Nhp6A-(1–54)-PK-GFP exits the nucleus, treating cells with Ophiobolin A should increase the rate at which the nuclear pool of this fusion becomes depleted. However, Ophiobolin A did not significantly affect this rate (Fig. 5C). Thus, calmodulin is required for efficient import of Nhp6Ap fusion into the nucleus but not nuclear export.

FIGURE 4. Nhp6Ap nuclear import requires calmodulin. A, cells expressing either Nhp6A-PK-GFP under the GAL1 promoter or constitutively expressed SV40-NLS-GFP were grown in medium containing raffinose at 30 °C. Galactose was added to the medium with either 50 μM Ophiobolin A (from a stock in Me2SO) or Me2SO only (— Ophiobolin A), and the cells were visualized after 1 h. B, cells containing either a temperature-sensitive (cmd1-1) or wild-type CMD1 alleles were grown in raffinose medium at 25 °C. They also contained either a plasmid encoding Nhp6A-PK-GFP under the GAL1 promoter or a plasmid that constitutively expressed SV40-NLS-GFP. Galactose was added to the medium, and the cells were shifted to 37 °C and visualized after 2 h. C, cells containing either temperature-sensitive (cmd1-1 or cmd1-7) or wild-type CMD1 alleles and constitutively expressing Nhp6A-(1–36)-PK-GFP were grown in at 30 °C and visualized. They were then shifted to 37 °C for 1 h and visualized again. The ratio of fluorescence intensity in the nucleus and the cytoplasm was calculated as described under “Experimental Procedures.” The error bars indicate the S.E., n = 15–20 cells.

(red boxes) or an unbleached portion of the cytoplasm (blue boxes) were depleted. In cells expressing SV40-NLS-GFP, both the cytoplasmic and nuclear pools of the fusion protein were rapidly bleached (Fig. 5A). This was not unexpected, because the size of this fusion (42.8 kDa) is below the diffusion limit of the nucleus, and it can probably rapidly diffuse out of the nucleus. In cells expressing Nhp6A-PK-GFP or H2B-GFP, there was no fluorescence loss in the nucleus even after hundreds of seconds (data not shown), probably because both
modulin. Taken together, our findings indicate that the Nhp6Ap NLS directly binds calmodulin and that this binding of Nhp6Ap is strictly required for its nuclear entry. In every case, Nhp6Ap fusions that do not interact with calmodulin are also not imported into the nucleus.

Because treating cells with Ophiobolin A blocked the nuclear entry of Nhp6Ap, we wondered whether treating calmodulin in vitro would prevent it from binding Nhp6Ap.

FIGURE 5. Calmodulin inhibition does not stimulate Nhp6A-PK-GFP export. A, cells constitutively expressing SV40-NLS-GFP were subjected to fluorescence loss in photobleaching analysis as described under “Experimental Procedures.” The location of the bleach is shown with a green box.

FIGURE 6. Mutations that block Nhp6A-PK-GFP nuclear import. A, the first 36 residues of Nhp6Ap. The locations of two point mutations that block the nuclear import of Nhp6A-PK-GFP are shown. B, cells expressing the indicated fusions. The arrows indicate the locations of the nucleus. C, structure of Nhp6Ap bound to DNA (28). The Nhp6Ap NLS (residues 1–36) is shown in gold. The locations of the two residues where amino acid substitutions (E34D and Y28C) block Nhp6Ap nuclear import are shown in red. D, cells expressing the indicated fusions were immunoblotted with anti-GFP antibodies to rule out that the Nhp6A portions of the mutant fusions were degraded.

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Because treating cells with Ophiobolin A blocked the nuclear entry of Nhp6Ap, we wondered whether treating calmodulin with this drug in vitro would prevent it from binding Nhp6Ap.

The relative fluorescence over time was quantitated in the cytoplasm (blue box) and nucleus (red box). Images of the cells at the indicated times are shown above. The results were corrected for the small amount of bleaching that occurred in nearby untreated cells over time (typically 2–5%). The percent of the initial fluorescence (%Fo) is shown. B, as in A except with cells constitutively expressing Nhp6A-(1–54)-PK-GFP. C, as in B except that cells were treated with 50 μM Ophiobolin A for 30 min prior to the experiment.
Calmodulin-driven Nuclear Import of Nhp6Ap

A

![Coomasie staining](image)

B

![GST-Nhp6A](image)

C

![GST-Nhp6A](image)

FIGURE 7. The Nhp6Ap NLS binds to calmodulin. A, GST fusions to Nhp6Ap were immobilized on glutathione-Sepharose in the presence (+) or absence (−) of Ca²⁺. Biotinylated bovine brain calmodulin was allowed to bind to the fusions, which were then washed and eluted with glutathione. The amounts of eluted calmodulin were determined by Western blot (top panel), and the amount of eluted fusion proteins was visualized by Coomassie staining (bottom panel). B, as in A except that the calmodulin was either treated with 50 μM Ophiobolin A (+) or mock treated (−) before the binding assay. C, as in A except that before elution the samples were incubated with 5 μg of plasmid DNA for 1 h at room temperature.

After incubating biotinylated bovine brain calmodulin with Ophiobolin A, it was no longer able to bind Nhp6Ap (Fig. 7B). Thus, Ophiobolin A likely blocks the nuclear import of Nhp6Ap in cells by preventing calmodulin from binding to Nhp6Ap.

Because Nhp6Ap tightly binds DNA after it enters the nucleus, we wondered whether it could bind DNA and calmodulin simultaneously. After allowing biotinylated bovine brain calmodulin to bind the GST-Nhp6Ap fusion, we added plasmid DNA and found that the fusion no longer bound calmodulin (Fig. 7C). In contrast, Nhp6A-R23A,R36A, which is defective for DNA binding, was still able to bind calmodulin in the presence of DNA. Thus, calmodulin binding and DNA binding by Nhp6Ap are mutually exclusive. After entering the nucleus, calmodulin is likely displaced from Nhp6Ap when the transcription factor binds DNA. These findings are consistent with the NMR solution structure of Nhp6Ap bound to DNA (Fig. 6C), which shows the calmodulin-binding segment 1–36 highlighted in gold. This segment makes numerous contacts with the major groove of B-DNA (gray).

DISCUSSION

Our findings suggest that a novel, Ran-independent nuclear import pathway traffics Nhp6Ap into the nucleus. This pathway requires calmodulin, which directly binds the Nhp6Ap NLS; mutations in Nhp6Ap that block nuclear entry ablate calmodulin binding. In contrast, NLS motifs in Ran-dependent substrates are recognized by importins/karyopherins. Thus, calmodulin-dependent nuclear import appears to be totally distinct from the canonical pathway involving nucleotide-bound forms of Ran and importins. The finding that Nhp6Ap import dramatically slows in ΔFG strains suggests that an unidentified FG-interacting protein may also be required for Nhp6Ap nuclear entry.

How calmodulin binds Nhp6Ap or other HMGB proteins remains to be determined. The two Nhp6Ap residues that we found were required for calmodulin-binding are not conserved in mammalian HMGB proteins, suggesting that a structure rather than a specific sequence may be recognized by calmodulin. It is possible that calmodulin binds Nhp6Ap in a manner distinct from the standard wrap-around mode, because it seems unlikely that all cytosolic proteins that interact with calmodulin are imported into the nucleus. Indeed, recent evidence suggests that calmodulin can interact with some transcription factors in an unusual dimer conformation (23). Once bound, the complex of calmodulin and Nhp6Ap probably recruits additional proteins needed to facilitate its movement across the nuclear pore. This larger complex would enter the nucleus and upon interaction with DNA, calmodulin would dissociate, thus providing directionality to the transport pathway.

Why Nhp6A would use this novel nuclear import pathway is not yet known. Because calmodulin binding to the Nhp6Ap NLS is calcium-dependent, calcium signaling could regulate Nhp6Ap nuclear entry. However, Nhp6Ap seems to be constitutively in the nucleus, and there is no evidence that Nhp6Ap cycles between the nucleus and cytoplasm. It is also possible that calmodulin, together with other unknown proteins, could be required for proper loading of Nhp6Ap onto chromatin and so must accompany Nhp6Ap into the nucleus.

The yeast calmodulin-dependent import pathway utilized by Nhp6Ap is similar to a pathway we previously described in permeabilized mammalian cells (9). In this study, calmodulin was sufficient to mediate the import of large proteins bearing a number of basic peptides. This pathway was Ran-independent and was suggested to be an alternative import pathway important during cell activation (9). It was not clear from our previous study what types of nuclear proteins might be transported in a calmodulin-dependent fashion. More recently, calmodulin has been implicated in the nuclear transport of the HMGB proteins...
SOX9 (11) and SRY (19). These architectural transcription factors play key roles in numerous cellular differentiation pathways including sex determination. These proteins contain a calmodulin-binding site in addition to sequences recognized by importin β. A defect in calmodulin-dependent import was shown to be the underlying cause of at least one form of human sex reversal, a syndrome called Campomelic dysplasia/autosomal sex reversal (11, 12). A similar autosomal sex reversal phenotype occurs when three insulin-related receptors are ablated in mice, suggesting that intracellular signaling cascades may impact the normal functions of SRY and SOX9 (24, 25).

Based on sequence similarities, it is likely that the entire family of mammalian HMGB transcription factors use calmodulin as a means of regulating their nuclear import. In addition, the family of transcription factors using this pathway may extend to the helix-loop-helix family of transcription factors that also bind to calmodulin (23, 26, 27). The entry of nuclear proteins by this pathway may extend to members of the helix-loop-helix family of transcription factors as a means of regulating their nuclear import. In addition, the yeast Nhp6Ap provides a unique model for studying this evolutionarily conserved nuclear import pathway.

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