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Exportin 1 (Crm1p) Is an Essential Nuclear Export Factor

Katrin Stade,* Charleen S. Ford,† Christine Guthrie,* and Karsten Weis,†‡
*Department of Biochemistry and Biophysics
†Department of Microbiology and Immunology
University of California at San Francisco
San Francisco, California 94143-0414

Summary

Nuclear protein export is mediated by nuclear export signals (NESs), but the mechanisms governing this transport process are not well understood. Using a novel protein export assay in S. cerevisiae, we identify *CRM1* as an essential mediator of nuclear protein export in yeast. Crm1p shows homology to importin β-like transport factors and is able to specifically interact with both the NES motif and the Ran GTPase. A mutation in the shuttling protein Crm1p affects not only protein export, but also mRNA export, indicating that these pathways are tightly coupled in S. cerevisiae. The presented data are consistent with the conclusion that Crm1p is a carrier for the NES-mediated protein export pathway. We propose *CRM1* be renamed exportin 1 *(XPO1)*.

Introduction

The existence of a cell nucleus in eukaryotes causes the spatial separation of cellular processes such as DNA transcription and mRNA translation. As a consequence, a large number of different macromolecules have to be exchanged bidirectionally between the nuclear and cytoplasmic compartments. The site of exchange is the nuclear pore complex (NPC), a multiprotein structure, consisting of probably more than 100 different polypeptides (Rout and Wente, 1994; Goldberg and Allen, 1995; Doye and Hurt, 1997). Each NPC is capable of translocating hundreds of macromolecules per minute in both directions (Görlich and Mattaj, 1996). Export cargoes comprise different classes of RNA molecules including messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), and uridine-rich small nuclear RNAs (UsnRNAs; Izaurralde and Mattaj, 1995). In the opposite direction, a large number of proteins and matured UsnRNP particles are actively imported into the nucleoplasm (Görlich and Mattaj, 1996; Nigg, 1997). In addition, a number of proteins have been shown to continuously shuttle between the nucleus and the cytoplasm, and examples for both slow and rapid shuttling proteins have been identified (Nigg, 1997).

Import and export processes through the NPC are generally signal dependent and saturable and thus mediated by carriers (Görlich and Mattaj, 1996; Nigg, 1997). The best characterized transport event is the nuclear import of proteins, which has been studied in great detail with the help of in vitro transport systems (Newmeyer et al., 1986; Adam et al., 1990). The conventional nuclear localization signal (NLS) that directs protein import is characterized by either one or two short stretches of positively charged amino acids (Dingwall and Laskey, 1991). This NLS motif is recognized by a cytoplasmic NLS receptor complex consisting of two subunits, importin α and importin β (reviewed by Melchior and Gerace, 1995; Görlich and Mattaj, 1996; Nigg, 1997). The α subunit binds directly to the NLS and interacts with the β subunit via a short amino-terminal domain (Görlich and Mattaj, 1996; Nigg, 1997). Two additional factors are required for NLS-mediated protein import in vitro: the Ran GTPase (Moore and Blobel, 1993; Melchior et al., 1993) and a small homodimeric protein called p10 or NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995). Ran, like other GTPases, cycles between a GTP- and a GDP-bound state, and the effects on its targets are regulated by this cycle (Rush et al., 1996). GTP hydrolysis by Ran has been shown to be required for the translocation reaction through the nuclear pore (Schlenstedt et al., 1995; Palacios et al., 1996; Weis et al., 1996).

Recently, additional protein import pathways have been elucidated. One pathway is exemplified by the rapidly shuttling protein hnRNP A1 (Pollard et al., 1996). The import signal for the hnRNP A1 protein consists of a 38–amino acid motif, which has been termed the M9 domain (Siomi and Dreyfuss, 1995; Weighard et al., 1995). A factor that binds to the M9 motif and mediates its import in an in vitro transport system was identified and named transportin (Pollard et al., 1996). The yeast homolog of transportin, Kap104, was independently characterized and shown to bind to two yeast mRNA binding proteins, Nab2p and Nab4p (Aitchison et al., 1996). Both transportin and Kap104 show homology to importin β but, unlike importin β, directly interact with their import substrates without an adaptor such as importin α (Aitchison et al., 1996; Pollard et al., 1996; Bonifaci et al., 1997). Two additional importin β homologs, karyopherin β3/Kap123 and Pse1, have recently been characterized in yeast and metazoans and were shown to bind to ribosomal proteins, suggesting that they function as specialized carriers in the nuclear import of ribosomal proteins (Rout et al., 1997; Yaseen and Blobel, 1997).

In comparison to our knowledge on nuclear protein import, less is currently known about the functional mechanisms governing the export of macromolecules from the nucleus. With the lack of a functional in vitro export system, yeast genetics and microinjection experiments in Xenopus oocytes have been the main approaches used to characterize nuclear export pathways. Kinetic competition experiments in Xenopus oocytes have shown that different classes of RNA, including mRNA, snRNA, tRNA, and rRNA, are exported via distinct pathways (Zasloff, 1983; Bataille et al., 1990; Jarmolowski et al., 1994; Pokrywka and Goldfarb, 1995). Because most, if not all, RNAs associate with proteins in the nucleus, it has been postulated that RNA export events are mediated by proteins containing appropriate

‡ To whom correspondence and requests for materials should be addressed.

export signals (Izaurralde and Mattaj, 1995; Nigg, 1997). Support for this model comes from the identification of small transferable signals in proteins that cause their active and rapid export from the nucleus. The first nuclear export signals (NESs) were identified in the inhibitor of the cAMP-dependent protein kinase, PKI, and in the HIV REV protein (Fischer et al., 1995; Wen et al., 1995). This signal is characterized by a short leucine-rich sequence motif, and similar sequences have now been identified in proteins including TFIIIA, IκBα, yeast Gle1p and in the yeast importin β protein (Fischer et al., 1995; Fritz and Green, 1996; Iovine and Wente, 1997; Murphy and Wente, 1996). Proteins that bind to this NES in two hybrid assays have been identified in both yeast (yRip1p; Stutz et al., 1995) and humans (hRIP/RAB1; Bogerd et al., 1995; Fritz et al., 1995). Both yRip1p and hRIP/RAB1 contain FG-rich sequence elements similar to repeat regions found in a class of nuclear pore proteins. Recently it was shown that the leucine-rich NES also interacts with FG repeats of several nucleoporins, and it was suggested that translocation through the NPC into the cytoplasm requires a sequential binding of the NES to the FG repeats (Fritz and Green, 1996; Stutz et al., 1996). The NES of REV is able to competitively inhibit the export of both U1 snRNA and 5S RNA but does not affect the export of mRNA and tRNA when injected into Xenopus oocytes (Fischer et al., 1995). This suggests that the REV protein exploits a cellular export pathway that is normally used by U1snRNA and 5S RNA.

A different nuclear export motif was identified in the hnRNP A1 protein (Michael et al., 1995). The M9 domain required for the nuclear import of the shuttling hnRNP A1 protein confers rapid export upon an otherwise nuclear localized reporter protein (Michael et al., 1995). Since hnRNP A1 binds to mRNA, it was proposed that it plays a role in the export of mRNAs. In support of this, microinjection experiments, using saturating amounts of the M9 domain, block mRNA export in Xenopus oocytes; however, the export of other RNA classes remains unaffected (Izaurralde et al., 1997).

Here, we report the characterization of an essential nuclear export factor, which we name exportin 1 (Crm1p/Xpo1p). Using a novel protein export assay, we show that Xpo1p functions in the NES-dependent export pathway in S. cerevisiae.

Results

Crm1p (Xpo1p) Shares Significant Homology with Importin β-like Transport Factors

To date, four importin β-like nuclear transport factors have been characterized in S. cerevisiae: Kap104/transportin, Kap123, Pse1, and importin β/Kap95 itself. Evidence was provided for all four factors that they function in nuclear import pathways of different classes of protein cargoes (reviewed by Nigg, 1997; Rout et al., 1997). Sequence analysis of the S. cerevisiae genome revealed several additional genes that share significant similarity with importin β and the other three family members (Fornerod et al., 1997; K. W., unpublished data). The S. pombe homolog of one of these genes, *CRM1* (*c*hromosomal *r*egion *m*aintenance), was originally identified as a gene mutated in a cold-sensitive strain showing abnormal chromosomal structures at the nonpermissive temperature (Adachi and Yanagida, 1989). We propose *CRM1* be renamed *XPO1* (exportin 1) because of its function in nuclear export (see below).

The Xpo1p protein has an overall identity of 16.3% and ~35% similarity to importin β over its entire length (data not shown). In addition, it shows 15%–20% identity and approximately 30%–40% similarity to the other three importin β family members in a pairwise FASTA alignment (data not shown). A high level of homology can be identified in an amino-terminal region representing amino acids 63–142 of Xpo1p, which had been previously designated as the "CRIME-domain" (Crm1, importin β, etc.; Fornerod et al., 1997). Like the other family members, Xpo1p is an acidic protein (calculated pI 5.32) and has a calculated molecular mass of 124,104. Because of its sequence similarity to nuclear transport factors, we decided to study the function of Xpo1p in more detail.

As previously reported, *XPO1* is an essential gene in S. cerevisiae (Toda et al., 1992; our unpublished data). To generate conditional *XPO1* alleles, low fidelity PCR mutagenesis was employed, and mutagenized plasmids were introduced by plasmid shuffling into an *XPO1* deletion strain covered by the wild-type *XPO1* allele. One plasmid that allowed growth at RT but not at 37°C was chosen for further analysis and the mutant allele was designated *xpo1-1* (Figure 1A).

To better analyze the temperature-sensitive phenotype of *xpo1–1*, cell growth of the deletion strain with plasmids containing either the wild-type *XPO1* gene or the *xpo1–1* allele was compared (Figure 1B). The *xpo1–1* strain grew slightly more slowly than the wild-type strain at RT and ceased growth rapidly after the shift to the nonpermissive temperature (Figure 1B). The *xpo1–1* mutant is recessive for growth since no growth phenotype was detected in a diploid strain containing the *xpo1–1* allele (data not shown). As expected, no growth defect was observed in an *XPO1* deletion strain carrying the wild-type plasmid (Figure 1B).

Xpo1p (Crm1p) Is a Nuclear Protein That Shuttles between the Nucleus and the Cytoplasm

In order to analyze the subcellular localization of Xpo1p, a Xpo1p-GFP fusion was generated by introducing the *GFP* gene in-frame at the carboxyl terminus of *XPO1*. The fusion was functional since it was able to rescue a strain deleted for *XPO1* at all temperatures tested (data not shown). Analysis of the GFP fluorescence at either RT (Figure 2A), 30°C (data not shown), or 37°C (Figure 2B) demonstrated that Xpo1p is mainly localized in the nucleoplasm, but in some cells a faint accumulation at the nuclear periphery was also detectable. We next wanted to determine the intracellular distribution of the temperature-sensitive xpo1–1 protein at different growth temperatures. As for the wild-type protein, an in-frame GFP-fusion was generated at the carboxyl terminus of xpo1–1p. A plasmid expressing this xpo1–1-GFP protein still conferred a temperature-sensitive growth phenotype to the deletion strain (Figure 1A), and cell growth

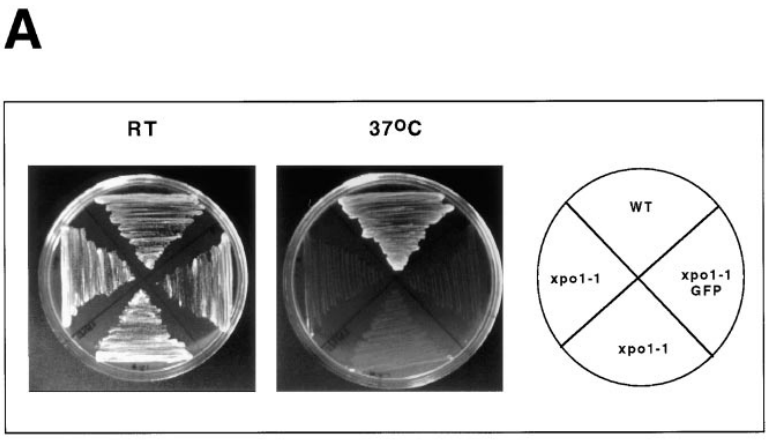

Figure 1. Growth Analysis of the Temperature-Sensitive *xpo1–1* Allele

(A) Cells derived from an *XPO1* deletion strain covered by plasmids expressing either the wild-type (*XPO1*), the *xpo1–1* mutant, or the xpo1–1 GFP fusion protein were streaked on plates and grown at the permissive (RT) or the nonpermissive temperature (37°C).

(B) Overnight cultures from either wild-type cells (squares) or *xpo1–1* (triangles) cells were diluted and grown at RT. After 9 hr cells were split and either shifted to 37°C or maintained at RT. The increase of the optical density at 600 nm is plotted against time. The time point at which the cells were shifted to 37°C is indicated by the arrow.

was similar in cells expressing untagged or GFP-tagged xpo1–1p (data not shown). In xpo1–1-GFP cells grown at RT, the GFP signal was indistinguishable from the one observed in wild-type cells and mainly nucleoplasmic fluorescence was detected (Figure 2C). However, after the shift to the nonpermissive temperature, the xpo1–1-GFP protein rapidly mislocalized and accumulated in several clusters at the nuclear periphery (Figure 2D). This redistribution was accompanied by a loss of nucleoplasmic signal, suggesting that a large fraction of the xpo1–1 protein was relocalized to these peripheral structures. This redistribution was reversible because, after shifting back to the permissive-temperature, most of the xpo1–1-GFP signal returned to the nucleoplasm (data not shown).

Since the peripheral accumulation of the xpo1–1-GFP protein was reminiscent of the nuclear pore–clustering phenotype previously described for a class of nucleoporin mutants (reviewed by Doye and Hurt, 1997), the distribution of the nuclear pore protein Nup188 (Nehrbass et al., 1996; Zabel et al., 1996) and of the NPC-associated Gle1/Brr3 protein (Murphy and Wente, 1996; T. Awabdy and C. G., unpublished data) was analyzed in the *xpo1–1* strain at the nonpermissive temperature (Figures 2E and 2F). Neither mislocalization of Gle1/Brr3 nor NPC clustering could be detected when either Nup188-GFP (Figure 2E) or Gle1-GFP (Figure 2F) was expressed in *xpo1–1* cells at 37°C. Thus, the xpo1–1 protein does not cause an NPC-clustering phenotype. In addition, the mislocalized xpo1–1-GFP protein did not colocalize with nucleolar markers such as Nop1p (data not shown). We conclude that Xpo1p is a soluble protein that is localized to the nucleus at steady state. In addition, the rapid and reversible relocalization of the xpo1–1 protein at the nonpermissive temperature suggests that Xpo1p can undergo dynamic changes in its localization.

To further examine the dynamic behavior of Xpo1p, a shuttling assay was employed that utilizes the nup49–313 strain to uncouple nuclear protein export from reimport (Lee et al., 1996). The Xpo1p-GFP fusion protein was expressed in wild-type NUP49 cells and in nup49–313 cells defective in protein import at the nonpermissive temperature (Doye et al., 1994; Lee et al., 1996). After shift to 37°C and inhibition of de novo protein synthesis by cycloheximide, the distribution of the GFP-fusion protein was analyzed (Figures 2G and 2H). Whereas the Xpo1-GFP protein stayed entirely nuclear in *NUP49* cells (Figure 2G), a strong cytoplasmic accumulation of the GFP signal could be detected in *nup49–313* cells (Figure 2H). Typically 30%–50% of the *nup49–313* cells showed a strong cytoplasmic signal after 5 hr at the nonpermissive temperature. No cytoplasmic accumulation was detectable in *nup49–313* cells grown at RT (data not shown). We therefore conclude that Xpo1p is able to leave the nucleus. It accumulates in

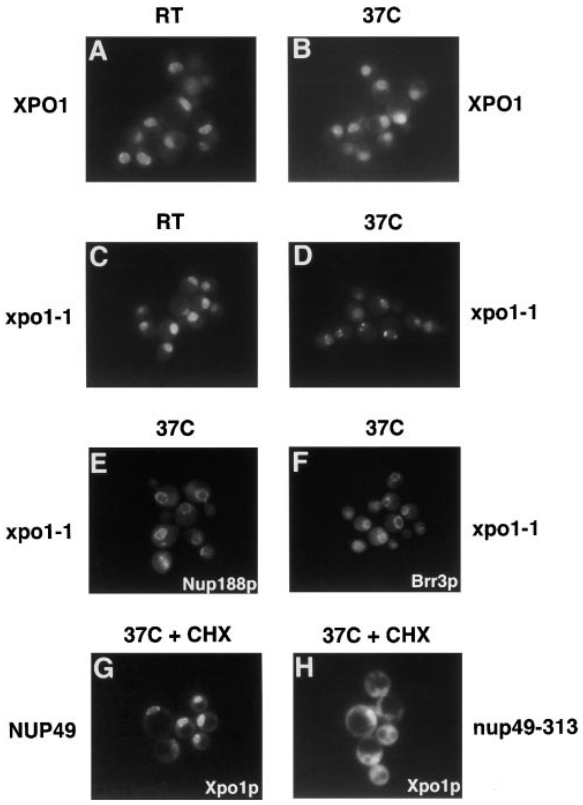

Figure 2. Xpo1p Is Nuclear at Steady State but Shuttles between the Nucleus and the Cytoplasm

The subcellular localization of the XPO1-GFP (A and B) and the xpo1–1-GFP fusion protein (C and D) was analyzed either at RT (A and C) or after a shift to 37°C (B and D). In order to compare the xpo1–1p localization with the nuclear pore distribution in the *xpo1–1* mutant, the localization of a Nup188-GFP fusion protein (E) or a Brr3-GFP fusion protein (F) was analyzed in *xpo1–1* mutant cells grown for 30 min at the nonpermissive temperature. To further examine the dynamic behavior, the XPO1-GFP fusion protein was also expressed in NUP49 wild-type cells (G) and in nup49–313 mutant cells (H) and its localization analyzed by epifluorescence. Cells were grown for 5 hrs at the nonpermissive temperature, which causes a block in protein import in nup49–313 cells (Doye et al., 1994; Lee et al., 1996). To inhibit de novo protein biosynthesis, cycloheximide was added during the temperature shift.

the cytoplasm of *nup49–313* cells presumably as a result of the inhibition of its nuclear reimport at the nonpermissive temperature. Thus, Xpo1p can shuttle between the nucleus and the cytoplasm.

A Novel Protein Export Assay in S. cerevisiae

The shuttling behavior of Xpo1p prompted us to analyze a possible role of Xpo1p in nuclear export. To study nuclear protein export, a novel assay was devised in S. cerevisiae (Figure 3). A competition assay was developed in which a GFP reporter was fused to both the SV40 large T NLS and to the leucine-rich NES of PKI. To prevent passive diffusion through the NPC, two GFP moieties were used, resulting in a reporter protein with a total calculated molecular weight of 68,219. At steady state, this NES-GFP-NLS reporter was localized to the cytoplasm, and nuclear exclusion of the reporter protein could be observed in some cells (Figure 3A). However, when the PKI-NES was replaced with the PKI-P12 mutant (which contains a single L to A mutation in the PKI NES motif; Wen et al., 1995), the GFP reporter protein exclusively localized to the nucleoplasm (Figure 3D). This demonstrates that the PKI-NES is a functional export signal in S. cerevisiae, and, furthermore, it suggests that in yeast the PKI-NES is stronger than the SV40 NLS. The NES-GFP-NLS fusion protein is likely to shuttle continously between the cytoplasm and the nucleus but is enriched in the cytoplasmic compartment at steady state, presumably because the rate of its export is higher than the rate of import. This assay should thus provide a simple and sensitive visual screen to identify cells that are either defective in or have a reduced rate of nuclear protein export, since in both cases a nuclear accumulation of the NES-GFP-NLS reporter protein would be expected.

Xpo1p (Crm1p) Is a Nuclear Protein Export Factor

To examine the role of Xpo1p in nuclear protein export, the NES-GFP-NLS reporter was introduced into the *XPO1* deletion strain expressing either the wild-type or the *xpo1–1* allele (Figures 4A–4D). As expected, the NES-GFP-NLS reporter was localized in the cytoplasm of wild-type cells that were grown either at RT (Figure 4A) or 37°C (Figure 4B). In the *xpo1–1* strain grown at the permissive temperature, most of the GFP signal was detected in the cytoplasm; however, in some cells, a nuclear accumulation of the reporter could be observed (Figure 4C). This nuclear accumulation was drastically enhanced when the *xpo1–1* cells were shifted to the nonpermissive temperature (Figure 4D). After 5 min, a strong nuclear accumulation was visible in most cells (data not shown), and after 15 min at 37°C the NES-GFP-NLS was exclusively localized to the nucleus (Figure 4D). This indicates that nuclear protein export is severely impaired in *xpo1–1* cells.

Previous studies have shown that the yRip1 protein is able to bind to the leucine-rich NES in the two-hybrid assay (Stutz et al., 1995). To examine the phenotype of an *RIP1* deletion strain in our NES-dependent protein export assay, the NES-GFP-NLS reporter was expressed in a Δ*RIP1* strain (Figure 4F) and in the corresponding wild-type background (Figure 4E). No nucleoplasmic accumulation of the reporter protein could be detected when the GFP signal was analyzed in cells lacking the *RIP1* gene.

From these data we conclude that *xpo1–1* has a strong protein export phenotype. The onset of the phenotype is extremely rapid and is apparently specific for *xpo1–1*, since another strain previously implicated in nuclear export events was not defective in the export of the NES-GFP-NLS reporter (Figure 4F and data not shown).

Xpo1p (Crm1p) Binds to the NES and to Ran in the Two-Hybrid Assay

The two-hybrid assay was chosen to test whether Xpo1p is able to interact with the NES motif. Both the PKI NES and the P12 mutant were fused to the LexA-DNA binding

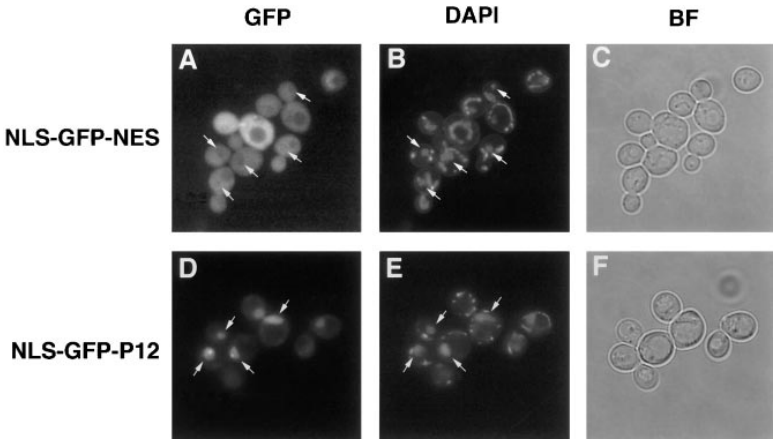

Figure 3. A Novel Protein Export Assay in S. cerevisiae

Wild-type cells expressing either an NES-GFP-NLS (A–C) or a P12-GFP-NLS fusion protein (D–F) were examined by fluorescence (A, B, D, and E) and by bright field microscopy (C and F). GFP or DAPI signals were visualized in the fluorescein (A and D) or UV channel (B and E), respectively. Note that DAPI preferentially stains mitochondrial DNA in living cells. The position of the nuclei is indicated by arrows. Nuclear exclusion of the GFP signal can be detected in cells expressing the NES-GFP-NLS reporter (A) whereas the P12-GFP-NLS reporter accumulates in the nuclei (D). No GFP signal is detected in vacuoles under these growth conditions (A and D).

domain and tested for their interaction with a Xpo1-transactivation-domain fusion protein in liquid β-galactosidase assays (Figure 5A). Strong transactivation was observed when the NES and the Xpo1p hybrid proteins were coexpressed (Figure 5A, lane 1). The transactivation was approximately 26 times higher than the one measured with the P12 mutant (Figure 5A, lane 2) or in a control without plasmid (data not shown). A similar low background transactivation was found when either the NES (Figure 5A, lane 3) or the P12 mutant hybrid (Figure 5A, lane 4) were tested with vector controls. Thus, Xpo1p specifically interacts with the PKI-NES in the two-hybrid assay.

It has been shown for three importin β-like proteins that they bind to the Ran GTPase (Bonifaci et al., 1997; Nigg, 1997; Rout et al., 1997). We therefore wanted to test if Xpo1p could bind to Ran (Figure 5B). Again, the two-hybrid assay was used to analyze the interaction between a LexA-Xpo1p hybrid and a Ran-transactivation-domain fusion protein (Figure 5B, lane 1). We observed a strong interaction between Xpo1p and Ran whereas no interaction was detected when the cytoplasmic GTPase Rab3A was used as a bait (Figure 5B, lane 2). Furthermore, the LexA-Xpo1 hybrid alone did not show transactivation (Figure 5B, lane 3), demonstrating that the Xpo1p interaction with Ran is specific.

xpo1–1 Cells Are Not Defective in NLS-Mediated Nuclear Protein Import

All previously characterized importin β-like proteins have been implicated in nuclear protein import processes (Görlich and Mattaj, 1996; Nigg, 1997; Rout et al., 1997). The data from the NES/NLS competition assay presented above (Figure 4) suggested that the *xpo1–1* strain is not defective in protein import. We nevertheless sought to examine the behavior of the *xpo1–1* strain in a nuclear protein import assay (Shulga et al., 1996; Figure 6). Cells from the *nup49–313* strain, previously shown to be defective in protein import (Doye et al., 1994) and from the *xpo1–1* strain were transformed with a NLS-GFP reporter and grown at the permissive temperature (Figures 6A and 6D). Cells were then metabolically poisoned to allow the equilibration of the NLS reporter across the nuclear envelope (Figures 6B and 6E). After the shift to the nonpermissive temperature and the release of the metabolic block, the reimport of the NLS reporter was examined (Figure 6C and 6F). Whereas protein import was clearly inhibited in *nup49–313* cells at the nonpermissive temperature (Figure 6C), no import inhibition could be detected in *xpo1–1* cells after a 30 min shift to 37°C (Figure 6F). Interestingly, after longer shifts to the nonpermissive temperature (i.e., longer than 1 hr) we could observe an inhibition of nuclear protein import in this assay (data not shown). However, this import defect occurs much later than the export block, and the two defects can clearly be discriminated temporally.

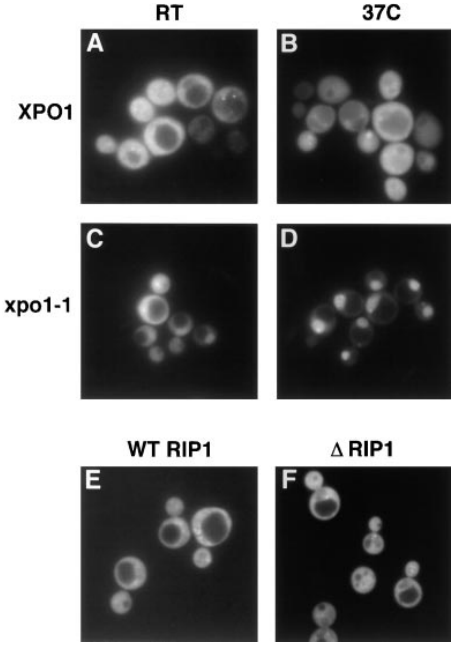

Figure 4. NES-Mediated Protein Export Is Inhibited in *xpo1–1* Cells

The NES-GFP-NLS reporter was expressed in *XPO1* wild-type (A and B) or in *xpo1–1* mutant cells (C and D) that were grown either at RT (A and C) or shifted to 37°C for 15 min (B and D). As a test for the specificity of the export phenotype in *xpo1–1* cells, the localization of the protein export reporter was also examined in an RIP1 deletion strain (F) and in the corresponding wild-type strain (E).

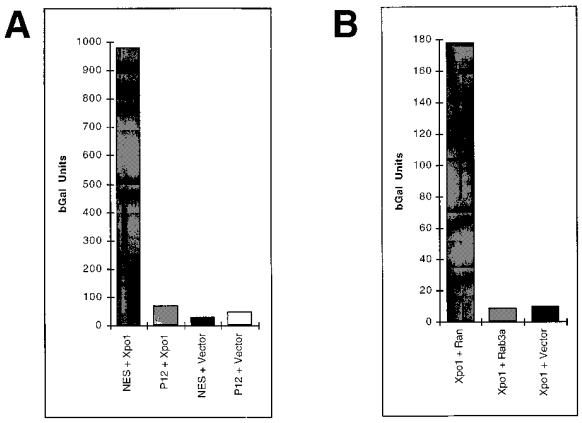

Figure 5. Xpo1p Interacts Specifically with the PKI NES and with Ran in the Two-Hybrid Assay

(A) The two-hybrid interactions between a LexA-NES_{PKI} (lanes 1 and 3) and a LexA-P12 hybrid protein (lanes 2 and 4) with either a Xpo1-B42 hybrid (lanes 1 and 2) or the B42 transcriptional activation domain alone (lanes 3 and 4) were analyzed by liquid β-galactosidase assays. For each experiment, the β-galactosidase activity was determined four times with four individual cultures.

(B) The histogram shows the result of the two-hybrid assay with an Xpo1-LexA fusion protein as a bait and either Ran-B42 (lane 1), Rab3a-B42 (lane 2), or a vector control (lane 3) as preys. The experiments were performed with four individual cultures and average β-galactosidase units were calculated.

We therefore conclude that the *xpo1–1* strain does not have a primary import defect in this NLS-mediated protein import assay. The result that protein import is impaired at later time points can be most easily explained by the failure to recycle an essential import factor in the export deficient *xpo1–1* cells.

Polyadenylated RNA is Rapidly Accumulated in Nuclei of *xpo1–1* Cells at the Nonpermissive Temperature

To test if Xpo1p plays a role in mRNA export in S. cerevisiae, the distribution of polyadenylated RNA was analyzed in *xpo1–1* cells (Figure 7). An *XPO1* deletion strain covered by plasmids expressing either the wild-type (Figures 7A and 7B) or the *xpo1–1* allele (Figures 7C–7F) was examined at both RT (Figure 7C) and 37°C (Figure 7A and 7E) by in situ hybridization with an oligo dT50 probe. To localize nuclei, DAPI staining was performed (Figures 7B, 7D, and 7F). As expected, polyadenylated RNA was detected in the cytoplasm of *XPO1* cells grown at either RT (data not shown) or 37°C (Figure 7A). Similarly, no nuclear accumulation of polyadenylated RNA was observed in *xpo1–1* cells grown at the permissive temperature (Figure 7C). However, in the *xpo1–1* strain shifted to the nonpermissive temperature, a strong nuclear oligo-dT signal was detected as early as 15 min after the shift to 37°C (Figure 7E).

These results demonstrate that export of mRNA is impaired in *xpo1–1* cells. The temporal appearance of the mRNA export phenotype is similar to the onset of the NES-protein export defect, suggesting that these two export events are tightly coupled in S. cerevisiae.

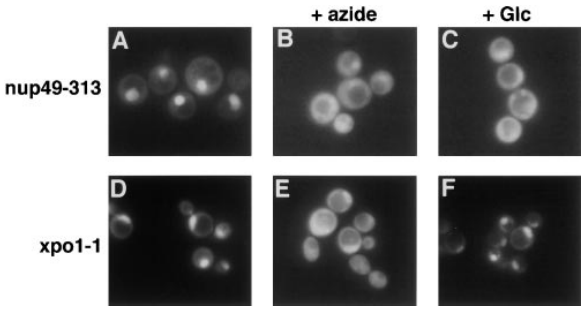

Figure 6. Protein Import Is Not Inhibited in *xpo1–1* Cells

A diffusible NLS-GFP reporter was expressed in either nup49–313 (A–C) or *xpo1–1* cells (D–F). At the permissive temperature, the reporter is localized in the nucleus of both strains (A and D). Cells were then metabolically poisoned to allow equilibration of the reporter through the nuclear envelope (B and E). Import was initiated by removal of the poison and addition of glucose and the localization of the NLS-GFP reporter was analyzed at the nonpermissive temperature (C and F).

Discussion

In this study we have characterized Crm1p as a novel importin β-like transport factor functioning in the export of NES-containing proteins and mRNA. We propose *CRM1* be renamed exportin 1 (*XPO1*) because of its function in nuclear export. The extremely rapid onset and strength of the observed export phenotypes in the temperature-sensitive *xpo1–1* allele suggest to us that Xpo1p is likely to play a direct role in nuclear export. This is supported by two-hybrid data, which demonstrate that Xpo1p can specifically interact with both an NES-containing export substrate and the Ran GTPase. Xpo1p is localized in the nucleus at steady state but is able to shuttle between the nucleus and the cytoplasm. These data are consistent with exportin 1 acting as a direct carrier that mediates the export of NES-containing proteins to the cytoplasm.

The S. pombe homolog of *XPO1* was previously identified as *crm1*$^+$ (*c*hromosomal *r*egion *m*aintenance) since a cold-sensitive mutant revealed abnormal chromosomal structures at the nonpermissive temperature (Adachi and Yanagida, 1989). In addition to the reported changes in chromosome structure, mutations in the fission yeast *crm1*$^+$ gene have been shown to cause upregulation of the transcription factor pap1 (Toda et al., 1992) and can lead to a multidrug resistance phenotype, including resistance to caffeine and leptomycin B (Nishi et al., 1994; Turi et al., 1994; Kumada et al., 1996). Similar pleiotropic phenotypes, including morphological changes in chromosome structure, have been previously described for mutations in other nuclear transport factors, such as RCC1 or Ran/TC4 (Dasso, 1993; Rush et al., 1996), and the role of *XPO1* in nuclear export could account for the complexity of its mutant phenotype. However, we cannot exclude the possibility that *xpo1–1* shows additional phenotypes to those analyzed in this study or that other *XPO1*-alleles reveal novel functions of *XPO1*. Among the reported mutant phenotypes, the leptomycin B resistance is particularly interesting, since a recent study has demonstrated that this drug can

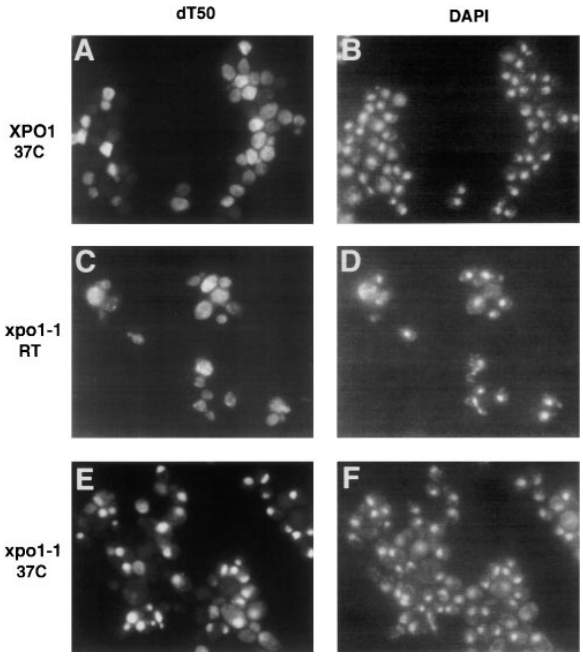

Figure 7. Polyadenylated RNA Rapidly Accumulates in *xpo1–1* Cells at the Nonpermissive Temperature

Wild-type *XPO1* cells grown at 37°C (A and B) and mutant *xpo1–1* cells grown at either RT (C and D) or shifted for 15 min to 37°C (E and F) were fixed and analyzed by in situ hybridization with an oligo dT50 probe (A, C, and E). Nuclear DNA was stained with DAPI and the signal was visualized by epifluorescence (B, D, and F).

block the nuclear export of the HIV REV protein in mammalian cells (Wolff et al., 1997). Together with our data, this suggests that one cellular target of leptomycin B is *XPO1*. The observed block of REV export in leptomycin-treated cells is consistent with the cellular function of *XPO1* demonstrated here.

The human homolog of Xpo1p, hCRM1, was recently identified as a factor interacting with the nucleoporin CAN/Nup214 (Fornerod et al., 1997). In this study, the authors demonstrated that hCRM1 interacts with the FG-repeat region of CAN and provided evidence that hCRM1 can additionally bind to other FG repeat-containing NPC proteins. Furthermore, it was shown that hCRM1 is a dynamic factor that is mainly localized in the nucleoplasm and on both sides of the NPC (Fornerod et al., 1997). These properties suggest that some of the functions of exportin 1 are conserved between yeast and humans. It will be interesting to test whether hCRM1 indeed functions in the same export pathways as its yeast ortholog Xpo1p.

As expected from the export phenotype of *xpo1–1*, *XPO1* is an essential gene in S. cerevisiae (Toda et al., 1992; K. S. and K. W., unpublished data), and the encoded protein shares significant regions of homology to other importin β-like transport factors (Fornerod et al., 1997; data not shown). All previously characterized transport factors of this protein family have been shown to function in nuclear protein import events (Görlich and Mattaj, 1996; Nigg, 1997; Rout et al., 1997). Exportin 1 is thus a novel family member that acts as an export factor but, like its homologs, can interact with its cargo and the Ran GTPase. The homology among all these factors suggests that they are derived from a common ancestor. Such an ancestral factor could have been a shuttling protein that interacted with the NPC, with the Ran GTPase, and presumably with its cargoes. Later in evolution, the family might have diverged to fulfill more specialized functions and to transport specific cargoes in a vectorial fashion. In any event, it can be concluded from the homology between these import factors and exportin 1 that the two assymetric processes of protein import and export have some common features, and it can be anticipated that certain paradigms will be shared between these transport processes.

The export phenotype of *xpo1–1* is apparently specific since no block in NLS-mediated protein import could be detected either in the NES/NLS competition assay (Figure 4) or in a protein import assay at short times after a temperature shift (Figure 6). Interestingly, protein import does seem to be impaired after longer shifts to the nonpermissive temperature (data not shown). We interpret this as a secondary effect of the block in protein export. Since all nuclear transport factors are expected to shuttle continously between the nucleus and the cytoplasm, it is not surprising that a block in protein export would eventually lead to an arrest of protein import. A leucine-rich NES was identified in the protein import factor importin β (Iovine and Wente, 1997), and it is therefore conceivable that the reexport of importin β is impeded in *xpo1–1* cells. It will be interesting to examine the dynamic behavior of importin β in the *xpo1–1* strain.

We were unable to uncouple the protein export and mRNA export phenotypes in *xpo1–1* mutant cells. Both export events are equally affected in the *xpo1–1* mutant, and, most significantly, the phenotypes are apparent in less than 15 min after the shift to the nonpermissive temperature. In the simplest case, this could reflect a direct coupling of the two processes, as seen in the REV-dependent export of RRE-containing viral mRNA. Indeed, a yeast protein containing a leucine-rich NES was recently shown to be required for mRNA export and has been proposed to be a cellular counterpart of Rev (Murphy and Wente, 1996). However, Gle1/Brr3 is predominantly localized at the NPC (Murphy and Wente, 1996; T. Awabdy and C. G., unpublished data) (Figure 2F), and we could not observe any change in the localization of a Gle1-GFP protein when analyzed in *xpo1–1* cells grown at the nonpermissive temperature (Figure 2F). It is presently unclear whether Gle1p shuttles between the nucleus and the cytoplasm, and we cannot exclude the possibility that Gle1 acts downstream of *XPO1* or uses an *XPO1*-independent export pathway. Additional studies are needed to address these questions and to clarify the role of Gle1p and its relationship with Xpo1p.

The tight coupling of mRNA export and protein export observed in *xpo1–1* cells is in contrast to NES-competition experiments in Xenopus oocytes, in which these two export pathways could be clearly distinguished (Fischer et al., 1995). Several alternative explanations could account for this apparent difference in the systems. For example, one could postulate an adaptor upstream of *XPO1* that functions in mRNA export and interacts with *XPO1* independently of a leucine-rich

NES. Alternatively, the difference in the two systems could reflect a variation in the mRNA export pathways employed in yeast and metazoans. Strong evidence has been provided that hnRNP-like proteins, such as hnRNPA1, are involved in mRNA export in metazoan cells (reviewed by Izaurralde and Mattaj, 1995; Nigg, 1997), including the competitive inhibition of mRNA export by the hnRNP A1 M9-domain in Xenopus (Izaurralde et al., 1997). However, no direct homologs for this class of proteins can be found in the S. cerevisiae genome. Instead, factors such as npl3, nab2, or nab4/Hrp1p have been implicated as mediators of mRNA export (Nigg, 1997). To date, an M9-like export motif has not been identified for any of these factors. It will now be important to identify the adaptor(s) mediating mRNA export in yeast. Based on the results presented here, we predict that such a factor would function via the *XPO1* export pathway.

The yRip1p protein has been previously identified as a factor that binds to NES sequences in the two-hybrid assay (Stutz et al., 1995). Surprisingly, we were unable to detect an export phenotype in an *RIP1*-deletion strain when tested in our NES-dependent export assay (Figure 4E). Since *RIP1* is not essential (Stutz et al., 1995), it is possible that other factors might be able to compensate for the loss of the *RIP1* gene; however, alternative explanations cannot be excluded at present. It is interesting in this regard that recent data suggest that Rip1p functions in the selective export of heat shock mRNA (Cole and Saavedra, 1997) and might be involved in alternative export pathways.

We have presented a novel in vivo assay to study nuclear protein export in S. cerevisiae. The assay is based on the competition between an NES and an NLS motif, both fused to a GFP-reporter protein. At steady state, this reporter protein is localized to the cytoplasm, most likely because its export rate is higher than its rate of import. The reporter presumably shuttles continously between the cytoplasm and the nucleus and undergoes a constant, futile cycle between import and export. A small decrease in the export rate can therefore cause a change in the localization equilibrium. This competition assay is thus a very sensitive tool to analyze nuclear export in vivo. For example, it will allow the examination of the functionality and/or the strength of export signals in yeast. Furthermore, it provides a simple visual screen to identify mutants that are defective in nuclear protein export. Yeast genetics can now be used to identify additional protein export factors.

Experimental Procedures

Plasmid Construction

The plasmid pKW435 containing the *LEU2* gene inserted into the *XPO1* locus was constructed as follows: the *LEU2* gene was amplified by PCR from yeast-genomic DNA and cloned into a BGlLII/HinDIII cut pBluescript KS+ (Stratagene) vector carrying the *XPO1* gene fused in-frame to a C-terminal *GFP* (S65T; F64L) gene (see below). The wild-type *XPO1* allele was amplified from yeast-genomic DNA using the PCR Expand system (Boehringer Mannheim) with oligonucleotides KS#8 [5′-GCCCGTCGACCC AGT TAC TGT CAA TCC TCT CC] and UC#29 [5′-GGGGATCCGCCTCTAATCATCAAG]. The fragment was cut with SalI and BamHI and ligated into a pRS313 vector. To generate an XPO1-GFP fusion, the *XPO1* gene was amplified from genomic-yeast DNA using primer KS#8 [5′-GCC CGT CGA CCC AGT TAC TGT CAA TCC TCT CC] and KS#2: [5′-CGC GGA TCC TAG AATTCGGAAGGTTTTAATAACCCACC]. The PCR product was digested with SalI/EcoRI and ligated into pBluescript KS+ containing a *GFP* (S65T,F64L) gene between the EcoRI/BamHI restriction sites to generate plasmid pKW465. To allow for fusion protein expression in yeast, pKW465 was digested with SalI and BamHI releasing the XPO1-GFP insert. This insert was cloned into either a YCp50 vector (*URA3*) or pRS313 vector (*HIS3*), which had been cut with the same enzymes to generate plasmids pKW466 or pKW470, respectively.

The *xpo1–1* temperature-sensitive allele was generated by low-fidelity PCR mutagenesis (Dieffenbach and Dveksler, 1995) using primers KS#1 [5-ACGCGTCGACATAAGTTATGAGAAGCTGTCATCG] and KS#2. The PCR fragment was subcloned with SalI/EcoRI into the pBluescript-GFP vector as described above. SalI/BamHI fragments were then subcloned into pRS313, and plasmids were introduced into the *XPO1* deletion strain by plasmid shuffling. Plasmid pKW456 was chosen for further analysis. The GFP moiety in pKW456 was replaced by an oligonucleotide linker (oligos UC#32 [5′-AATTCTGAT AG], UC#33 [5-GATCCTATCAG]) to construct an untagged *xpo1–1* allele (pKW457).

The LexA-NES_{PKI} hybrid (pKW483) and the LexA-P12 hybrid (pKW484) were constructed by cloning oligos UC#5 [5′-AATTCAATGA ATTAGCCTTGAAATTAGCAGGTCTTGATATCAACAAGACAGG]/UC #6 [5-GATCCCTGTCTTGTTGATATCAAGACCTGCTAATTTCAAGGCTA ATTCATTG] or UC7 [5′-AAT TCA ATG AAT TAG CCT TGA AAT TAG CAG GTG CTGATATCAACAAGACAGG]/UC#8 [5′-GAT CCC TGTC TTG TTG ATA TCAGCACCTGCTAATTTCAAGGCTAATTCATTG] into plasmid pEG202 (Gyuris et al., 1993). The LexA-XPO1 fusion (pKW442) was constructed by cloning an EcoRI/BamHI PCR product into pEG202 (Gyuris et al., 1993) using primers KS#9 [5′-CCG AAT TCA AAC ATA ATA TGG AAG GAA TTT TGG] and UC#29 [5′-GGGGATCCGCCTCTAATCATCAAG]. The fusion protein is functional since pKW442 is able to rescue a *XPO1* deletion strain (data not shown). The B42-XPO1 fusion (pKW471) was generated by subcloning the EcoRI/BamHI fragment of pKW442 into pJG4–5 (Gyuris et al., 1993). The plasmid pKW430 containing the NLS-GFP-NES fusion was constructed using the following strategy: the ADH promoter (SacI/HindIII), the SV40 NLS (HindIII/EcoRI, including some additional vector sequence), the PKI NES (EcoRI/BamHI; [oligos UC#5/6]), and two GFP moieties (BamHI/XhoI) were subcloned in-frame into pRS426 (2 μ, *URA3*) using the indicated sites. For plasmid pKW431, expressing a NLS-GFP-P12, fusion protein, the PKI-NES was replaced with the PKI-P12 mutant (oligos UC#7/8).

Yeast Strains

To construct the *XPO1*::*LEU2* deletion strain, the following strategy was employed: Plasmid pKW435 was cut with SalI and NcoI releasing the *LEU2* gene and *XPO1* flanking sequences. This fragment was used to transform W303 (*Mat a/α*, *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 can 1–100*) cells according to Guthrie and Fink (1989). The integration of the *LEU2* marker at the *XPO1* locus was confirmed by PCR and Southern analysis (data not shown). This diploid strain was transformed with plasmid pKW466 and sporulated. LEU+, URA+ spores were selected and tested for plasmid-dependent growth on 5′-FOA plates. Plasmids pKW456, 457, and 470 were introduced into the *XPO1* deletion strain covered with pKW466 by plasmid shuffling on 5′-FOA plates.

Protein Shuttling Assay

Shuttling assays were performed essentially as described (Lee et al., 1996), with the following modifications: the NUP49 wild-type and nup-313 mutant strains were transformed with a YCp 50 plasmid containing the *CRM1-GFP* fusion gene under its own promotor (pKW466). Cycloheximide was added to a final concentration of 0.1 mg/ml, and the cultures were either shifted to the nonpermissive temperature or kept at RT for 5 hr. The distribution of the Crm1-GFP fusion protein was analyzed with a fluorescence microscope as described below.

Two-Hybrid Analysis

Vector system and yeast strains were used as described by Brent and coworkers (Gyuris et al., 1993) and β-galactosidase assays were performed as reported (Kandels-Lewis and Seraphin, 1993).

Protein Export Assay

To analyze protein export in different yeast strains, NES-GFP2-NLS (pKW430) or P12-GFP2-NLS fusion proteins (pKW431) were expressed. Cultures were grown in SC medium lacking uracil to mid-log phase at the indicated temperatures and GFP signals were analyzed in living cells.

Protein Import Assay

Nuclear protein import assays were performed as described (Shulga et al., 1996) with the following modifications: *XP01* and *xpo1–1* cells were transformed with a constitutively expressed NLS-GFP reporter construct (Shulga et al., 1996) and were grown to mid-log phase in SC-URA, 2% glucose medium at RT. To allow for the equilibration of the reporter between cytoplasm and nucleus, cells were transferred into 10 mM Na-azide (Sigma) and 10 mM deoxy-glucose (Sigma) in SC-URA medium. After 1 hr, cultures were shifted to the nonpermissive temperature for the times indicated. Reimport was induced by addition of glucose to a final concentration of 2%.

dT50 In Situ Hybridization

dT50 in situ hybridization was essentially performed as described (Amberg et al., 1992; Kadowaki et al., 1994). A detailed description of the protocol will be presented elsewhere (A. deBruyn-Kops, K. S., and C. G., unpublished data).

Fluorescence Microscopy and Image Analysis

Fluorescence samples were observed with a Leica DMLB microscope using a 100× PL Fluotar oil immersion objective. Images were aquired with an Optronics DEI-750 CCD camera using the Scion Image Software program. Panels were assembled with Adobe Photoshop software.

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