Nuclear Import of the Yeast AP-1-like Transcription Factor Yap1p Is Mediated by Transport Receptor Pse1p, and This Import Step Is Not Affected by Oxidative Stress*

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The yeast AP-1-like transcription factor, Yap1p, is essential for the oxidative stress response in budding yeast. Yap1p is located predominantly in the cytoplasm; however, upon imposition of oxidative stress, Yap1p concentrates in the nucleus and activates target genes. Yap1p is constitutively transported in and out of the nucleus. Oxidative stress inhibits the Crm1p/Xpo1p-dependent nuclear export step, resulting in nuclear accumulation of Yap1p. In this study, we examined the mechanism for Yap1p nuclear import, and determined whether the import step is affected by oxidative stress. The nuclear accumulation of Yap1p required the activity of the small GTPase, Ran/Gsp1p. Under conditions in pse1–1 cells carrying a temperature-sensitive mutation of the importin β family member PSE1/KAP121, nuclear translocation of Yap1p was inhibited dramatically. In an in vitro assay, we showed that Yap1p could directly bind to Pse1p and that this interaction was dissociated by Ran-GTP. These results indicate that Pse1p is the nuclear import receptor for Yap1p. In addition to Pse1p, we suggest that Kap123p, which is homologous to Pse1p, has a minor effect on the nuclear import of Yap1p. Furthermore, we identified the nuclear localization signal of Yap1p and demonstrated that the nuclear import of Yap1p was not affected by oxidative stress.

Regulation of the nucleocytoplasmic transport of transcription factors is, in most cases, a crucial step for the transmission of extracellular signals such as stress and growth stimuli to the nucleus. When such information is received, transcription factors in the cytoplasm translocate into the nucleus and become ready to activate specific gene expressions.

Macromolecules are transported in and out of the nucleus through the nuclear pore complex (NPC)† embedded in the nuclear envelope. Transport through the NPC is mediated by a family of transport receptors (importin β/karyopherin β family; importins and exportins) that share homology in the Ran binding domain. The transport receptors recognize cargos through transport signals such as the nuclear localization signal (NLS) and the nuclear export signal. It has been shown that multiple pathways of nucleocytoplasmic transport are carried out by utilizing different transport receptors and transport signals (see Ref. 1 for review). 14 transport receptors (the importin β family proteins) have been identified from the genome information of the budding yeast, *Saccharomyces cerevisiae* (1–3). To date, those include nine import receptors, four export receptors, and one other that is uncharacterized. Intriguingly, only four of the yeast transport receptors are essential, and the other receptors are not essential despite the fact that they transport essential cargos. One explanation for this is that multiple transport receptors may mediate the transport of a single specific cargo.

The direction of the nucleocytoplasmic transport is thought to be mediated by the function of small GTPase Ran (4, 5). Ran GTase-activating protein and its coactivator, Ran-binding protein 1, are localized in the cytoplasm (6, 7). On the other hand, the guanine nucleotide exchange factor for Ran is exclusively in the nucleus (8). This distribution predicts that Ran is in the GDP-loaded form (Ran-GDP) in the nucleus and in the GDP-loaded form in the cytoplasm. According to the predicted gradient of different nucleotide-loaded forms of Ran, it has been suggested that these different forms of Ran can regulate the association and dissociation of the receptor-cargo complex.

The binding of Ran-GTP to the import receptor facilitates the dissociation of the import receptor-cargo complex and releases cargo into the nucleus (4, 9, 10). In contrast, the binding of Ran-GTP to the export receptor is required for the formation of the export receptor-cargo-Ran-GTP complex (11–13). When this trimeric complex is recognized by Ran GTase-activating protein and Ran-binding protein 1 in the cytoplasm, it dissociates and releases the cargo into the cytoplasm (12, 14). Thus, the Ran-GTP gradient is a key determinant for transport event between the nuclear and cytoplasmic compartments.

It is becoming clear that nuclear translocation of transcription factors depends on whether transport signals for the receptors are presented rather than on changing activity of the receptor. Some examples indicate that the NLSs of transcription factors, which are normally covered with a higher ordered structure, can be exposed by extracellular signals transmitted through phosphorylation array (see Ref. 15 for review).

The yeast AP-1-like transcription factor, Yap1p, which has a basic leucine-zipper domain and forms a homodimer, is crucial for the oxidative stress response in the budding yeast (16). In response to oxidative stress, Yap1p is activated to induce mul-
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Multiple target genes, which are critical in the cellular defense system for oxidative stress to increase the levels of reduced thioredoxin and reduced glutathione in the cells (see Ref. 17 for review). We have shown previously that Yap1p is mainly localized in the cytoplasm and translocates into the nucleus under oxidative stress (16). Interestingly, the nuclear translocation of Yap1p is regulated at the step of nuclear export. Yap1p is likely to be constitutively imported into the nucleus and exported to the cytoplasm by the export receptor Crm1p/Xpo1p. When oxidative stress is imposed, the later step, that is, the interaction of Crm1p with the cysteine-rich domain at the C terminus of Yap1p, is inhibited, resulting in increased Yap1p levels in the nucleus (18, 19). Consistent with this result, Yap1p derivatives lacking the cysteine-rich domain are constitutively localized in the nucleus, and the resulting Yap1p-dependent transcription of the reporter gene is increased. In addition to this elevated level of transcription, however, oxidative stress can further induce reporter gene expression, suggesting that an additional mode of the regulatory system is responsible (16). We have so far found no evidence that the import step is not responsible for the oxidative stress-induced nuclear localization of Yap1p.

Here we address the molecular mechanism of the nuclear import of Yap1p to determine whether oxidative stress affects the Yap1p import step. Our results indicate that importin β family member Pse1p/Kap123p is the nuclear import receptor for Yap1p. In addition to Pse1p, we suggest that Kap123p, which is related closely to Pse1p, has a minor effect on the nuclear import of Yap1p. Moreover, we identified the Yap1p NLS and showed that oxidative stress does not affect the nuclear import pathway of Yap1p.

**Experimental Procedures**

**Yeast Strains and Cultures**—The yeast strains used in this study are listed in Table I. Yeast cells were grown in yeast extract, pepton, adenine, dextrose or synthetic medium supplemented with amino acids (20). Yeast cells were exposed to oxidative stress by the addition of diamide to a final concentration of 1.5 mM or of H2O2 to a final concentration of 0.5 mM, when required, as described previously (16).

**Construction of Plasmids**—Expression plasmids for the green fluorescent protein (GFP) fused to Yap1p were constructed as follows: GFPcoat-fused Yap1p expression plasmids, pRS cpGFP-YAP1, and pRS cpGFP-yap1(1–244) (TRP1 CEN) (16) were digested with Sal I and NcoI and inserted into the corresponding sites of pRS315 (21) to generate pRS315-GFP-YAP1 and pRS315-GFP-yap1(1–244), respectively.

To make the plasmid to express Pse1p as a fusion protein to glutathione S-transferase (GST), a BamHI-Msc1 fragment corresponding to an N-terminal region of Pse1p from pPS1567 (22) and an Msc1-XhoI fragment corresponding to a C-terminal region of Pse1p from pPS1066 (22) were ligated into BamHI and SalI sites of pGEX-6P-2 (Amersham Pharmacia Biotech). The resulting GST-Pse1p expression plasmid was designated pGEX-PSE1.

The pGEX-KAP123 was constructed as follows: A 5′-coding region of KAP123 was amplified from pPS1067 containing KAP123 (22) by polymerase chain reaction (PCR) using 5′-GTCAGCTCGAGTGAGTCAACAATTTCTAAGTCA-3′ and 5′-TTCAGCAAGAAGCAGTTGGCATG-3′ as primers (SalI and XcmI sites are underlined) and digested SalI and XcmI; a 3′-coding region of KAP123 was isolated from pPS1067 with XcmI and AvaII, where the AvaII site was blunt-ended; and these fragments were inserted between SalI and blunt-ended NotI of pGEX-6P-2.

To generate hemagglutinin (HA)-tagged GFP-Yap1p(1–244), a BamHI-BstEII fragment corresponding to a C-terminal region of Yap1p from pRS cpGFP-HA-YAP1 (16) was replaced by that containing yap1(1–244) from pRS cpGFP-yap1(1–244) (16). The resulting plasmid pRS cpGFP-HA-yap1(1–244) was digested with PvuII and SalI and inserted into the blunt-ended EcoRI and SalI sites of pET28b(+) (Novagen) to construct the His-tagged Yap1p(1–244) expression plasmid, pET-yap1(1–244).

By utilizing pPS965 (23) as a template for GSP1, a G21V mutation was introduced by PCR using the following primers: primer 1, 5′-GATATAGACCATATGCTTCGAAAGTGGT-3′; primer 2, 5′-TC_CAGAAGTGGTGAATGTTGACGCAGTTGGCATG-3′; and primer 3, 5′-GCTACTGTCGAGATGTTGACGCAGTTGGCATG-3′. A 5′-coding region of GSP1 was isolated by PCR using primers 1 and 2 (NdeI and SpeI sites are underlined) and digested with Ndel and SpeI. A 3′-coding region of GSP1, in which the 21st codon of GGT (Gly) was changed to GTC (Val) (G21V mutation), was isolated by PCR using primers 2 and 3 (SpeI and SalI sites are underlined) and digested with SpeI and SalI. Both of these fragments were ligated between the Ndel and SalI sites of pET15b (Novagen) to generate the His-tagged Gsp1pG21V expression plasmid. The resulting plasmid was designated pET-GSP1G21V.

In the construction of plasmids to identify Yap1p NLS, the following GFP fusion vector was generated. Plasmids having genes encoding the duplicate infame fusion protein of GFP36 (24) as well as the HA tag were constructed under the regulated constitutive cup1 promoter (16) or inducible met3 promoter (18) in plasmid pRS15 (21) and were designated pRS15 cup1-2xGFP-HA and pRS15 met3-2xGFP-HA, respectively. To generate a series of Yap1p derivatives on the N-terminal region of Yap1p (see Fig. 5A), PCRs using corresponding synthetic oligonucleotides are carried out or restriction enzymes are used. The resulting fragments corresponding to various N-terminal regions of Yap1p were inserted in the 3′-coding region of duplicated GFP. Expected fusion proteins expressed by these plasmids are more than 60 kilodaltons.

**In Vivo Import Assay**—To determine the minor transport activity of Yap1p and the effect of oxidative stress on the nuclear import of Yap1p, we performed an in vivo nuclear import assay using the inducible met3 promoter to express 2xGFP-fused Yap1p(1–571) or Yap1p(2–59). Yeast cells were cultured under a condition in which the met3 promoter was suppressed in the presence of methionine until the mid-log phase. The cells were then collected by centrifugation, washed once with methionine-depleted medium, and incubated in the methionine-depleted medium to induce the expression of 2xGFP-Yap1p derivatives by the met3 promoter. Oxidative stress and/or temperature shift from the permissive temperature (25 °C) to the restrictive temperature (37 °C) were performed at the same time as induction by the met3 promoter. Expression analysis was carried out as described previously (16). Briefly, yeast cells were grown to the mid-log phase, and the localization of GFP in live yeast cells was observed using a confocal laser scanning microscope (MRC1024, Bio-Rad).

**Recombinant Protein Expression and Purification**—Purification of GST fusion proteins was performed as described by Isoyama et al. (25). Escherichia coli BL21 cells were transformed with the pGEX-PSE1 or pGEX-KAP123 plasmids to express fusion proteins to GST and grown at 37 °C in the presence of ampicillin. At an A600 of 0.5, isopropyl-β-D-thiogalactoside was added to the culture at a final concentration of 1 mM and further cultured at 37 °C for 4 h. The cells were harvested by
centrifugation, resuspended in phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄), and disrupted by sonication. The cell lysates were mixed with Triton X-100 to a final concentration of 1% and incubated at 4 °C for 30 min. The fusion proteins were purified by using glutathione-Sepharose 4B according to the manufacturer’s instructions (Amersham Pharmacia Biotech). To isolate His-Yap1p(1–244) or His-Gsp1pG21V, E. coli BL21(DE3) cells carrying pET-yap1(1–244) or pET-GSP1G21V were grown in the presence of kanamycin or ampicillin, respectively, and the expression was induced with isopropyl-β-D-thiogalactoside at a final concentration of 1 mM at 20 °C for 20 h. The cells were harvested and sonicated in lysis buffer (50 mM Na₂HPO₄, pH 8.0, 300 mM NaCl, 1 mM 2-mercaptoethanol, and 20 mM imidazole). After the addition of Triton X-100 to a final concentration of 1%, the lysates were loaded into a column of nickel-nitrilotriacetic acid agarose according to the manufacturer’s instructions (Qiagen). The column was washed, and the fusion proteins were eluted with elution buffer (lysis buffer containing 250 mM imidazole).

Purified fusion proteins were concentrated by VIVASPIN (Vivascience) and further purified by chromatography on a Superdex 200 in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 2 mM 2-mercaptoethanol using the SMART system (Amersham Pharmacia Biotech).

**Binding Assay—** For the assay of Yap1p binding to Pse1p or Kap123p, fusion proteins were expressed and purified from bacterial lystate as described above. 5 μg of GST-Pse1p, GST-Kap123p, or GST were bound to 20 μl of the 50% slurry of glutathione-Sepharose beads by incubating in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 10% glycerol, and 0.5 mg/ml bovine serum albumin) for 2 h at 4 °C. The beads bound to GST fusion proteins were washed with washing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, and 10% glycerol) and incubated with 5 μg of His-Yap1p(1–244) in 250 μl of binding buffer for 3 h at 4 °C. After washing extensively, bound proteins were eluted with 10 mM reduced glutathione and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie staining or immunoblotting. His-Yap1p(1–244) was detected with anti-His-Tag (MBL) and peroxidase-conjugated goat anti-rabbit immunoglobulins (DAKO) as primary and secondary antibodies, respectively. ECL Western blotting detection reagents were used according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

**Dissociation Experiment—** His-Gsp1pG21V was incubated in the presence of 2 mM GTP, 20 mM EDTA, and 2 mM dithiothreitol for 1 h at room temperature. After the addition of MgCl₂ to a final concentration of 50 mM, the reaction mixture was incubated further on ice for 20 min. His-Gsp1pG21V loaded with GTP (His-Gsp1pG21V·GTP) was purified by chromatography on Fast Desalting (Amersham Pharmacia Biotech) in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM MgCl₂, and 2 mM 2-mercaptoethanol. 10 μg of His-Yap1p(1–244) was incubated with 10 μg of GST-Pse1p or GST-Kap123p prebound to 20 μl of glutathione-Sepharose beads in a reaction volume of 500 μl for 3 h at 4 °C. The beads were washed with washing buffer, divided into two equal parts, and incubated with 5 μg of His-Gsp1pG21V·GTP or buffer for 30 min at room temperature. After centrifugation, the supernatant was collected as unbound protein. Then, the beads were washed several times with washing buffer, and the bound protein was dissolved in SDS sample buffer. The samples were separated by SDS-PAGE and immunoblotted with anti-His-Tag antibodies as described above.

**RESULTS**

**Nuclear Accumulation of Yap1p Requires Ran/Gsp1p—** In most cases, nuclear transport events require the activity of the small GTPase Ran (4, 9, 10). Therefore, we first examined if the nuclear import of Yap1p also requires the activity of Ran using a previously characterized temperature-sensitive mutant (gsp1–1) of GSP1 encoding the budding yeast homologue of Ran (23). The constitutive nuclear-localized mutant of Yap1p, GFP-Yap1p(1–571), was expressed in gsp1–1 cells, and the cells were cultured at the permissive temperature of 25 °C. As we previously observed in the wild-type cells (16), GFP-Yap1p(1–571) was localized in the nucleus (Fig. 2A, panel 1). In contrast, this nuclear accumulation was inhibited at the restrictive temperature (37 °C) (Fig. 1), indicating clearly that the nuclear import of Yap1p is mediated in a Ran-dependent manner.

**Nuclear Accumulation of Yap1p Requires Pse1p—** The above data suggested that, like many other NLS-containing cargo proteins, Yap1p was imported into the nucleus by a transport receptor(s) that requires Ran for its transport activity. To identify such a receptor(s), we examined how the nuclear accumulation of Yap1p was affected using a panel of yeast strains carrying each mutation of transport receptors including the importin β family members as well as importin α. Two different conditions were used to induce the nuclear accumulation, or in other words, to inhibit the nuclear export step of Yap1p. First, the constitutive nuclear-localized mutant of Yap1p (GFP-Yap1p(1–571)) was expressed in mutant cells, and we observed which mutation affected its localization. Second, wild-type Yap1p (Yap1pWT) fused with GFP was expressed in each mutant cell, and the specific nuclear export step of Yap1pWT was inhibited by oxidative stress to induce nuclear accumulation of Yap1p (18).

Yeast cells carrying a mutation of importin α (erp1–31) or importin β (trs1–4) showed no defect in nuclear translocation of Yap1p (data not shown), demonstrating that the nuclear import of Yap1p does not depend on the classical NLS import pathway. Other strains carrying mutations of importin β family member axm1Δ, nmd3Δ, or mtr10Δ showed no defect in the nuclear accumulation of Yap1p (data not shown). However, we found that yeast localization of GFP-Yap1p(1–571) was significantly inhibited in the temperature-sensitive PSE1 mutant (pse1–1) cells under the restrictive temperature of 37 °C (Fig. 2A, compare panels 2 and 3). In addition, unlike Pse1p, Kap123p did not affect the nuclear localization, although Kap123p is related closely to Pse1p (Fig. 2A, panel 4).

Intriguingly however, most of the GFP-Yap1p(1–571) was spread in the cytoplasm in pse1–1 cells at the restrictive temperature and residual fluorescence could be observed in the nucleus (Fig. 2A, panel 3). One possible explanation for this was that preexisting GFP-Yap1p(1–571) in the nucleus was not degraded within 1 h after shifting to the restrictive temperature. To examine this possibility, nuclear accumulation of Yap1pWT was induced by the imposition of oxidative stress in pse1–1 cells at the same time as the temperature shift to the restrictive temperature. Again, the nuclear accumulation of Yap1pWT induced by diamide was drastically inhibited only in pse1–1 cells at the restrictive temperature (Fig. 2B, compare panels 4 and 6 with panels 3 and 5) but not other mutant cells (Fig. 2B, compare panels 7 and 8; data not shown). Nevertheless, as shown in Fig. 2B, panel 6, the residual nuclear fluorescence of GFP-Yap1pWT still could be observed in this condition.

We then speculated that other transport receptors could confer the weak activity for nuclear import of Yap1p in addition...
Yap1p (Yap1pWT, B21866) in yeast cells carrying a pse1–1 in vivo of 2xGFP-Yap1p(1–571) still could be observed in the shift to the restrictive temperature (37 °C) as described under depleting methionine from the medium at the same time of the was repressed with the addition of methionine and induced by possible artifacts caused by the oxidative stress and protein stability described above. Expression of 2xGFP-Yap1p(1–571) was observed in wild-type, pse1–1, or kap123Δ cells transformed with pRS315-GFP-Yap1p. The cells were incubated at 30 °C (panels 1 and 2, wild-type cells; panels 7 and 8, kap123Δ cells), the permissive temperature of 25 °C (panels 3 and 4, pse1–1 cells), or the restrictive temperature of 37 °C (panels 5 and 6, pse1–1 cells) with 1.5 mM diamide (+, panels 2, 4, 6, and 8) or without diamide (−, panels 1, 3, 5, and 7) for 1 h. The fluorescent and transmitted images were detected as described in the legend to Fig. 1.

Previous studies have indicated that Pse1p and its closely related transport receptor Kap123p (Yrb4p) can confer overlapping functions for nuclear import, e.g., ribosomal protein L25 is transported mainly by Kap123p; however, Pse1p can substitute for Kap123p (10, 26). Therefore, we predicted that Kap123p might be responsible for the residual nuclear localization of Yap1p in pse1–1 cells at the restrictive temperature. To test this possibility, we observed the nuclear accumulation of Yap1p in yeast cells carrying a KAPI23 disruption mutant in the background of pse1–1 (pse1–1 kap123Δ cells). In this case, we performed an in vivo import assay to eliminate the effect of possible artifacts caused by the oxidative stress and protein stability described above. Expression of 2xGFp-Yap1p(1–571) was repressed with the addition of methionine and induced by depleting methionine from the medium at the same time of the shift to the restrictive temperature (37 °C) as described under “Experimental Procedures.” Although the residual fluorescence of 2xGFp-Yap1p(1–571) still could be observed in the pse1–1 cells (Fig. 3, panel 2), it was strongly suppressed in pse1–1 kap123Δ cells at the restrictive temperature (Fig. 3, compare panels 2 and 4). Taken together, these results indicate that Pse1p is a nuclear import receptor responsible for Yap1p; however, Kap123p also has a minor potential function when the activity of Pse1p is repressed under restrictive conditions.

Direct Binding of Yap1p to Pse1p—Previous studies have indicated that transport receptors can directly bind to the import cargos. To test whether this was the case for the interaction between Pse1p or Kap123p and Yap1p in an in vitro binding assay, His-tagged Yap1p(1–244), which has an NLS and can be constitutively localized in the nucleus (16), and GST-fused Pse1p were expressed in E. coli and purified. His-Yap1p(1–244) was mixed with GST-Pse1p or GST-Kap123p prebound to glutathione-Sepharose beads, and protein bound to the beads was resolved in SDSPAGE followed by Coomassie staining to detect Pse1p and Kap123p or immunoblotting with anti-His-tag antibodies to detect Yap1p. We found that His-Yap1p(1–244) could bind to GST-Pse1p as well as GST-Kap123p, but not to GST alone (Fig. 4A), indicating that Yap1p directly interacts with Pse1p and Kap123p. As shown in Fig. 4, lane 3, degradation products of GST-Kap123p, which were purified by glutathione-Sepharose beads, were detected. We speculated that these products did not bind to Yap1p because the degradation might have been limited in the C-terminal region of Kap123p, which is generally required for cargo binding. Therefore, it was presumed that His-Yap1p(1–244) bound only to full-length GST-Kap123p. Despite the minor function of Kap123p on the nuclear import of Yap1p as described above, the amount of Yap1p(1–244) binding to Pse1p was similar to that binding to Kap123p in vitro (Fig. 4A, compare lanes 2 and 3).

Yap1p-Pse1p Complex Is Dissociated by Gsp1p-GTP—Previous studies indicate that Ran-GTP can dissociate import receptor-cargo interaction (4, 9, 10). To determine whether the interaction between Yap1p and Pse1p or Kap123p could be disrupted by Gsp1p-GTP, we prepared the G21V mutant of Gsp1p (Gsp1pG21V), where GTP hydrolysis was disrupted by the mutation (27). As shown in Fig. 4B, Yap1p(1–244) bound to Pse1p and Kap123p were clearly dissociated by the addition of Gsp1pG21V-GTP (compare lanes 2 and 4 with lanes 1 and 3).
His-Yap1p (1–244) were incubated and washed extensively. The dissociation reaction was carried out with His-Gsp1p G21V-GTP (dissociated the Yap1p-Pse1p and Yap1p-Kap123p complexes. GST fusion proteins of import receptors bound to glutathione-Sepharose beads and degradation products of Kap123p, because these were detected by immunoblotting with anti-GST antibodies (data not shown).

Figure 4. Yap1p interacts directly with Pse1p and Kap123p, and the interaction is dissociated by Gsp1p-GTP. A, Yap1p associated with Pse1p and Kap123p. GST, GST-Pse1p, or GST-Kap123p purified from E. coli were bound to glutathione-Sepharose beads, incubated with His-Yap1p (1–244) also purified from E. coli, and washed extensively. Bound proteins were eluted with 10 mM glutathione, and eluted proteins were separated on 12% SDS-PAGE and subjected to Coomassie staining (upper panel) or immunoblotting with anti-His-tag antibodies (lower panel). His-Yap1p (1–244) bound to GST, GST-Pse1p, and GST-Kap123p are shown in lanes 1, 2, and 3, respectively. The bands representing GST, GST-Pse1p, GST-Kap123p, and His-Yap1p (1–244) are indicated by arrows. The positions of molecular mass markers are shown on the left of the figure in kilodaltons. The protein of 67 kDa is bovine serum albumin. It is also noted that some bands (from 55 to 70 kDa) appearing in lanes 3 and 4 were not that from 10 to 49, could be deleted without having an effect on NLS activity. Although the deletion of four N-terminal amino acids did not affect NLS activity, the deletion of nine amino acids further abrogated NLS activity. Two lines of evidence indicate that Pse1p mediates the nuclear import of Yap1p. First, the nuclear transport of Yap1p was strongly prevented in pse1Δ cells at the restrictive temperature, whereas none of the other mutant cells carrying each gene under the inducible met3 promoter was not affected by the oxidative stress induced by either diamide (Fig. 6) or H2O2 (data not shown). Therefore, we conclude that oxidative stress does not affect the nuclear import of Yap1p.

Identification of Yap1p NLS—We have shown previously that the Yap1p NLS is located within amino acids 1–244 (16), which contain the basic DNA binding region as well as the dimerization domain (basic leucine-zipper). To identify the Yap1p NLS, genes corresponding to the series of N-terminal regions of Yap1p were fused to duplicate copies of GFP536 genes under the inducible met3 promoter as described under “Experimental Procedures.” As shown in Fig. 5, Yap1p (2–72), Yap1p (2–66), and Yap1p (2–59) were localized in the nucleus (A and B, panel 2). However, when this C-terminal deletion was extended further to amino acid position 50 (Yap1p (2–49)), the nuclear localization of Yap1p was abolished (Fig. 5B, panel 3), suggesting that Yap1p amino acids 50–59 are essential for the NLS function. Actually, Yap1p amino acid region from 50 to 59 (KKKGSKTSKK) is similar to the classical NLS (28). Therefore, we tested whether this short amino acid sequence could act as an NLS. Unexpectedly, it failed to confer NLS activity when fused to duplicate copies of GFP (Fig. 5B, panel 4). Next we demonstrated the effect of N-terminal deletion for the NLS activity. Although the deletion of four N-terminal amino acids did not affect NLS activity, the deletion of nine amino acids strongly abrogated NLS activity (Fig. 5B, panels 5 and 6). Further deletion did not recover the NLS activity of Yap1p (Fig. 5A). However, the internal amino acid region from 17 to 49, not from 10 to 49, could be deleted without having an effect on NLS activity (Fig. 5, A and B, panels 7 and 8). Taken together, we conclude that two amino acid regions of Yap1p, 5–16 and 50–59, are required for the NLS activity. In addition, when the two basic amino acid residues in this N-terminal region were substituted to alanine (K7A, R8A), the NLS activity was abrogated (data not shown).

Nuclear Import of Yap1p Is Not Affected by Oxidative Stress—We have shown previously that the constitutive nuclear-localized Yap1p (1–571) can still confer further elevation of the transcriptional level of the reporter gene in response to oxidative stress (16). Therefore we performed an in vivo import assay of Yap1p (1–571) and Yap1p (2–59) under oxidative stress to examine whether oxidative stress could affect the nuclear import of Yap1p. The nuclear accumulation of Yap1p (1–571) or Yap1p (2–59) observed at 30, 60, and 90 min after induction of the met3 promoter was not affected by the oxidative stress imposed by either diamide (Fig. 6) or H2O2 (data not shown). Therefore, we conclude that oxidative stress does not affect the nuclear import of Yap1p.

DISCUSSION

Regulation of the Yap1p transcription factor is mediated mainly at the level of nuclear localization. Inhibition of the nuclear export step by oxidative stress results in an increased level of Yap1p in the nucleus, leading to the activation of target gene transcription. It has been suggested that an additional activation mechanism confers enhancement of Yap1p-dependent transcription because the constitutive nuclear-localized Yap1p mutant (Yap1p (1–571)) can be further activated by oxidative stress (16). Here we examined the molecular mechanism of the import step of Yap1p and determined whether the import step of Yap1p was affected by oxidative stress.

Two lines of evidence indicate that Pse1p mediates the nuclear import of Yap1p. First, the nuclear transport of Yap1p was strongly prevented in pse1Δ cells at the restrictive temperature, whereas none of the other mutant cells carrying each one of the genes encoding an importin β family member as well as an adaptor molecule (importin α) that we tested affected the nuclear import of Yap1p (Fig. 2). In addition, our genetic studies also showed that the activity of the small GTPase Ran was required for the nuclear accumulation of Yap1p, similar to many other nuclear transport cargos (Fig. 1). Second, we demonstrated that Yap1p can directly interact with Pse1p in vitro, and Ran-GTP can specifically inhibit this interaction (Fig. 4).

In addition to the Pse1p-mediated nuclear import of Yap1p, we observed a minor effect of Kap123p on the nuclear import of Yap1p when the two basic amino acid residues in this N-terminal region were substituted to alanine (K7A, R8A), the NLS activity was abrogated (data not shown). However, the internal amino acid region from 17 to 49, not from 10 to 49, could be deleted without having an effect on NLS activity (Fig. 5, A and B, panels 7 and 8). Taken together, we conclude that two amino acid regions of Yap1p, 5–16 and 50–59, are required for the NLS activity. In addition, when the two basic amino acid residues in this N-terminal region were substituted to alanine (K7A, R8A), the NLS activity was abrogated (data not shown).
Yap1p (Fig. 3). More residual nuclear-localized Yap1p was detected in pse1–1 cells than in pse1–1 kap123A cells at the restrictive temperature. In addition, we showed that Kap123p can also bind directly to Yap1p (Fig. 4A), and the other group showed that Kap123p can interact with Yap1p by the two-hybrid assay (19). However, our observation indicated the discrepancy between the in vitro binding ability and the nuclear import ability of Pse1p and Kap123p. Such a discrepancy may be caused by the inefficiency of the other nuclear import step after Kap123p–Yap1p binding, e.g. transport through the NPC and dissociation of the receptor-cargo complex in the nucleus. Thus, we conclude that Pse1p is a major import receptor for Yap1p, and Kap123p has a minor potential function. A similar example has been indicated for the case of ribosomal protein L25 (10). Ribosomal protein L25 can bind to Kap123p and Pse1p in vitro. Disruption of KAP123 inhibits the nuclear localization of ribosomal protein L25, indicating that Kap123p is a major import receptor for the ribosomal protein. However, it has been suggested that Pse1p is a minor import receptor for ribosomal protein L25 because overexpression of Pse1p induces the nuclear localization of the ribosomal protein (26).

Yap1p now can be included in the list of cargos transported by Pse1p, which include the transcription factor Pho4p and ribosomal protein L25. In the case of Pho4p, the import is solely mediated by Pse1p but not by Kap123p (29). Thus, Yap1p, Pho4p, and ribosomal protein L25 apparently have different specificities in terms of receptor selectivity. Among these homologous import receptors (Pse1p and Kap123p), only Pse1p is essential for yeast cell growth. Although ribosomal proteins are essential for protein synthesis, cell growth is not affected in the KAP123 disruption mutant. This was explained by the fact that the nuclear transport of ribosomal proteins is substituted by Pse1p as described above. PSE1 is an essential gene for yeast cell growth, whereas Yap1p and Pho4p transported by Pse1p are not essential, suggesting that there should be some other essential cargos that definitely require Pse1p as a nuclear import receptor.

We identified bipartite NLS in Yap1p amino acid regions 5–16 and 50–59, where those respective regions include two basic residues (KR) and a cluster of basic residues (KKKG) with a 41-amino acid-long spacer (Fig. 5). There is no sequence homology between the NLS of Yap1p and that of Pho4p except that the basic amino acids are distributed in Pho4p NLS (amino acids 140–166) (29). It is becoming clear that many NLSs, which are recognized directly by the importin β homologue, consist of longer amino acid regions rather than a short peptide motif like the classical NLS recognized by the importin α/β complex (1, 28). This may be explained by the hypothesis that import receptors have a second function, i.e. receptors bind to cargos and somewhat cover the cargo molecules to prevent inappropriate interaction with the cellular components before the cargos reach their final destination. This hypothesis is consistent with the recent finding that Ran-GTP-mediated dissociation of the interaction between the TATA-binding protein and its import receptor, Kap114p, is stimulated by TATA-containing DNA (30). Interestingly, in the case of many transcription factors including Yap1p, NLS is located next to the DNA binding domain (for example, Gal4 DNA binding domain (31), the basic leucine-zipper domain including the CAAT/enhancer-binding protein (32) and v-jun (33), basic helix-loop-helix domain (34, 35), homeodomain (36, 37), and high-mobility group domain (38)). We therefore speculate that the association of the respective import receptor to the NLS of these transcription factors might protect their DNA binding domains and might inhibit the DNA binding activity until these factors reach an appropriate DNA binding site, and the binding of the transcription factor to the DNA binding site might accelerate the dissociation of the receptor-cargo interaction.

Here, we demonstrated that the nuclear import of Yap1p(1–571) as well as Yap1p(2–59) are neither repressed nor enhanced by oxidative stress (Fig. 6), suggesting that the enhancement of the transcriptional activity of the constitutive nuclear-localized Yap1p(1–571) by oxidative stress should be caused by other step, e.g. the enhancement of DNA binding activity (39) or transcriptional activity by modification. It has been shown that Pse1p interacts with a number of nucleoporins that constitute the NPC, and these interactions can be dissociated by Ran-GTP (40–42). These events are supposed to be the mechanism of nuclear transport through the NPC. Therefore, our results indicate that the course of nuclear import of Yap1p, that is, binding of Yap1p to Pse1p, interaction of Pse1p with nucleoporins, and the dissociation of Yap1p from Pse1p, is not affected by oxidative stress caused by diamide and H₂O₂.

Regulation of the nuclear import step is generally crucial for most transcription factors that are activated by extracellular
signals. In contrast, we now provide evidence that Yap1p nuclear localization is entirely regulated at the step of nuclear export. The interaction between Yap1p and its export receptor Crm1p is regulated by oxidative stress (18, 19). This unique strategy for Yap1p regulation might be significant because this regulatory mechanism seems to be conserved in Yap1p homologues Pap1 of the fission yeast (43) and Cap1 of *Candida albicans* (44). We have shown recently that Yap1p can directly sense an oxidative stress (redox) signal, and the nuclear accumulation starts at 1 min. This rapid response might be carried out by inhibition of the nuclear export of preexisting Yap1p in the nucleus because H$_2$O$_2$ and diamide might spread rapidly in the cells. Alternatively, it may be required for yeast cells to sense the oxidative stress (redox) signal in the nucleus under certain conditions.

In summary, we identified Pse1p as the nuclear transport receptor of Yap1p and showed that the nuclear import step of Yap1p was not affected by oxidative stress. Our results and previous studies clearly indicate that Yap1p subcellular localization in response to oxidative stress is regulated solely at the step of Crm1p-dependent nuclear export.

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Nuclear Import of the Yeast AP-1-like Transcription Factor Yap1p Is Mediated by Transport Receptor Pse1p, and This Import Step Is Not Affected by Oxidative Stress

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