Nuclear import of the histone acetyltransferase complex SAS-I in *Saccharomyces cerevisiae*

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Summary

The protein complex SAS-I links histone acetylation to the assembly of repressed chromatin in *Saccharomyces cerevisiae*. Sas2p, the histone acetyltransferase subunit of SAS-I, forms a complex with Sas4p and Sas5p, which are both required for maximal complex activity. In this study, we found that Sas4p was the central subunit of the SAS-I complex, bridging Sas2p and Sas5p. We demonstrated that the nuclear import of Sas2p and Sas5p was mediated by two karyopherins/importins, Kap123p and Pse1p, and both were associated in vivo with these importins. By contrast, Sas4p was not a substrate of Kap123p or Pse1p, suggesting that the nuclear import of the SAS-I subunits occurred independently of each other. Several other non-essential karyopherins were not involved in the nuclear import of SAS-I subunits. When the putative nuclear localization signal (NLS) of Sas2p was deleted, nuclear accumulation of Sas2p was significantly decreased. By contrast, deletion of the proposed NLS of Sas4p had no influence on its nuclear localization. An unknown signal region was located in the N-terminal domain of Sas5p and was responsible for the nuclear import by Kap123p and Pse1p. We found a striking similarity between the NLS sequences of Sas2p and those of histones H3 and H4, which were recently reported to be further import substrates of Kap123p and Pse1p. A database search based on the aligned consensus sequence revealed potential new import substrates of the Kap123p and Pse1p nuclear import pathways, which are connected to chromatin function.

Key words: Importin/karyopherin, *KAP123*, SAS2, Silencing

Introduction

The transport of macromolecules between the nucleus and the cytoplasm occurs through nuclear pore complexes, which are large multiprotein structures embedded within the nuclear envelope (Rout et al., 2000; Chook and Blobel, 2001). The nuclear transport of proteins is mediated by a family of transport receptors called karyopherins (Kap). Fifteen karyopherins have been identified in yeast, ten of which have import receptor function (importins), four are export receptors (exportins) and one (Kap142p) appears to be involved in both pathways (Stage-Zimmermann et al., 2000; Chook and Blobel, 2001). Nuclear import likely occurs by multiple transient low-affinity interactions between the importin and the nuclear pore complex, thereby translocating the importin-cargo complex through the nuclear pore (Rout and Aitchison, 2001). Inside the nucleus, the importin binds the small GTPase Ran, which results in release of its cargo protein and termination of the import cycle (Kuersten et al., 2001).

Importins can associate directly with their import cargoes, which they recognize by specific nuclear localization signals (NLSs). The most common type of NLS is a short stretch of basic amino acids that introduce an overall net positive charge crucial for nuclear targeting properties of these sequences (reviewed by Chook and Blobel, 2001). In the case of classical nuclear protein import, karyopherin/importin α/β heterodimers (Kap60p/Kap95p) bind NLS-containing proteins in the cytosol and target them to the nucleus: an NLS within the cargo protein is recognized by the adapter protein importin α, whereas importin β enhances the affinity of α for the NLS and mediates docking of importin-NLS protein complexes to nucleoporins (nuclear pore complex proteins) (Enenkel et al., 1995; Görlich et al., 1995). Classical NLSs are categorized as either monopartite, containing a single cluster of basic amino acid residues (K/R), or bipartite, containing two clusters of basic amino acids separated by a linker of 10-12 non-conserved amino acid residues (Kalderon et al., 1984; Robbins et al., 1991). In yeast, the import receptor of the karyopherin α/β type is the Kap95p/Kap60p heterodimer (Kap60p is also termed Srp1p), which recognizes proteins with ‘classical’ basic NLSs (Pemberton et al., 1998). However, this is not a strict rule, as some proteins with a ‘classical’ NLS are not imported via the Kap95p/Kap60p pathway (e.g. import of the TFIIA subunit Tof1p is mediated by Kap122p) (Titov and Blobel, 1999).

The NLSs of most proteins are poorly defined, primarily because few cargoes have been determined for each importin (Chook and Blobel, 2001). Some cargoes can be imported by several different importins, indicating a redundancy of NLS recognition by importins (Rout et al., 1997; Jäkel and Görlich, 1998; Mosammaparast et al., 2001). Furthermore, importins can recognize a large number of different cargo proteins. For example, Kap123p binds to the ribosomal proteins Rps1p, Rpl4p, Rpl15p, Rpl16p, Rpl18p, Rpl25 and Rpl41p (Rout et al., 1997) and mediates import of ribosome-associated proteins
Egd1p and Egd2p (Franke et al., 2001), but also mediates the import of histones H3 and H4 (Mosammaparast et al., 2002a), and binds to histones H2A and H2B when KAP144 is deleted (Mosammaparast et al., 2001).

Although several studies describe the nuclear import of proteins and which importins recognize their NLs (reviewed by Chook and Blobel, 2001), little information is available on the nuclear import of entire protein complexes. The question arises whether a complex is imported as a whole (assembly in the cytoplasm), or whether single subunits are imported independently of each other (assembly in the nucleoplasm). Nuclear import of proteins appears to happen in small units rather than large assemblies, e.g. ribosomal proteins versus whole ribosomes, or single histones versus histone octamers (Rout et al., 1997; Mosammaparast et al., 2001).

In this study, we investigated the nuclear import of a protein complex, the histone acetyltransferase complex SAS-I. SAS-I import is necessary to guarantee the accurately timed reestablishment of histone acetylation patterns after DNA replication (Meijsing and Ehrenhofer-Murray, 2001). SAS-I consists of at least three subunits, Sas2p, Sas4p and Sas5p (Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001). Sas2 encodes a protein of the MYST family of histone acetyltransferases (HATs), which includes the yeast homologs Sas3p and Esa1p, *Drosophila* MOF and Chm, and human MOZ, MORF, Tip60 and HBO1. Sas4 encodes a putative cullin family protein, and Sas5 encodes a homolog of TAF130.

SAS2 was originally identified in two screens involved in transcriptional silencing (Reifsnyder et al., 1996; Ehrenhofer-Murray et al., 1997), and deletions of SAS4 and SAS5 show the identical silencing phenotypes as sas2Δ (Xu et al., 1999). Gene silencing in yeast results from the generation of heterochromatin-like structures that prevent gene expression, and is found at the two silent mating-type loci HML and HMR, at the telomeres and the rDNA locus (Rusche et al., 2003). Sas2 mutants show improved silencing at a mutated HMR locus and at the rDNA locus, but loss of silencing at the telomeres and at HML when SIR1 is deleted (Reifsnyder et al., 1996; Ehrenhofer-Murray et al., 1997; Meijsing and Ehrenhofer-Murray, 2001). A point mutation (K16R) in the histone H4 N-terminal tail phenocopies the effects of sas2 mutants on silencing: like a deletion of SAS2, the H4 K16R mutation is defective in telomeric silencing and improves silencing at a mutated HMR (Meijsing and Ehrenhofer-Murray, 2001). Purified SAS-I complex acetylates H4 K16 and H3 K14 in vitro; recombinant Sas2p shows in vitro HAT activity only in the presence of Sas4p, and this activity is stimulated by Sas5p (Sutton et al., 2003). In vivo, H4 K16 acetylation by SAS-I is required in subtelomeric regions to prevent gene silencing from spreading inappropriately into euchromatic regions. A functional link between SAS-I and chromatin assembly is

| Table 1. Yeast strains used in this study |
|------------------------------------------|
| **Strain** | **Genotype** | **Source** |
| CG1945 | MATα gal4Δ-542 gal80-538 ade2-101 his3-200 leu2-3,112 lys2-801 trp1-901 ura3-52 URA3::GAL4-LacZ::CyC1-6XHisΔ-LacZ | Clontech |
| Y187 | MATα gal4Δ gal80A ade2-101 his3-200 leu2-3,112 trp1-901 ura3-52 URA3::GAL1-UAS-GAL1-UAS-LacZ met-101 | Clontech |
| AEY2 | MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 (=W302-3B) | W302-3B |
| AEY266 | AEY2 sas2Δ::TRP1 | D. Rivier† |
| AEY1492 | W303 MATα sas4Δ::kanMX | E. W. Jones‡ |
| AEY1493 | W303 MATα sas5Δ::HIS3 | D. Rivier† |
| AEY3464 | AEY266 sas5Δ::kanMX | D. Rivier† |
| AEY1558 | MATα leu2 trp1 ura2-52 prr1-407 pep4-3 prb1-112 | Clontech |
| AEY1559 | AEY1558 sas2Δ::TRP1 | E. W. Jones‡ |
| AEY2424 | AEY1559 sas4Δ::kanMX | D. Rivier† |
| AEY2461 | AEY1558 sas4Δ::kanMX | D. Rivier† |
| AEY2465 | AEY1558 sas5Δ::kanMX SAS5-HA::TRP1 | E. W. Jones‡ |
| AEY2650 | AEY1558 sas5Δ::kanMX | D. Rivier† |
| AEY3095 | AEY1558 SAS5-myc::TRP1 | E. W. Jones‡ |
| AEY3450 | AEY1558 PSE1-HA::URA3 | D. Rivier† |
| AEY3451 | AEY3095 PSE1-HA::URA3 | D. Rivier† |
| AEY3454 | AEY1558 KAP123-HA::URA3 | D. Rivier† |
| AEY3456 | AEY3095 KAP123-HA::URA3 | D. Rivier† |
| AEY2956 | AEY2957 pse1-1 | P. A. Silver§ |
| AEY2957 | MATα leu2Δ1 trp1Δ2 his3-52 | P. A. Silver§ |
| AEY2958 | AEY2959 kap123Δ::HIS3 | P. A. Silver§ |
| AEY2959 | MATα his3Δ200 leu2Δ1 trp1 ura3-52 | P. A. Silver§ |
| AEY3153 | AEY3155 spt1-31 | M. Nomura‡ |
| AEY3154 | AEY3155 spt1-49 | M. Nomura‡ |
| AEY3155 | MATα ade2 his3 leu2 trp1 ura3 | M. Nomura‡ |

*Unless indicated otherwise, strains were constructed during the course of this study or were taken from the laboratory strain collection.
1. D. Rivier, University of Illinois, Urbana, IL.
2. E. W. Jones, Carnegie Mellon University, Pittsburgh, PA.
3. Harvard Medical School, Boston, MA.
4. University of California, Irvine, CA.
given by the observation that SAS-I interacts with the histone deposition proteins Asl1p and Cac1p, a subunit of the CAF-I complex (Meijisen and Ehrenhofer-Murray, 2001; Osada et al., 2001). Thus, SAS-I is a global HAT that likely acetylates H4K16 in the wake of chromatin assembly.

Here, we show that the nuclear import of two subunits of the SAS-I complex, Sas2p and Sas5p, was mediated by two importins, Kap123p and Pse1pKap121p. Both SAS-I subunits associated with Kap123p and Pse1p. GFP-Sas2p and GFP-Sas5p reporter fusion proteins were mislocalized to the cytosol in strains with mutations in the PSEI gene or a deletion of KAP123, while GFP-Sas4p remained nuclear in all kapa strains tested, and was still nuclear in the absence of SAS2 and SAS5. Nuclear import of each Sas protein occurred independently of the other SAS-I components, indicating that assembly of the complex takes place predominantly inside the nucleoplasm and after translocation of the individual subunits through the nuclear envelope. Deletions of other known non-essential karyopherins had no effect on the nuclear localization of Sas2p and Sas5p. Furthermore, an NLS was identified in Sas2p that showed significant sequence homology to the NLS of histones H3 and H4. A database search of the yeast genome, based on the derived consensus NLS, revealed potential new nuclear import substrates of the Kap123p and Pse1p import pathways.

Materials and Methods
Yeasts strains and media
Genomic taggings or plasmid transformations for the coinmunoprecipitation assays were performed in S. cerevisiae strain AEY1558 or its derivatives (Table 1). Strains used in the microscopic analysis were derivatives of W303 and BY4741 (Giaever et al., 2002). pse-I and kap123Δ strains were generous gifts of P. A. Silver (Harvard Medical School, Boston, MA), and srcp-31 and srcp-49 strains were kindly provided by M. Nomura (University of California, Irvine, CA). Other kap deletions used in this study were taken from the ResGen strain collection (Research Genetics, Invitrogen). Preparation of standard media and genetic manipulations including high-efficiency transformation of yeast cells were performed according to established procedures (Adams et al., 1997).

Two-hybrid analysis
For the two-hybrid analysis, SAS-I proteins were cloned in full-length into either pAS-BC (bait) or pACTIIst (prey) and transformed into yeast strains CG1945 or Y187, respectively (Tables 1 and 2). The two-hybrid procedure was performed as described (Fromont-Racine et al., 1997). The DNA library was kindly provided by M. Fromont-Racine (Institut Pasteur, Paris, France).

HA and myc tagging
Epitope tags were generated genomically with the exception of mycC-Sas2p and mycC-Sas4p, where strains transformed with plasmid pAE778 or pAE612 (Meijisen and Ehrenhofer-Murray, 2001) were used. A triple HA tag or a ninefold myc tag was added C-terminally by manipulating the corresponding loci genomically by transformation of a PCR-generated K. lactis TRP1 or URA3 marker cassette with 51 bp flanking homology regions as described previously (Schaper et al., 2001). All PCRs were performed with Herculase (Stratagene). The sequences of oligonucleotides are available from the authors on request. Correct genomic integration was verified by PCR on genomic DNA using primers outside the homology region and by immunoblotting using mouse monoclonal antibodies, anti-HA clone 12CA5 (Roche) or anti-myc antibody (Invitrogen), and the ECL detection system (Amersham Pharmacia Biotech).

Extract preparation and coinmunoprecipitations
Yeast extracts were prepared and coinmunoprecipitations for the determination of interactions within the SAS-I complex were performed exactly as described (Meijisen and Ehrenhofer-Murray, 2001). For coinmunoprecipitations of SAS-I interactions, strains AEY1558, AEY1559, AEY2461, AEY2465 or AEY2650 (Table 1) were transformed with pAE240 (Sas2p), pAE778 (myc6-Sas2p) or pAE612 (myc6-Sas4p) (Table 2). For coinmunoprecipitations of Pse1p-HA3 and Kap123p-HA3 with myc-tagged SAS-I subunits, strains expressing Pse1p-HA3, Kap123p-HA3, Pse1p-HA3/Sas5p-mycD and Kap123p-HA3/Sas5p-mycD were generated by genomic manipulation, Pse1p-HA3/mycC-Sas2p and Kap123p-HA3/mycC-Sas2p by transformation of strains AEY3450 and AEY3454 with pAE778 (Table 1 and 2). The following modifications were applied to the protein extraction and coinmunoprecipitation protocol: NP-40 was omitted from all buffers and the salt concentration was adjusted to 150 mM NaCl, except for the last washing step, where 210 mM NaCl was used. Protein extracts containing mycC-Sas2p or Sas5p-mycD were complexed with anti-myc antibody (Invitrogen) and immobilized to protein G agarose (Sigma). Precipitates were resuspended in SDS sample buffer and analyzed by immunoblotting.
GST-pulldown assays

GST-Sas2p, -Sas4p and -Sas5p fusions were generated in Escherichia coli by cloning of each SAS gene into pGEX-4T-1 (Amersham Pharmacia Biotech). GST-Sas fusion proteins were purified by affinity chromatography with glutathione agarose (Sigma) according to standard procedures. The SAS genes were also cloned into pBluescript KS (Stratagene). Radiolabeled Sas2p, Sas4p, Sas5p and luciferase were generated by in vitro transcription/translation using the transcription and translation (TNT)-coupled reticulocyte lysate system (Promega), the pBluescript-Sas vectors and [-35S]-methionine (Amersham) according to the manufacturer’s instructions. Binding reactions were carried out in buffer A100 (100 mM Tris-Cl pH7.5, 100 mM NaCl, 5 mM MgCl2, 10 mM EGTA, 10% glycerol, 0.3% Triton X-100, 1 mM DTT, 1 mM p-aminobenzamide), using 25 μl beads with the coupled GST-fusion protein and 9 μl TNT-radiolabeled protein. Binding reactions were incubated for 2 hours at room temperature, subsequently washed three times with buffer A250 (buffer A with 250 mM NaCl) and eluted with 30 μl sample buffer. Eluates were applied onto 12.5% SDS polyacrylamide gels and bands were visualized using a PhosphoImager (Molecular Dynamics).

Cell culture and fluorescence microscopy

GFP reporter constructs were based on plasmid pUG36 (kindly provided by J. H. Hegeman, University of Düsseldorf, Germany), where GFP is fused N-terminally to the protein of interest and under control of the inducible MET25 promoter (Niedenthal et al., 1996). A detailed description of the exact cloning procedure is available from the authors on request. Cells were grown overnight in SC-Met-Ura media, diluted into fresh medium to an optical density of 0.35 and grown at the temperatures indicated for 2-3 hours; all subsequent steps were performed as described previously (Franke et al., 2001). For image acquisition and manipulation, a Zeiss Axiosplan microscope with a 100× objective and the Axiovision 4.0 software were used. For each figure, images were taken at the same exposure settings.

Gel filtration

pse1-1 or wild-type cells were grown at 24°C to an optical density (600 nm) of 1.0. Whole cell lysates for size fractionation experiments were prepared exactly as described (Meijssing and Ehrenhofer-Murray, 2001). A HiPrep 16/60 Sephacryl S-300 HR gel filtration column (Pharmacia) was equilibrated in buffer A (125 mM Tris-Cl at pH 7.5, 1 mM EDTA, 10 mM Mg-acetate, 200 mM NaCl, 0.1% NP-40, 1 mM DTT). The elution profiles of HA-Sas5p from wild-type and pse1-1 extracts were compared with the elution of commercially available marker proteins (Pharmacia). Proteins from every other fraction were used. For each figure, images were taken at the same exposure settings.

Results

Sas4p was the connective core subunit of the SAS-I complex

The yeast SAS-I complex consists of at least three subunits, as was reported previously (Meijssing and Ehrenhofer-Murray, 2001; Osada et al., 2001). In order to investigate more closely the protein-protein interactions within the SAS-I complex, we used several different assays. In a yeast two-hybrid assay, a Sas4 fusion to the Gal4 DNA-binding domain interacted with the preys Sas2p and Sas5p (Fig. 1A), but not with the empty vector. Using Sas5p as the bait in a two-hybrid screen (Fromont-Racine et al., 1997), SAS4 was isolated six times on three overlapping fragments, limiting the region of interaction with Sas5p to the C-terminal part (amino acids 318-381; data not shown).

No direct interaction was detected between Sas2p and Sas5p in the two-hybrid assay, suggesting that Sas4p was the bridging subunit between Sas2p and Sas5p. This observation was confirmed by two other assays. Coimmunoprecipitation of Sas2p with Sas5p occurred only when Sas4p was present, whereas deletion of Sas5p or Sas2p had no influence on the interaction between Sas4p and Sas2p or Sas4p and Sas5p, respectively (Fig. 1B). This observation was supported by TAP-purifications of the SAS-I complex with extracts prepared from wild-type and sas4A strains in which SAS5 was genomically TAP-tagged. In wild-type extracts, Sas2p and Sas4p co-purified together with Sas5-TAP; by contrast, Sas2p was not recovered in the absence of Sas4p (data not shown). Additionally, in a GST pulldown assay with in vitro-translated radiolabeled SAS-I subunits, we observed direct interactions between GST-Sas2p and radiolabeled Sas4p, between GST-Sas4p and both Sas2p and Sas5p, and between GST-Sas5p and Sas4p (Fig. 1C). GST-Sas2p and GST-Sas4p also appeared to interact with themselves (radiolabeled Sas2p and Sas4p, respectively), but not with the negative control (Luciferase, Fig. 1C). Sas2p interacts with itself in two-hybrid assays (Meijssing and Ehrenhofer-Murray, 2001); moreover, we cannot rule out the possibility that Sas4p dimerizes under certain conditions. Little radiolabeled Sas2p or Sas5p was bound by GST-Sas5 or GST-Sas2p, respectively. Together, these data further support the notion that Sas4p is the connecting piece between Sas2p and Sas5p.

Sas2p and Sas5p interacted with the importins Pse1p and Kap123p

We used a yeast two-hybrid screen (Fromont-Racine et al., 1997) to identify proteins that interact with Sas5p. Among the positive clones that interacted with the bait Sas5p, we isolated eight times the ORF of YMR308c, which corresponds to the PSE1 gene. Interaction of Sas5p and Pse1p occurred within three overlapping C-terminal fragments of Pse1p (Fig. 2A). Pse1p belongs to the family of nuclear transport receptors called karyopherins (Kaps) or importins and was previously reported to support the nuclear import of a number of transcription factors (Chook and Blobel, 2001). Pse1p is homologous to Kap123p and, like Kap123p, is functionally redundant in mediating nuclear import of ribosomal proteins (Rout et al., 1997), ribosome associated proteins (Franke et al., 2001) and histones H3 and H4 (Mosammaparast et al., 2002a).

Hence, we were also interested to test for a physical interaction between the SAS-I complex and Kap123p. We performed coimmunoprecipitation experiments in order to verify the two-hybrid interaction between Sas5p and Pse1p in vivo, and to test for the interactions of Sas2p and Sas5p with Kap123p or Pse1p. For this purpose, subunits of the SAS-I complex, Pse1p or Kap123p were epitope tagged. In coimmunoprecipitation assays with myc-tagged SAS-I subunits immobilized on beads, significant coprecipitates of Pse1p-HA3 and Kap123p-HA3 were detected with Sas5p-myc9 and myc6-Sas2p, respectively, but not with myc6-Sas4p (Fig. 2B, and data not shown). Some non-specific binding of Pse1p-HA3 and Kap123p-HA3 to the myc-antibody was also
observed, but to a much lesser extent than the coimmunoprecipitation (Fig. 2B). These observations suggested that Sas2p and Sas5p were potential import substrates of Pse1p and Kap123p.

Pse1p and Kap123p were required for the nuclear import of Sas2p and Sas5p

To determine the role of Pse1p, Kap123p, and potentially other karyopherins in the import of Sas2p and Sas5p in vivo, GFP fusions of the SAS-I proteins were generated and their subcellular localizations were analyzed in cells harboring mutations or deletions in specific KAP genes. GFP fusions of Sas2p, Sas4p, and Sas5p showed predominantly nuclear localizations in the wild-type strain; GFP-Sas4p was exclusively localized in the nucleus, whereas Sas2p and Sas5p exhibited an additional slightly elevated GFP signal in the cytoplasm (Fig. 3). The nuclear accumulation of GFP-Sas2p and GFP-Sas5p, but not of GFP-Sas4p, was clearly decreased in *kap123Δ* or *pse1-1* cells (Fig. 3). Interestingly, these effects were already visible in *pse1-1* cells at the permissive temperature (23°C), and a shift towards the nonpermissive temperature had no further consequences. By contrast, the nuclear localization of GFP-Sas4p in *pse1-1* cells did not change even under nonpermissive conditions (data not shown). However, the nuclear import of Sas2p and Sas5p was not completely blocked in *pse1-1* and *kap123Δ* strains, as some nuclear GFP-Sas2p and GFP-Sas5p signal still remained in these *kap* mutants. In agreement with this, *pse1-7* and *kap123Δ* strains did not show one of the *sas* silencing phenotypes (data not shown), the suppression of an *HMR* silencing defect (Ehrenhofer-Murray, 1997). We used *pse1-7* in this assay, because crosses with the *pse1-1* mutant showed very poor spore viability, such that *pse1-1* was not manageable in these genetic assays (data not shown). The absence of a silencing phenotype was not surprising, given that Pse1p and Kap123p

![Fig. 1. Protein-protein interactions within the SAS-I complex.](image-url)

(A) Sas4p interacted with Sas2p and Sas5p in the two-hybrid system as shown by growth or no growth of the reporter strain in the absence of histidine. GBD, Gal4 DNA-binding domain (pAS-BC); GAD, Gal4 activation domain (pACTIIst); see Table 2 for plasmid details.

(B) Coimmunoprecipitation analysis of Sas5p-HA3/myc6-Sas2p, Sas4p-myc9/Sas2p and Sas5p-HA3/myc6-Sas4p interactions. Bound proteins were analyzed on 12.5% SDS polyacrylamide gels, immunoblotted and probed with α-myc or α-Sas2 antibodies.

(C) GST-pulldown assay to determine direct interactions within the SAS-I complex. Purified GST-Sas fusion proteins were immobilized on glutathione agarose beads and incubated with in vitro-translated radiolabeled SAS-I proteins (abbreviated 2, 4, and 5) or luciferase (L, negative control). An asterisk indicates degradation products of radiolabeled Sas4p. Washed beads were eluted with sample buffer, separated on 12.5% SDS polyacrylamide gels, and bands were visualized using a PhosphoImager.
have overlapping functions and that one can substitute for the other. Alternatively, other importins may take over function when the Pse1p and Kap123p import pathways are disturbed. Additionally, we generated pse1-1sas4Δ and kap123Δsas4Δ double mutant strains, in which we analyzed the subcellular localizations of GFP-Sas2p and GFP-Sas5p. We did not detect a significant difference in the localizations of Sas2p and Sas5p in the double mutant strains when compared with those of each pse1-1 or kap123Δ single mutant (data not shown).

In order to test whether the nuclear import of Sas2p and Sas5p was specifically mediated by Kap123p and Pse1p, we studied subcellular localization of the SAS-I proteins in various other kap mutants. Five more deletions of KAP genes, KAP108/SXM1, KAP114, KAP119, KAP122/PDR6 and KAP142/MSN5, were at our disposal. We found no significant decrease in the nuclear accumulation of GFP-Sas2p, GFP-Sas4p and GFP-Sas5p in cells lacking Kap108p, Kap114p, Kap122p or Kap142p (Fig. 4). Taken together, these results showed that the transport receptors Kap123p and Pse1p were required for the nuclear import of Sas2p and Sas5p, but not Sas4p, whereas the other karyopherins tested were not involved in the transport.

Identification of potential nuclear localization signals within the SAS-I complex

In order to identify putative NLSs in the three SAS-I subunits, we analyzed the amino acid (aa) sequences of Sas2p, Sas4p and Sas5p using the Prosite patterns/profiles motif scan program (Falquet et al., 2002). Bipartite NLSs were predicted for Sas2p (aa 19-36) and Sas4p (aa 336-353, Fig. 5A), whereas no such sequence was proposed for Sas5p. Additionally, the Prosite motif scan revealed a cullin homology region for Sas4p (aa 91-275, Fig. 5A), a motif frequently found in proteins involved in cell cycle transitions. A sequence analysis with SMART, a web tool (http://smart.embl.de/) for the identification of protein domains (Letunic et al., 2004), displayed the known Sas2p histone acetyltransferase (HAT) domain of the MYST family (aa 126-314, Fig. 5A). A zinc finger precedes the HAT domain and mediates the interaction of Sas2p with Sas4p (Meijsing and Ehrenhofer-Murray, 2001). SMART analysis of Sas5p revealed a region common to the YEATS family of proteins, which includes YNK7, ENL, AF-9 and TFIIIF (small subunit) that are implicated in stimulation of transcription (aa 6-114, Fig. 5A).

Several deletion mutants of the SAS-I proteins were created in order to test which parts of the proteins were necessary for nuclear accumulation and whether or not the proposed NLSs were genuine (Fig. 5). Nuclear accumulation was significantly decreased in deletions of a large N-terminal region of Sas2p (aa 1-146) as well as a smaller region, which both encompass the putative NLSs (Fig. 5B), suggesting that Sas2p (aa 1-48) indeed contained an NLS. Various N-terminal deletions of Sas4p had no effect on the nuclear localization, not even a deletion covering the entire N-terminus including the cullin homology region (aa 1-287, Fig. 5B). Deletion of the C-terminal aa 328-481 also had no effect on the nuclear signal of Sas4p, although a suggested bipartite NLS (aa 336-353) lies...
within this deletion. Only a remaining C-terminal part of Sas4p (aa 377–481) was not exclusively localized to the nucleus, although it cannot be ruled out that this fusion was no longer retained in the nucleus because of back-diffusion due to its small size. Together, the region of Sas4p responsible for the nuclear accumulation was presumably located within aa 288–327 (Fig. 5B). Finally, a deletion of the C-terminal half of Sas5p (aa 124–248) did not significantly decrease the nuclear signal of the GFP-Sas5p fusion, whereas deletion of the N-terminal half (aa 1–123) significantly reduced the nuclear accumulation (Fig. 5B), pointing to a yet unidentified signal sequence for nuclear import in the N-terminus of Sas5p.

The fact that Sas2p contained a predicted bipartite NLS of the ‘classical’ type prompted us to assay the GFP-Sas fusions for nuclear localization in the kap60 temperature-sensitive alleles srp1-31 and srp1-49. However, the nuclear accumulation of GFP-Sas2p, GFP-Sas4p and GFP-Sas5p was not decreased in srp1-31 or srp1-49 mutants, not even at non-permissive temperature (data not shown), indicating that the Kap95p/Kap60p import receptor was not required for SAS-I import.

Interestingly, the three GFP-Sas reporters displayed a nuclear localization even when the other SAS-I subunits were deleted; for instance, GFP-Sas4p remained nuclear in the absence of SAS2 or SAS5 or both (data not shown), suggesting that the subunits were imported independently of each other.

Sas5p participated in protein complexes of different molecular weight in pse1-1 and wild-type cells

We performed size fractionation experiments of total protein lysates from pse1-1 and its isogenic wild-type strain, which were previously transformed with pRS426-HA-Sas5 (pAE625, see Table 2) and cultured at permissive temperature (24°C). Analysis of the gel filtration fractions from both experiments revealed that the two HA-Sas5p elution profiles differed in two ways: first, the pse1-1 elution profile contained a new peak of Sas5p (fractions 67–75), corresponding to proteins of significantly lower molecular weight and lacking in the wild-type profile, while the fractions corresponding to the SAS-I complex (fractions 57–65) were similar to wild-type (Fig. 6). Second, a minor part of Sas5p was also found in higher molecular mass fractions of the wild-type profile (fractions 37–43), indicating that Sas5p was also a component of larger protein complexes in wild-type extracts, but not in the pse1-1 mutant (Fig. 6). Interestingly, it was previously reported that Sas5p is part of larger protein complexes (Meijsing and Ehrenhofer-Murray, 2001). The nature of this higher molecular weight assembly is not known, but further investigations will show whether its formation may be dependent on the nuclear import of Sas5p.

The putative Sas2p NLS showed similarity with those of histones H3 and H4

Two other nuclear proteins were recently reported to be substrates of the Kap123p and Pse1p import pathways, the histones H3 and H4 (Mosammaparast et al., 2002a). Both H3
and H4 contain an NLS at their N-terminal domains; the minimal NLS of H3 was localized to residues 1-28, while that of H4 was limited to residues 1-21 (Mosammaparast et al., 2002a). Even though this H4(1-21)-GFP2 fusion did not appear entirely nuclear, its nuclear signal was easily detectable above the cytoplasmic background. We generated an alignment of these minimal NLSs with the proposed NLS of Sas2p and found a considerable agreement among these sequences. Ten out of 22 aligned residues were highly conserved in the three proteins (Fig. 7). This accordance prompted us to search for proteins containing a consensus NLS based on our alignment that could serve as a potential signal sequence for the Kap123p and Pse1p nuclear import pathways. Using the BLASTP tool (Altschul et al., 1997) and each of the aligned NLS as the input query, we identified three other nuclear proteins with significant matches to the consensus NLS: histone H1, Snf12p/Swp73p, a subunit of the SWI/SNF chromatin remodeling complex, and the histone H2A variant Htz1p, the latter having the best agreement to the consensus sequence (Fig. 7). Remarkably, all aligned NLSs are located in the very N-terminal ends of these proteins. The presence of a similar consensus sequence in Snf12p, histones H1 and Htz1p raises the question whether these nuclear proteins may constitute further import substrates of Kap123p or Pse1p. However, Sas5p (this study), a number of ribosomal proteins (Pemberton et al., 1998) and ribosome associated proteins (Franke et al., 2001) are also substrates of these import pathways and do not display any sequence homology with this consensus NLS.

Discussion

In this report, we have investigated two aspects of the yeast histone acetyltransferase complex SAS-I, its architecture and the nuclear import of its components. A direct interaction was not observed between Sas2p and Sas5p, showing Sas4p to be the central bridging subunit of the complex. Interestingly, the chromatin assembly factor Asf1p was previously found to interact directly with Sas4p in a pulldown assay, but not with Sas2p and Sas5p (Osada et al., 2001). Hence, Sas4p constitutes the central core of the complex that interacts with various surface proteins.

Importantly, we found two of the SAS-I components, Sas2p and Sas5p, to interact with two import receptors, Pse1p/Kap121p and Kap123p. We showed that Sas2p and Sas5p import was dependent on both importins. Nuclear accumulation of all three GFP-Sas reporter fusions was not affected by deletions of the other SAS genes. As Sas2p and Sas5p did not interact with each other in the absence of Sas4p, we conclude that the Kap123p/Kap121p mediated nuclear import pathways of Sas2p and Sas5p occurred independently of each other, and also independently of Sas4p. This indicates that assembly of the SAS-I complex is a predominantly nuclear event, even though it cannot be ruled out that a minor fraction of the complex is also assembled in the cytoplasm. The size fractionation analysis performed with wild-type and pse1-1 extracts may support the previous findings, as a shift of HA-Sas5p towards lower molecular weight fractions (<140 kDa) was already observed at permissive temperature in the pse1-1 mutant, but not in wild-type cells.
Our in vivo localization studies demonstrated that Sas2p contained at least one NLS in its N-terminal part (amino acids 1-48), a region that was suggested to encompass a putative NLS by the Prosite Profiles server. By contrast, an NLS activity could not be related to the C-terminal part of Sas2p, neither experimentally nor by sequence searches. Similarly, we found the N-terminal half of Sas5p to be necessary for nuclear import, although a ‘classical’ NLS could not be identified by the Prosite motif scan. A mild sequence similarity was asserted between Sas5p amino acids 60-81 (lying within the suggested YEATS domain) and amino acid stretches in the factors Pho4p, Spo12p, Yap1p and Pdr1p (not shown), which are cargoes of the Pse1p import receptor (reviewed by Chook and Blobel, 2001). Future deletion analyses will reveal whether Sas5p(60-81) is responsible for the nuclear accumulation.

Surprisingly, none of the karyopherins tested were required for the nuclear import of Sas4p, since the deleted kap genes did not interfere with its nuclear import. Hence, Sas4p import might be mediated by the essential importins Kap104p or Kap111p, or by a yet unidentified import receptor. Sas4p may also be co-imported together with another factor, e.g. an unknown component of the SAS-I complex. Basic bipartite NLSs were proposed for Sas2p and Sas4p, but mutations in Kap60p/Srp1p did not affect their nuclear accumulation, although the Kap95p/Kap60p-mediated import is the main pathway for proteins containing ‘classical’ NLSs (Enenkel et al., 1995). Furthermore, the proposed NLS of Sas4p was not a genuine one, as the deletion of the entire C-terminus (including the putative NLS) did not affect nuclear localization of Sas4p. It seems likely that amino acids 288-327 of Sas4p may harbor a signal for nuclear destination, because fragments containing these residues were still capable of reaching the nucleus. Interestingly, the Sas5p-interaction domain of Sas4p (amino acids 336-353) was found to be necessary for nuclear accumulation.

Fig. 5. Deletion analysis of Sas2p, Sas4p and Sas5p and their cellular localizations.

(A) Predicted domains, motifs and nuclear localization signals (NLS) of Sas2p, Sas4p and Sas5p. The indicated domains/motifs were found by sequence analysis with the Prosite motif scan and the SMART domain search program. NLSs are depicted as green triangles; an asterisk indicates that the NLS could not be verified experimentally. In Sas2p, the histone acetyltransferase (HAT) domain is shown (red), preceded by a zinc finger motif (yellow). A cullin homology domain (orange) was found in Sas4p. Sas5p has sequence homology to the YEATS family of proteins (blue hexagon). (B) The N-terminal domains of Sas2p and Sas5p were necessary for their nuclear accumulation. Full-length or fragments of Sas2p, Sas4p and Sas5p (pAE1098-pAE1108, Table 2) were expressed as fusions to GFP in wild-type yeast cells and visualized by fluorescence microscopy. The start and end point of each fusion is indicated by their corresponding amino acids to the left of each image. Bar, 5 µm.
acids 318-381) fell into nearly the same region and may encompass the putative NLS. It may be concluded that when this region of Sas4p-Sas5p interaction was deleted, not only nuclear import of Sas4p was disturbed, but also assembly of the SAS-I complex, since an important interaction domain of Sas4p was absent.

The fact that the nuclear accumulation of Sas2p and Sas5p was significantly decreased in pse1-1 or kap123Δ cells raised the question whether these kap mutants show any silencing phenotypes. The HMR silencing defect, which is suppressed by the sas deletions, was not suppressed by pse1-7 nor kap123Δ suppressed suggesting that the amounts of Sas2p and Sas5p in the nucleus were still sufficient for silencing. One possibility is that Pse1p and Kap123p act in parallel, such that mutation of both would completely abrogate SAS-I accumulation in the nucleus, and thus would result in silencing phenotypes. However, this issue cannot be addressed, because pse1-1 shows a severe synthetic slow-growth phenotype in combination with kap114Δ (Mosammaparast et al., 2002a); and second, SAS-I complex subunit of the SAS-I complex was identified in this preparation as an acetyltransferase complex, but neither Sas2p nor any other histone H3, Kap123p, Kap121p and the Hat1p/Hat2p are modified post-translationally, one might hypothesize that Sas2p is acetylated, and that this acetylation might influence its nuclear import. Interestingly, Sas2p itself becomes acetylated by SAS-I in in vitro acetylation reactions. However, the histone acetylation function of Sas2p was not required for its nuclear import, as a non-functional HAT mutant of Sas2p displayed wild-type levels of nuclear accumulation (S.S., unpublished). Hence, autoacetylation of Sas2p is not a prerequisite for nuclear import and may be an artefact due to its high similarity to the N-terminal tail of histone H4, which is acetylated by Sas2p on lysine 16 (Meijsing and Ehrenhofer-Murray, 2001; Sutton et al., 2003).

As was recently demonstrated, Kap123p and Pse1p/Kap121p are involved in the nuclear import of all four core histones, H3 and H4, and H2A/H2B when KAP114 is deleted (Mosammaparast et al., 2001; Mosammaparast et al., 2002a). Since Sas2p is a histone acetyltransferase for histones H3 and H4 (Sutton et al., 2003), this raises the possibility that Sas2p is co-imported into the nucleus with H3/H4 by their main import receptor Kap123p. However, this seems unlikely because, first, a purified cytosolic H4 preparation contained histone H3, Kap123p, Kap121p and the Hat1p/Hat2p acetyltransferase complex, but neither Sas2p nor any other subunit of the SAS-I complex was identified in this preparation (Mosammaparast et al., 2002a); and second, SAS-I complex

![Fig. 6. Size fractionation of wild-type and pse1-1 protein extracts prepared from cells (AEY2956 or AEY2957) grown at 24°C and expressing HA-Sas5p (pAE625). The elution profiles of HA-Sas5p were analyzed by gel electrophoresis on 12.5% SDS polyacrylamide gels and immunoblotting of the indicated fractions using an anti-HA antibody. The elution peaks of marker proteins are labeled above.](image)

### Fig. 7. Alignment of the suggested NLSs of Sas2p and histones H3 and H4. Histone H1, Snf12p and the histone variant Htz1p were found by BLAST P queries using the NLSs of Sas2p, histones H3 and H4, respectively, and showed significant similarity to the consensus sequence.
Nuclear import of the SAS-I complex

like NLS sequences can use the Kap123p or Pse1p import pathways to enter the nuclear environment.

In summary, we have shown that the nuclear import of Sas2p and Sas5p was mediated by the importins Kap123p and Pse1p, and that the import of each SAS-I protein occurred independently of the others. Hence, we propose that SAS-I complex assembly takes place in the nuclear environment. Thus, we have identified a new example for protein complex components that appear to be translocated into the nucleus as single polypeptides, and not as an entire protein complex. It may be deduced from our data and the reports of others that nuclear import happens in import units of one or two proteins rather than large protein assemblies, whereas nuclear export has been reported on both single proteins and large protein-RNA complexes. For instance, Toa1p and Toa2p, the two subunits of TFIIB (imported by Kap122p), are rather small in size (Titov and Blobel, 1999), and many ribosomal proteins are imported individually (Rout et al., 1997), whereas Kap120p exports the entire 60S ribosomal subunit (Stage-Zimmermann et al., 2000). In yeast, a number of examples are reported to fit this rule (Chook and Blobel, 2001) but it is clearly an oversimplification and does not apply to numerous proteins that are involved in complex biogenesis pathways, for instance, in snRNP assembly. snRNP biogenesis involves nuclear export of snRNA, assembly of a stable core of seven Sm-subunits in the cytoplasm and re-import of the mature snRNP into the nucleus (Yong et al., 2004). By contrast, SAS-I complex assembly constitutes a supplemental example for the nucleus being a popular site of protein complex assembly.

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References

Adams, A., Gottschling, D. E., Kaiser, C. A. and Stearns, T. (1997). Methods in Yeast Genetics: a Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Baudin-Baillieu, A., Guillemet, E., Cullin, C. and Lacroute, F. (1997). Characterization and proteome exploration of the yeast genome through exhaustive two-hybrid screens. Nat. Genet. 16, 277-282.

Gierer, G., Chu, A. M., Nudler, E., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B. et al. (2002). Functional profiling of the Saccharomyces cerevisiae genome. Nature 418, 387-391.

Görlach, D., Vogel, F., Mills, A. D., Hartmann, E. and Laskey, R. A. (1995). Distinct functions for the two importin subunits in nuclear protein import. Nature 377, 246-248.

Jäkel, S. and Görlach, D. (1998). Importin, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. EMBO J. 17, 4491-4502.

Jäkel, S., Albig, W., Kutay, U., Bischoff, F. R., Schwamborn, K., Doenecke, D. and Görlach, D. (1999). The importin/importin 7 heterodimer is a functional nuclear import receptor for histone H1. EMBO J. 18, 2411-2423.

Kalderon, D., Roberts, B. L., Richardson, W. D. and Smith, A. E. (1984). A short amino acid sequence able to specify nuclear location. Cell 39, 499-509.

Kobor, M. S., Venkatasubramanayam, S., Meneghini, M. D., Gin, J. W., Jennings, J. L., Link, A. J., Madhani, H. D. and Rine, J. (2001). A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p directs histone variant H2A.Z into euchromatin. PLoS Biol. 2, e131.

Kuerten, S., Ohno, M. and Mattaj, I. W. (2001). Nuclear transport: Ran, beta and beyond. Trends Cell Biol. 11, 497-503.

Lutken, I., Copley, R. R., Schmidt, S., Ciccarelli, F. D., Doerks, T., Schultz, J., Ponting, C. P. and Bork, P. (2004). SMART 4.0: towards genomic data integration. Nucleic Acids Res. 32, D142-D144.

Meijling, S. H. and Ehrenhofer-Murray, A. E. (2001). The silencing complex SAS-I links histone acetylation to the assembly of repressed chromatin by CAI-1 and Asf1 in Saccharomyces cerevisiae. Genes Dev. 15, 3169-3182.

Mosammaparast, N., Jackson, K. R., Guo, Y., Brame, C. J., Shabanowitz, J., Hunt, D. F. and Pemberton, L. F. (2001). Nuclear import of histones H2A and H2B is mediated by a network of karyopherins. J. Cell Biol. 153, 251-262.

Mosammaparast, N., Guo, Y., Shabanowitz, J., Hunt, D. F. and Pemberton, L. F. (2002a). Pathways mediating the nuclear import of histones H3 and H4 in yeast. J. Biol. Chem. 277, 862-968.

Mosammaparast, N., Ewart, C.S. and Pemberton, L. F. (2002b). A role for nucleosome assembly protein 1 in the nuclear transport of histones H2A and H2B. EMBO J. 21, 6527-6538.

Niedenthal, R. K., Riles, L., Johnston, M. and Hegemann, J. H. (1996). Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. Yeast 12, 773-786.

Osada, S., Sutton, A., Muster, N., Brown, C. E., Yates, J. R., III, Sternglanz, R. and Workman, J. L. (2001). The yeast SAS (something about silencing) protein complex contains a MYST-type putative acetyltransferase and functions with chromatin assembly factor ASFI. Genes Dev. 15, 3155-3168.

Pemberton, L. F., Blobel, G. and Rosenblum, J. S. (1998). Transport routes through the nuclear pore complex. Curr. Opin. Cell Biol. 10, 392-399.

Reifsnyder, C., Lovell, J., Clarke, A. and Pilus, L. (1996). Yeast silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. Nat. Genetics 14, 42-49.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Séraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol. 17, 1030-1032.

Robbins, J., Dilworth, S. M., Laskey, R. A. and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell 64, 615-623.

Rout, M. P. and Aitchison, J. D. (2001). The nuclear pore complex as a transport machine. J. Biol. Chem. 276, 16593-16596.

Rout, M. P., Blobel, G. and Aitchison, J. D. (1997). A distinct nuclear import pathway used by ribosomal proteins. Cell 89, 715-725.

Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y. and Chaït, B. T. (2000). The yeast nuclear pore complex: composition, architecture and transport mechanism. J. Cell Biol. 148, 635-651.

Rusche, L. N., Kirchmaier, A. L. and Rine, J. (2003). The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu. Rev. Biochem. 72, 481-516.

Schaper, S., Fromont-Racine, M., Linder, P., de la Cruz, J., Namane, A. and Yaniv, M. (2001). A yeast homolog of chromatin assembly factor 1 is involved in early ribosome assembly. Curr. Biol. 11, 1885-1890.

Seedorf, M. and Silver P. A. (1997). Importin/karyopherin protein family
members required for mRNA export from the nucleus. *Proc. Natl. Acad. Sci. USA* 94, 8590-8595.

Stage-Zimmermann, T., Schmidt, U. and Silver, P. A. (2000). Factors affecting nuclear export of the 60S ribosomal subunit in vivo. *Mol Biol. Cell*. 11, 3777-3789.

Strahl, B. D. and Allis, C. D. (2000). The language of covalent histone modifications. *Nature* 403, 41-45.

Sutton, A., Shia, W. J., Band, D., Kaufman, P. D., Osada, S., Workman, J. L. and Sternglanz, R. (2003). Sas4 and Sas5 are required for the histone acetyltransferase activity of Sas2 in the SAS complex. *J. Biol. Chem.* 278, 16887-16892.

Titov, A. A. and Blobel, G. (1999). The karyopherin Kap122p/Pdr6p imports both subunits of the transcription factor II A into the nucleus. *J. Cell Biol.* 147, 235-246.

Xu, E. Y., Kim, S. and Rivier, D. (1999). *SAS4* and *SAS5* are locus-specific regulators of silencing in *Saccharomyces cerevisiae*. *Genetics* 153, 25-33.

Yong, J., Wan, L. and Dreyfuss, G. (2004). Why do cells need an assembly machine for RNA-protein complexes? *Trends Cell Biol*. 14, 226-232.