Cell, Vol. 101, 187–198, April 14, 2000, Copyright ©2000 by Cell Press

PHAX, a Mediator of U snRNA Nuclear Export Whose Activity Is Regulated by Phosphorylation

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Summary

In metazoa, assembly of spliceosomal U snRNPs requires nuclear export of U snRNA precursors. Export depends upon the RNA cap structure, nuclear cap-binding complex (CBC), the export receptor CRM1/Xpo1, and RanGTP. These components are however insufficient to support U snRNA export. We identify PHAX (phosphorylated adaptor for RNA export) as the additonal factor required for U snRNA export complex assembly in vitro. In vivo, PHAX is required for U snRNA export but not for CRM1-mediated export in general. PHAX is phosphorylated in the nucleus and then exported with RNA to the cytoplasm, where it is dephosphorylated. PHAX phosphorylation is essential for export complex assembly while its dephosphorylation causes export complex disassembly. The compartmentalized PHAX phosphorylation cycle can contribute to the directionality of export.

Introduction

The presence of the nucleus in a eukaryotic cell necessitates the existence of efficient mechanisms for bidirectional transport of macromolecules between the nucleus and the cytoplasm. Many proteins and RNAs are transported through the nuclear pore complexes (NPCs) that span the nuclear envelope. Identification of Importin β family members as major nucleocytoplasmic transport receptors has greatly enhanced our understanding of nuclear transport mechanisms and has enabled the development of robust models for the mechanism of nuclear import and export of relatively simple cargoes (Görlich, 1998; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998; Ohno et al., 1998; Hood and Silver, 1999).

The small GTPase Ran is a key regulator of directionality in these models. Like other small GTPases, Ran can switch between the GDP- and GTP-bound states, but to do so efficiently requires additional proteins. Due to the cytoplasmic localization of RanGAP and RanBP1/BP2, which promote GTP hydrolysis on Ran, Ran is predicted to be mainly the GDP-bound form in the cytoplasm. Conversely, due to the nuclear localization of Ran's guanine nucleotide exchange factor, RanGEF or RCC1, nuclear Ran should be mainly in the GTP-bound form. According to the current model for the nuclear export of a simple cargo, an export receptor binds in the nucleus to a cargo cooperatively with RanGTP, forming a trimeric export complex. This complex moves to the cytoplasm based on interactions between the receptor and components of the NPC and disassembles due to GTP hydrolysis triggered by RanGAP and RanBP1/BP2 (Cole and Hammell, 1998; Dahlberg and Lund, 1998; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998).

This model is sufficient to explain the export of tRNAs from the nucleus by the Exportin-tRNA/Xpo-t receptor and its yeast homolog Los1 (Arts et al., 1998; Hellmuth et al., 1998; Kutay et al., 1998; Sarkar and Hopper, 1998). The export of other cellular RNAs from the nucleus, however, appears to require more complex mechanisms. One example of this is represented by a subset of the U snRNAs. Major spliceosomal U snRNAs such as U1, U2, U4, and U5 are transcribed in the nucleus by RNA polymerase II and acquire a monomethylated m^7G-cap structure. In metazoa, this class of U snRNAs is then initially exported from the nucleus. The RNAs form complexes in the cytoplasm with a group of proteins called the Sm proteins. The monomethyl cap structure is subsequently hypermethylated, and the RNA–protein complexes are then imported back into the nucleus where they take part in pre-mRNA splicing reactions (Mattaj, 1988; Lührmann et al., 1990).

It has been shown that the m^7G-cap structure of the U snRNAs is an essential signal for their nuclear export (Hamm and Mattaj, 1990; Jarmolowski et al., 1994). Export is dependent on interaction of the U snRNA cap structure with the nuclear cap-binding complex or CBC (Izaurralde et al., 1994; 1995a). CBC is a heterodimeric complex composed of two subunits, CBP80 and CBP20, and both subunits are required for binding to the m^7G-cap structure (Izaurralde et al., 1994; 1995a; Kataoka et al., 1994; 1995). CBC associates with the cap structures of nascent RNA polymerase II transcripts (Visa et al., 1996). It has therefore been hypothesized that CBC carries U snRNAs to the cytoplasm and then shuttles back to the nucleus, and immuno-electron microscopy studies in *Chironomus tentans* have suggested that CBC accompanies the Balbiani ring mRNPs at least as far as the cytoplasmic face of the NPC (Visa et al., 1996).

The other factors implicated in U snRNA export are RanGTP and CRM1/Xpo1, which is also the export receptor for proteins carrying leucine-rich nuclear export signals (NESs). U snRNA export is specifically inhibited by inactivating CRM1 with leptomycin B, by saturating CRM1 with an excess of NES peptides (Fischer et al., 1995; Fornerod et al., 1997), and by reducing the nuclear concentration of RanGTP (Izaurralde et al., 1997). Based on this information, the simplest model for U snRNA export would be the following. CBC binds to a newly transcribed U snRNA via its cap structure, and CRM1/Xpo1 binds to CBC cooperatively with RanGTP. This complex then moves to the cytoplasm and disassembles.

In this study, we test this hypothesis and find that U snRNA export requires an additional protein. This protein acts as an adaptor between the CBC/RNA complex

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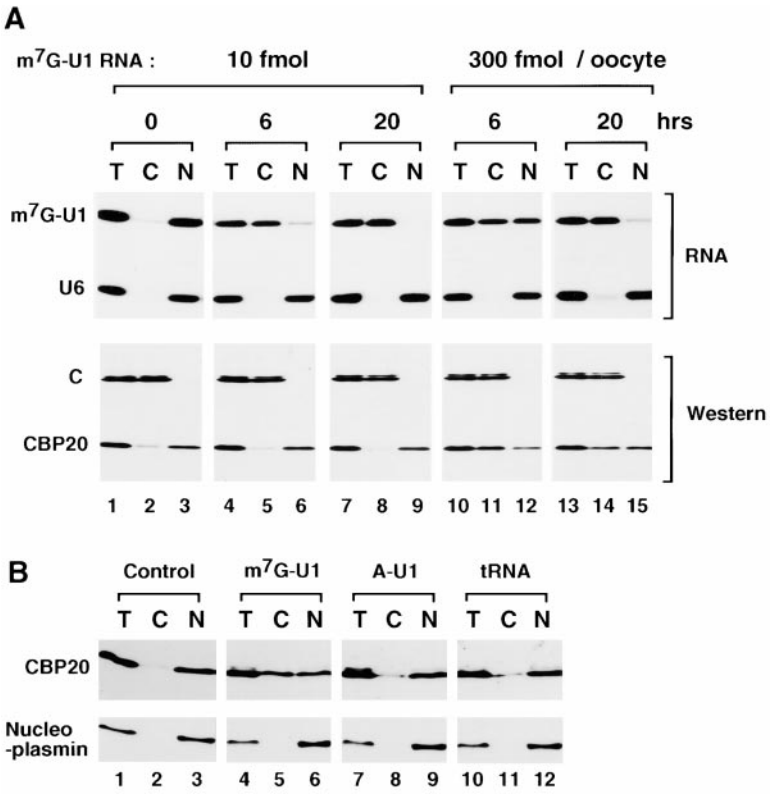


Figure 1. m7G-Capped RNA Induces Shuttling of CBC

(A) A mixture of ^{32}P-labeled and unlabeled m^{7}G-capped U1ΔSm RNA (10 fmol/oocyte, lanes 1–9 or 300 fmol/oocyte, lanes 10–15 of unlabeled RNA) and U6Δss RNA was injected into the nucleus of *Xenopus* oocytes, and RNA (upper panel) and protein (lower) were isolated from total oocytes (T) or from cytoplasmic (C) or nuclear (N) fractions either immediately (lanes 1–3) or 6 hr (lanes 4–6 and 10–12) or 20 hr (lanes 7–9 and 13–15) after injection. RNA and protein fractions were analyzed by denaturing PAGE followed by autoradiography or Western blotting, respectively. C is a fortuitously cross-reacting band that was used as a control for cytoplasmic loading.

(B) 300 fmol/oocyte of either m^{7}G-capped U1ΔSm RNA (lanes 4–6), A-capped U1ΔSm RNA (lanes 7–9), or human initiator methionyl tRNA (lanes 10–12) was injected into the nucleus of *Xenopus* oocytes, and the oocytes were fractionated and proteins isolated 2 hr after injection and analyzed by Western blotting using anti-CBP20 (upper; Izaurralde et al., 1995a) and, as a control, anti-nucleoplasmin (lower, Görlich et al., 1996) antibodies. Uninjected oocytes were used as a control (lanes 1–3).

and the CRM1/RanGTP proteins. The activity of the protein is regulated by phosphorylation, so it is named PHAX, for phosphorylated adaptor for RNA export.

Results

As described above, the working model for U snRNA export has CBC serving as an adaptor between a U snRNA and CRM/Xpo1. A critical aspect of the model is that CBC should accompany the transported RNA into the cytoplasm. The steady state localization of CBC in HeLa cells and *Saccharomyces cerevisiae* is mainly nuclear (Izaurralde et al., 1995b; Görlich et al., 1996). This was shown also to be the case in *Xenopus laevis* oocytes by Western blotting analysis using an anti-CBP20 antibody (Figures 1A and 1B, lanes 1–3). When a subsaturating amount (Jarmolowski et al., 1994) of m^{7}G-capped U1ΔSm RNA was injected into the oocyte nucleus together with a control RNA, U6Δss RNA, export of the U1 RNA was almost complete by 6 hr, whereas U6 RNA stayed in the nucleus (Figure 1A, lanes 1–9). Under these conditions, CBP20 remained nuclear (lanes 1–9). When a saturating amount of m^{7}G-capped U1 was injected, export of U1 RNA was still proceeding after 6 hr (Figure 1A, lanes 10–12, upper panel). Under these conditions, the majority of CBP20 had moved to the cytoplasm (lanes 10–12). After 20 hr, when U1 export was almost complete, the steady state location of CBP20 was already returning to its resting, nuclear, state (lanes 13–15). Since CBC is a stable complex, and interaction with a capped RNA requires both CBP80 and CBP20, this result strongly suggests that CBC moves to the cytoplasm together with U snRNA and subsequently recycles back to the nucleus. Control RNAs such as A-capped U1ΔSm RNA or tRNA did not induce CBC export (Figure 1B).

A Bridging Activity Required for U snRNA Export Complex Formation

Although the above results were consistent with the working model, attempts to reconstitute an export complex in vitro with capped RNA and recombinant proteins (CBC, CRM1/Xpo1, and RanGTP) were unsuccessful (data not shown, see below). A protein mobility shift assay was therefore developed to search for additional components. CBP80 was labeled with ^{35}S-methionine by translation in reticulocyte lysate and then mixed with unlabeled recombinant CBP20 to form a labeled CBC probe. Note that labeled CBC is contaminated with rabbit reticulocyte lysate carried over from the translation reaction. When CBC was incubated with recombinant CRM1/Xpo1 and RanGTP, a slow-migrating complex was formed (Figure 2, lanes 1–3). This complex formation was specific, since it did not form when RanGTP was replaced by RanGDP (lane 4) or when Xpo1 was replaced by Importin β (lane 6). Complex formation was inhibited both by leptomycin B (lane 5) and by an NES peptide from PKI (Wen et al., 1995) but not by an inactive NES mutant peptide, P10 (lanes 7–12).

Complex formation appeared to be mediated by a factor(s) in the reticulocyte lysate since it was not observed when bacterially produced components were employed (see below). In addition, adding increasing amounts of unprogrammed lysate enhanced complex formation (lanes 13–16). This suggested the existence of a bridging factor in the reticulocyte lysate.

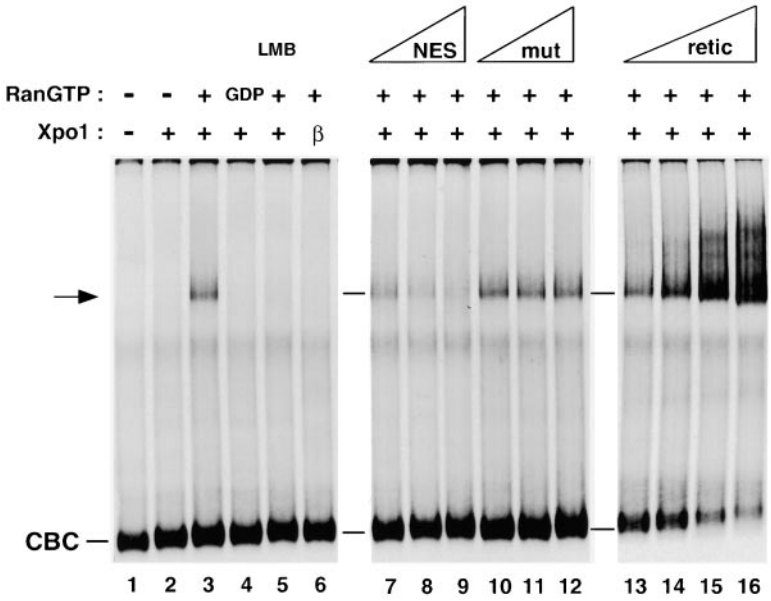


Figure 2. An Activity that Bridges CBC and CRM1/Xpo1

^{35}S-methionine-labeled CBC was incubated for 40 min at 25°C in the presence or absence of 1 μM RanQ69LGTP (RanGTP, +) or RanT24NGDP (GDP), 1 μM CRM1/Xpo1 or importin β (β), 5 μM leptomycin B (LMB), and PKI NES peptide (NES) or its inactive mutant version P10 (mut) at 17 μM (lanes 7 and 10), 50 μM (lanes 8 and 11), or 150 μM (lanes 9 and 12) as indicated above the lanes. Unprogrammed rabbit reticulocyte lysate was added in lanes 13–16: 0.1 μl, lane 13; 0.2 μl, lane 14; 0.4 μl, lane 15; or 0.8 μl, lane 16. The samples were fractionated by native 6% PAGE followed by fluorography. Free CBC and the RanGTP-dependent complex are indicated by "CBC" and an arrow, respectively.

Purification of the Bridging Factor

The bridging activity was purified from rabbit reticulocyte lysate over several chromatographic steps using the CBC mobility shift as an assay (Table 1). The activity peaked sharply in fraction #43 of the last purification step (Figure 3A, upper). Complete complex formation required CRM1/Xpo1, RanGTP, and fraction #43 (Figure 3B, lane 5). If any of these was omitted, the complex was not formed (lanes 1–4 and 6–11). The weak background of complex formation seen in lane 4 is due to the reticulocyte lysate in the labeled CBC. Note that smaller complexes were formed whenever fraction #43 was added to CBC (lanes 3, 7, 9, and 11). As described later, these represent interaction of CBC and the bridging factor.

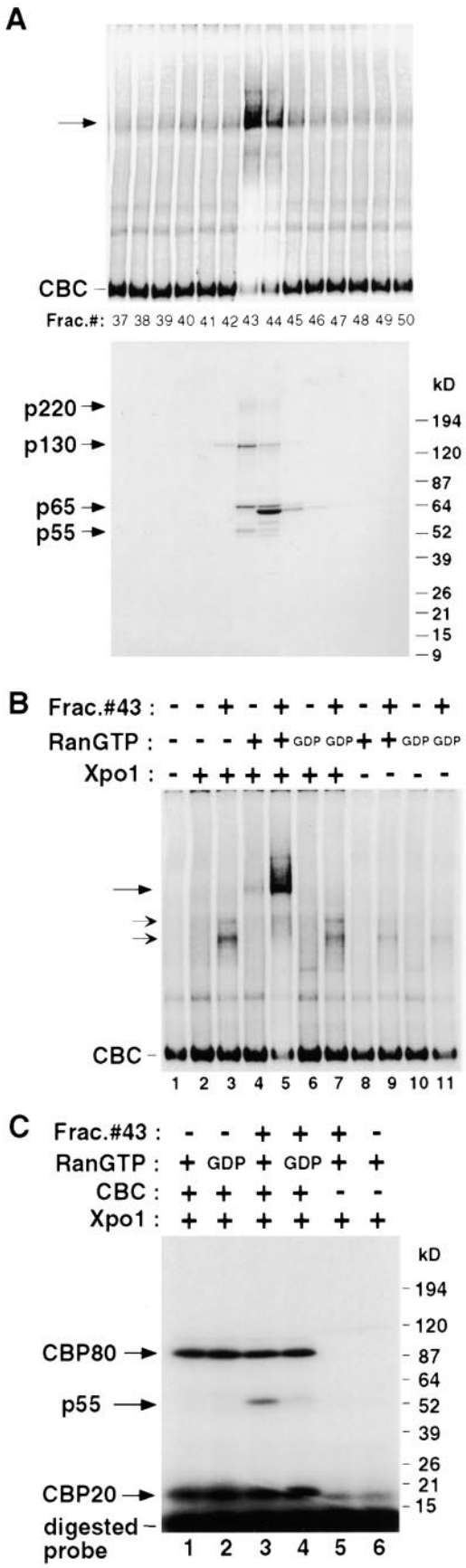
Four major protein bands were visible after SDS-PAGE of fraction #43 followed by Coomassie staining (Figure 3A, lower). The presence of all four proteins correlated well with complex-forming activity (Figure 3A). To examine the presence of these proteins in the CBC complex, a UV cross-linking experiment was performed. Fraction #43 was mixed with recombinant CBC, Xpo1, RanGTP, and a short m^{7}G-capped RNA that had been labeled with ^{32}P and (4-)thioUridine, and the mixture was exposed to UV irradiation. As seen in Figure 3C, not only CBP80 and CBP20, but also a 55 kDa band (p55), were cross-linked to the RNA. The cross-link occurred most efficiently when all the components were present (compare lane 3 with 1 and 2 and 4–6). p55 was also weakly cross-linked when RanGTP was replaced by RanGDP (lane 4). This weaker cross-link was also observed when only fraction #43, CBC, and the RNA probe were present (data not shown), suggestive of subcomplex formation. Since p55 cross-linking was not observed in these conditions in the absence of CBC (lanes 5 and 6), these results confirmed p55 as a candidate bridging factor. p55 does exhibit nonspecific RNA binding (data not shown), but this is masked in Figure 3C by the presence of tRNA competitor.

Peptide sequence information was obtained by mass spectrometric analysis of purified p55. Four peptide sequences matched database entries for a mouse cDNA homologous to rat resiniferatoxin-binding protein, a putative 26 kDa protein (RBP-26). The rat cDNA had been cloned by screening a phage expression library for interaction with resiniferatoxin (Ninkina et al., 1994). The mouse cDNA had a frame shift compared to the rat sequence leading to an extension of the reading frame and resulting in a putative protein of 385 amino acids with a predicted molecular weight of 43 kDa (Figure 5B). This protein has putative homologs in human, *Drosophila melanogaster*, zebrafish, newt, and *Caenorhabditis elegans* but no significant sequence similarity to other proteins in the databases. We obtained the mouse cDNA

Table 1. Purification of p55

Purification Step	Total Protein (mg)	Total Activity (× 10^3 U)	Yield (%)	Specific Activity (U/mg Protein)	Purification Factor (Fold)
Reticulocyte lysate	2312	40.2	100	17.4	1
Ammonium sulfate	13.5	14	35	1040	60
MonoQ (6 M urea)	4.27	11.2	28	2620	151
Heparin sepharose	0.09	7.0	17	76900	4420
MiniQ	0.02	2.4	6	120000	6900

The activity of 0.5 μl reticulocyte lysate was arbitrarily defined as one unit.



(IMAGE: 988750). Although the predicted molecular weight is significantly smaller than 55 kDa, the in vitro translated protein comigrated with purified p55, indicating that the cDNA was full length (data not shown).

p55 Activity Is Modulated by Phosphorylation

To determine whether p55 was part of the CBC export complex, we again utilized mobility shift experiments, but in this case with labeled p55. Various recombinant components and in vitro transcribed m^7G-capped U1 RNA (all unlabeled) were mixed with labeled p55. When p55 produced in reticulocyte lysate was used (Figure 4A, lanes 1–8), various RanGTP-dependent complexes were formed (lanes 1, 3, 5, and 7). In contrast, when p55 produced in *E. coli* S30 was used (Figure 4A, lanes 9–16), RanGTP complexes were not observed. The composition of the complexes was deduced by comparison with experiments where different components were labeled, where antibodies against various components were used to block or shift the complexes, or by comparison with fully recombinant complexes (data not shown, see below).

The lowest mobility complex (lane 7) contains U1 snRNA, CBC, RanGTP, and CRM1/Xpo1 (Figure 4A; data not shown), indicating that p55 is indeed part of the U snRNA export complex. The fact that *E. coli* p55 was incapable of forming this complex (lane 15) indicated either that an additional reticulocyte lysate component was required for complex formation or that *E. coli* p55 required modification to be active. Since we noted that *E. coli* and reticulocyte-produced p55 had different mobilities on SDS-PAGE, we investigated the latter possibility.

We first examined p55 phosphorylation. Purified recombinant p55 from *E. coli* was immobilized on Ni-NTA resin and incubated with fractionated reticulocyte lysate in the presence of ATP. The resin was washed extensively with 8 M Urea buffer and high salt and p55 was eluted by imidazol and renatured. p55 prepared in this way exhibited reduced SDS-PAGE mobility (Figure 4B, compare lanes 1 and 4). Excluding ATP and including the protein kinase inhibitor staurosporine during the incubation prevented the mobility shift (lane 2), and treatment of p55 with alkaline phosphatase partly reversed

Figure 3. The Purified Bridging Factor

(A) (Upper) Fractions from miniQ chromatography were assayed as in Figure 2. Note that all assays contain some reticulocyte lysate from the CBP80 synthesis, and thus all form a low amount of the complete complex (arrow).

(A) (Lower) The same fractions were analyzed by 5%–20% gradient SDS/PAGE followed by Coomassie staining. The four major proteins in fraction #43 and molecular size markers are indicated on the left and right sides, respectively.

(B) Full complex formation requires CBC, CRM1/Xpo1, RanGTP, and the bridging factor. Specificity of the activity of fraction #43 is examined as in Figure 2. Smaller complexes are marked by small arrows (see text).

(C) p55 cross-links to RNA in conditions that favor complex formation. A ^{32}P-labeled m^7G-capped RNA probe was incubated as indicated above the lanes in the presence or absence of fraction #43 (1 μl/reaction) and/or various recombinant components (1 μM), and the samples were UV irradiated, treated with RNase, and separated by SDS-PAGE. The cross-linked bands and molecular size markers are indicated on the left and right, respectively.

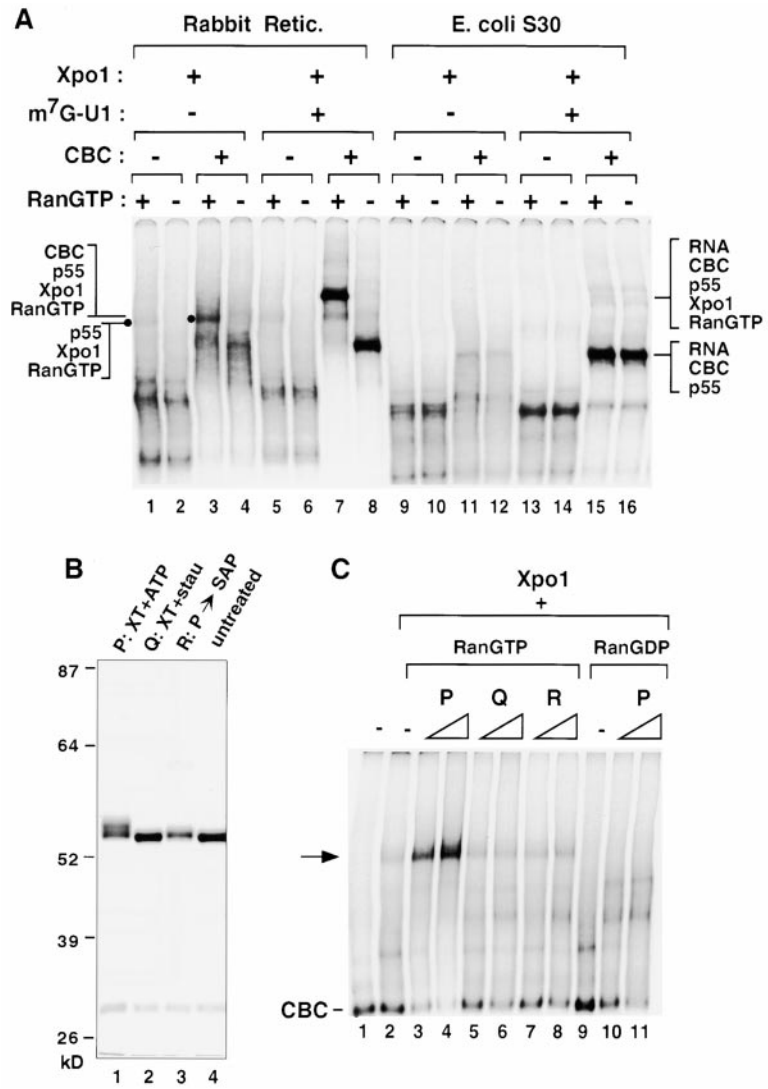


Figure 4. The Activity of p55 Is Regulated by Phosphorylation

(A) p55 produced in *E. coli* does not form a RanGTP and CRM1/Xpo1-containing complex. ^{35}S-methionine-labeled p55 produced by in vitro translation in rabbit reticulocyte lysate (left lanes) or in *E. coli* S30 (right lanes) was incubated in the presence or absence of m^{7}G-capped U1ΔSm RNA (1 μM) and/or other recombinant components (1 μM) as indicated above the lanes, and complex formation was analyzed by native PAGE. The composition of the major complexes is shown on the left and right. Lanes 3 and 4 should be compared with 11 and 12 and lanes 7 and 8 with lanes 15 and 16.

(B) p55 is a phosphoprotein. Purified recombinant p55 from *E. coli* (lane 4) was immobilized on Ni-NTA and incubated with fractionated rabbit reticulocyte lysate in the presence of ATP (1 mM) at 30°C for 30 min. The resin was washed extensively with a buffer containing 8 M Urea and high salt, and p55 was eluted in imidazole buffer containing 8 M Urea. The eluted p55 was renatured by slow dialysis and analyzed by SDS/PAGE and stained with Coomassie (P, lane 1). p55 was treated identically but in the absence of ATP and in the presence of the protein kinase inhibitor staurosporin (10 μM) (Q, lane 2). p55 from lane 1 was also treated with shrimp alkaline phosphatase (R, lane 3).

(C) p55 phosphorylation is necessary for complex formation. The three p55 preparations from (B) (P, Q, and R, approximately 30 or 100 ng/lane as indicated) were analyzed by CBC band shift as in Figure 1 (lanes 1–8). The complete complex is not seen in the presence of RanGDP (lanes 9–11).

the mobility shift (lane 3), indicating that p55 was phosphorylated. When the three p55 preparations were tested for complex formation, only the phosphorylated p55 was active (Figure 4C, lanes 3 and 4; note that lane 2 represents the background p55 activity present in reticulocyte lysate). p55 made in *E. coli* is competent to form p55-CBC-RNA complexes (Figure 4A, lanes 15 and 16) but not to interact with RanGTP and Xpo1, indicating that this latter interaction is dependent on the phosphorylation state of p55.

Assembly of U snRNA Export Complexes In Vitro

Although demonstrating a role for p55 phosphorylation, the above experiments do not rule out a requirement for additional reticulocyte components. To clarify this point, the phosphorylated recombinant p55 (Figure 4B, lane 1) was further purified by mono Q chromatography in the presence of 6 M Urea and tested for activity in complex formation. CBC made in *E. coli* binds to the capped RNA probe (Figure 5A, lane 9). Xpo1 and RanGTP bind to the p55-CBC-RNA precomplex to form a higher order complex (lane 15) whose formation is strictly dependent on RanGTP (lanes 14, 16). We refer to this complex as the U snRNA export complex. If unphosphorylated p55 is used in the same experiment, no export complex is formed (data not shown). Moreover, p55 can also be phosphorylated and activated by recombinant Casein Kinase II from *E. coli* (lanes 17–21), demonstrating that all the components required for export complex formation have been identified.

p55 Is Required for U snRNA Export

p55 contains several possible sequences that resemble known leucine-rich NESs. One of these sequences, underlined in Figure 5B, was mutated to generate p55ΔNES. When tested in the mobility shift assay, this mutant entered the p55-CBC-RNA complex but not the export complex (Figure 5C, compare lanes 1–4 with 5–8). This suggested that the ΔNES mutant might function in a dominant-negative way by binding to CBC-RNA and preventing Xpo1 interaction.

This possibility was tested by *Xenopus* oocyte microinjection. When a mixture of ^{32}P-labeled RNAs was injected into the nucleus, either alone or together with

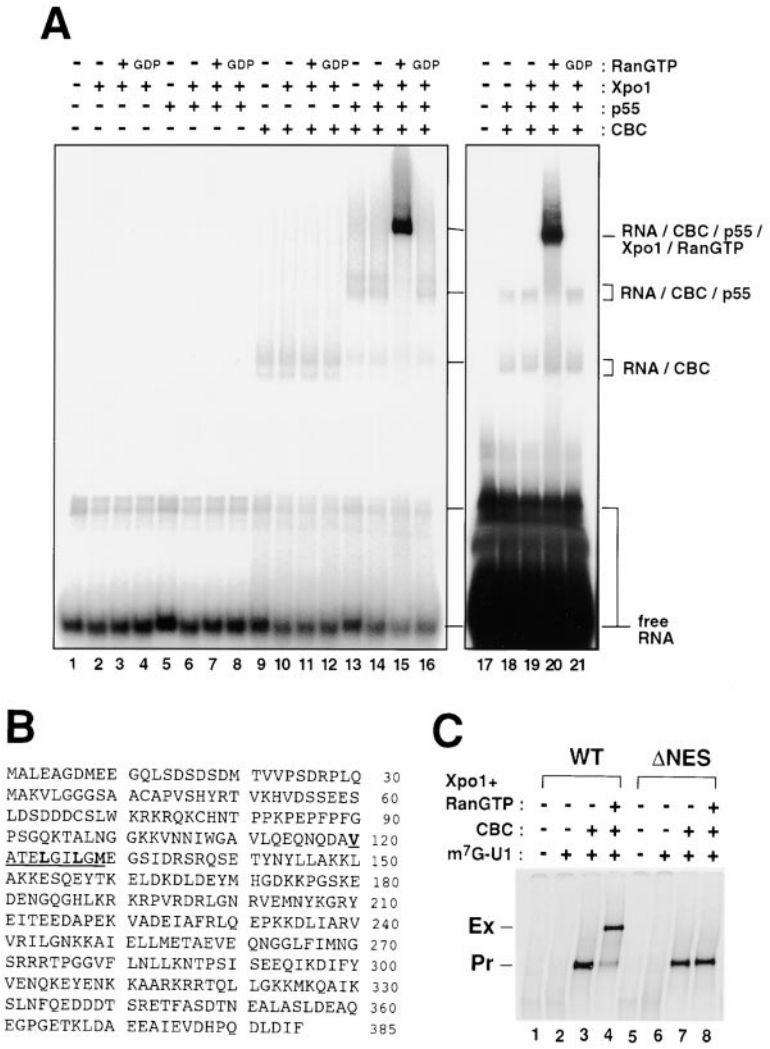


Figure 5. U snRNA Export Complex Assembly In Vitro

(A) A ^{32}P-labeled capped RNA probe was incubated for 40 min at 25°C in the presence or absence of 1 μM RanQ69LGTP (RanGTP) or RanT24NGDP (GDP), 1 μM CRM1/Xpo1, 1 μM p55 that had been phosphorylated either by fractionated rabbit reticulocyte lysate (lanes 5–8 and 13–16) or by recombinant Casein Kinase II (lanes 17–21), and 1 μM CBC as indicated above the lanes. The samples were fractionated by native 6% PAGE followed by autoradiography. Free RNA probe and major complexes are indicated on the right.

(B) Amino acid sequence of mouse p55. The major nuclear export signal is underlined, and the hydrophobic residues corresponding to the consensus sequence are in bold. In the p55 ΔNES mutant, both leucine residues and the methionine residue were mutated to alanine.

(C) ^{35}S-methionine-labeled wild-type p55 (lanes 1–4) or p55 ΔNES (lanes 5–8) produced in reticulocyte lysate were tested for activity in U snRNA export complex assembly together with the indicated components. Export complex (Ex) and precomplex (Pr) are labeled.

recombinant p55, export of both U1 and U5 was stimulated by p55 (Figure 6A, lanes 1–4, 7, and 8). Stimulation was by 23 ± 4.6% 1 hr after injection (data from three experiments) and by 24 ± 3.1% after 2 hr (data from two experiments). In contrast, neither tRNA nor DHFR mRNA export was affected (Figure 6A, lanes 1–4, 7, and 8). In contrast, when recombinant p55ΔNES was injected with the same mixture of labeled RNAs, U snRNA export was reduced whereas DHFR and tRNA were not affected (Figure 6A, lanes 5 and 6). U snRNA export was inhibited by 23.5 ± 6.9% after 1 hr (three experiments) and 37.5 ± 7.2% after 2 hr (two experiments). Neither protein inhibited the export of Rev (Figure 6B), although for reasons that are not understood, coinjection with p55 resulted in stabilization of the Rev protein. The lack of effect on Rev export indicated that p55 is not generally involved in CRM1/Xpo1 function but rather plays a specific role in U snRNA export. Further evidence for this conclusion came from analyzing the effect on RNA export of microinjection of affinity-purified anti-p55 antibody (Figure 6C). Anti-p55 antibodies inhibited export of U1 and U5 snRNAs but not of tRNA (Figure 6C, compare lanes 5 and 6 with lanes 1–4, 7, and 8).

To determine whether p55 accompanied the U snRNA export complex into the cytoplasm, the migration of p55 between the nucleus and cytoplasm was studied. When injected into the cytoplasm of *Xenopus* oocytes, p55 moved into the nucleus (Figure 6D, lanes 1–4). When injected into the nucleus, p55 did not apparently leave (lanes 5–8). Xpo1/RanGTP interacts weakly with p55 alone but strongly with the p55-CBC-RNA complex (Figure 4A, lanes 1 and 7). When a saturating amount of m^{7}G-capped U1 RNA was coinjected, p55 export was induced (Figure 6D, lanes 9 and 10), whereas A-capped U1 RNA did not induce p55 export (lanes 11 and 12). p55 therefore behaves identically to CBC in this respect (Figure 1). These results strongly suggest that U1 RNA, CBC, p55, Xpo1, and RanGTP move to the cytoplasm as a single entity and that p55 only interacts efficiently with Xpo1 in vivo in the presence of U1 snRNA.

Compartment-Specific Phosphorylation of p55

Phosphorylated p55 is required for U snRNA export complex assembly. To determine whether p55 phosphorylation in vivo is constitutive, we examined the fate of microinjected p55. Either unphosphorylated p55 made in *E. coli* S30 or phosphorylated p55 made in reticulocyte lysate (Figure 7A, lanes 1, 2, 13, and 14)

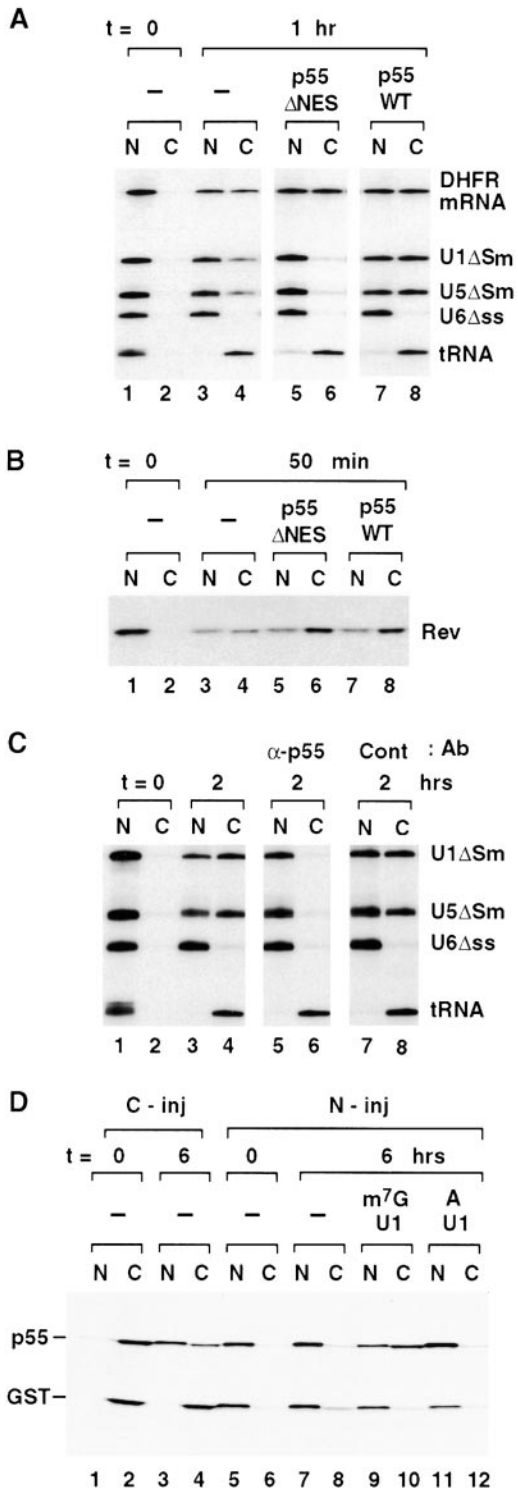


Figure 6. p55 Is Essential for U snRNA Export

(A) A mixture of ^{32}P-labeled RNAs, containing DHFR mRNA, U1ΔSm, U5ΔSm, U6Δss, and human initiator methionyl tRNA, was injected into the nucleus of *Xenopus* oocytes either alone (lanes 1–4) or together with 330 fmol/oocyte of recombinant p55ΔNES (lanes 5 and 6) or wild-type p55 (lanes 7 and 8). RNA was extracted immediately (lanes 1 and 2) or 1 hr (lanes 3–8) after injection and analyzed by denaturing PAGE.

(B) ^{35}S-labeled Rev protein was injected into the nucleus alone (lanes 1–4) or with 330 fmol/oocyte of recombinant p55ΔNES (lanes 5 and 6) or wild-type p55 (lanes 7 and 8), and protein was extracted immediately (lanes 1 and 2) or 50 min (lanes 3–8) after injection and analyzed.

(C) A mixture of ^{32}P-labeled RNAs, containing U1ΔSm, U5ΔSm, U6Δss, and human initiator methionyl tRNA, was injected into the nucleus alone (lanes 1–4) with affinity-purified anti-p55 antibody (lanes 5 and 6) or control IgG (lanes 7 and 8) and analyzed as in (A).

(D) m^{7}G-capped U1 snRNA induces p55 shuttling. A mixture of ^{35}S-labeled p55 and GST was injected into the cytoplasm (C-inj, lanes 1–4) or the nucleus (N-inj, lanes 5–12) of *Xenopus* oocytes either alone or together with RNA as indicated above the lanes. Protein from cytoplasmic (C) or nuclear (N) fractions was collected immediately (lanes 1, 2, 5, and 6) or 6 hr (lanes 3 and 4 and 7–12) after injection and analyzed by SDS-PAGE followed by fluorography. m^{7}G U1, m^{7}G-capped U1ΔSm RNA; A U1, A-capped U1ΔSm RNA (both 500 fmol/oocyte).

were used. On injection into the cytoplasm, *E. coli* p55 was initially unaffected (lanes 15 and 16). The fraction of p55 that entered the nucleus was however phosphorylated (lanes 17 and 18). Reticulocyte lysate p55, even when reisolated immediately after cytoplasmic injection, was partially dephosphorylated (lane 4), and prolonged incubation did not lead to further changes in the steady state level of phosphorylation of the cytoplasmic fraction of p55 (lane 6). Further experiments established that p55 is subject to rapid cycles of dephosphorylation and rephosphorylation in the cytoplasm (data not shown). In contrast, the fraction of p55 that entered the nucleus was rephosphorylated to a level indistinguishable from the injected material (lane 5). These results suggested that p55 is subject to net dephosphorylation in the cytoplasm and net phosphorylation in the nucleus.

This conclusion was examined by nuclear microinjection of the two forms of p55. Phosphorylated p55 remained phosphorylated after nuclear injection (Figure 7A, lanes 9–12), whereas unphosphorylated p55 underwent phosphorylation (lanes 21–24). Inducing p55 export from the nucleus by microinjection of saturating amounts of U1 snRNA resulted, as before, in partial redistribution of p55 to the cytoplasm (lanes 7, 8, 19, and 20), and this redistribution was accompanied by p55 dephosphorylation resembling that seen on direct microinjection of phosphorylated p55 into the cytoplasm (lanes 4, 6, 8, and 20). Endogenous p55 was shown by Western blotting to undergo a similar mobility shift on entering the cytoplasm (data not shown).

Dephosphorylation of p55 Results in Export Complex Disassembly

To examine the functional significance of cytoplasmic p55 dephosphorylation, its effect on U snRNA export complex disassembly was tested. Export complex disassembly is in general thought to involve RanBP1/BP2, which compete with export receptors for RanGTP binding, and RanGAP, which, together with RanBP1/2, induces GTP hydrolysis by Ran, thus rendering complex disassembly irreversible.

Export complexes were formed from RNA, CBC, p55, Xpo1, and RanQ69LGTP (Figure 7B, lane 2) along with precomplexes that lacked Ran and Xpo1 (lane 1). Since addition of RanBP1 and RanGAP to "recombinant" export complexes induced their disassembly, the hydrolysis-deficient RanQ69L mutant (Klebe et al., 1995) was

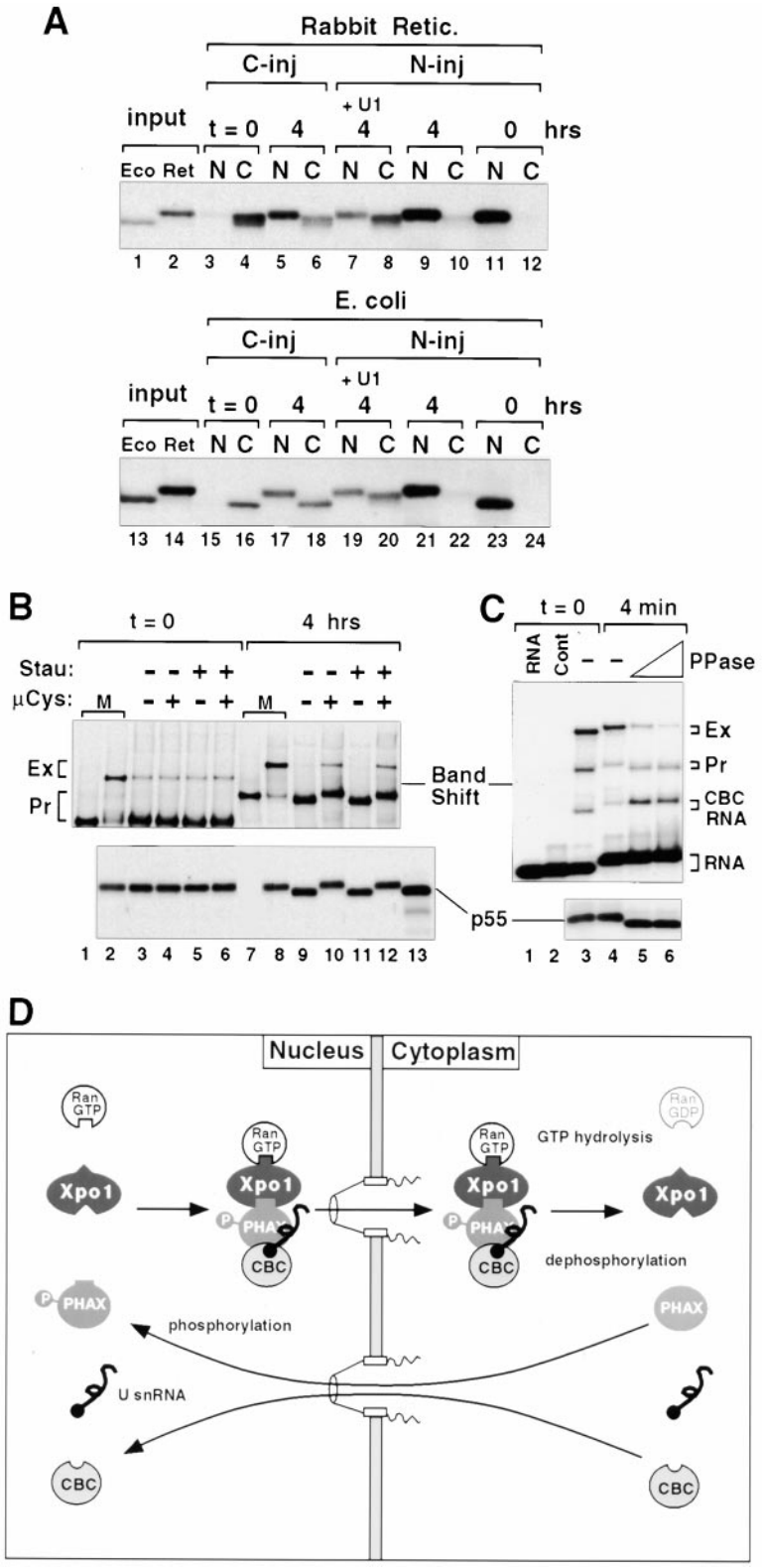


Figure 7. Phosphorylation/Dephosphorylation Cycle of p55/PHAX

(A) ^{35}S-labeled wild-type p55 translated either in rabbit reticulocyte lysate (lanes 2–12 and 14) or in *E. coli* S30 (lanes 1, 13, and 15–24), was injected into either the cytoplasm (lanes 3–6 and 15–18) or the nucleus (lanes 7–12 and 19–24) of *Xenopus* oocytes. For lanes 7, 8, 19, and 20, m^{7}G-capped U1ΔSm RNA (500 fmol/oocyte) was coinjected. Protein was recovered from cytoplasmic (C) or nuclear (N) fractions immediately (lanes 3, 4, 11, 12, 15, 16, 23, and 24) or 4 hr (lanes 5–10 and 17–22) after injection and analyzed by SDS/PAGE.

(B) Export complex (Ex) and precomplex (Pr) were assembled using ^{35}S-labeled p55 from rabbit reticulocyte lysate, m^{7}G-capped U1ΔSm RNA, CBC, Xpo1, and RanQ69LGTP. The mixture was subsequently mixed with an equal volume of *Xenopus* oocyte cytoplasmic extract in the absence or presence of staurosporine (Stau, 10 mM) and/or microcystin-LR (μCys, 2 mM), and incubated at 25°C for 0 (lanes 3–6) or 4 hr (lanes 9–12). Disassembly of complexes was monitored as in Figure 4A (upper) and dephosphorylation of p55 by SDS PAGE (lower). M, markers for Ex and Pr at each time point; note that the native gel (upper) was run continuously and lanes 7–12 loaded 4 hr after lanes 1–6. The markers for each group of lanes therefore run differently. Lane 13, p55 from *E. coli* S30 (unphosphorylated).

(C) Recombinant export complexes were assembled using a ^{32}P-labeled U1ΔSm RNA probe. Export complex (Ex), precomplex (Pr) and CBC/RNA complexes are labeled. Lane 1, free RNA probe. Lane 2, complexes were formed in the presence of 400 μM cap dinucleotide, which competes with the labeled RNA for complex formation. Lanes 3–6 (upper) complexes were preformed, then 400 μM cap dinucleotide was added. Lane 3 was loaded immediately onto a running native gel while lanes 4–6 were loaded 4 min later after control incubation (lane 4) or incubation with 100 Units or 200 Units of recombinant λ protein phosphatase (lanes 5 and 6). In parallel, proteins were extracted from an aliquot of the same incubations and separated by denaturing SDS-PAGE. p55 was detected by Western blot (lower panel).

(D) Model of U snRNA export. See text.

used to inhibit disassembly via the combined action of RanBP1/2 and RanGAP on addition of these complexes to *Xenopus* oocyte cytosolic extracts. The complexes were incubated in cytosol for various time periods either alone or together with the phosphatase inhibitor microcystin, the kinase inhibitor staurosporine, or both inhibitors.

If complexes were examined by native gel electropho-

resis immediately after addition to cytosolic extract, both export complexes and precomplexes were observed, and the presence of the inhibitors had no effect (Figure 7B, lanes 3–6). Denaturing SDS-PAGE revealed that p55 was phosphorylated in these conditions (lower panel, lanes 2–6). Upon incubation in the absence of inhibitors or, more rapidly, in the presence of kinase inhibitor, the export complex was disassembled (lanes 9 and 11; data not shown). Under these conditions, p55 was progressively dephosphorylated (lower panel). The addition of microcystin blocked both p55 dephosphorylation and export complex disassembly (lanes 10 and 12). The half-life of the export complex in the absence of cytosol was determined to be roughly 5 hr by dilution of preformed complex to a concentration at which complex formation could not occur and monitoring complex disassembly (data not shown). Neither p55 dephosphorylation nor export complex disassembly was observed in nuclear extract (data not shown).

Although these experiments strongly suggested that p55 dephosphorylation results in export complex disassembly, the presence of extract left open the possibility that p55 was not the sole phosphatase target involved. To clarify this point, export complexes were assembled from recombinant components. A large excess of cap dinucleotide was added either together with the labeled RNA probe (Figure 7C, lane 2) or after complex formation (lanes 3–6) to rule out detection of complexes that might reassemble from disassembled components. In these conditions, export complexes were stable (compare lanes 3 and 4). In contrast, when recombinant λ protein phosphatase was added, p55 was dephosphorylated and export complexes disassembled (Figure 7C, lanes 4–6, upper and lower panels) resulting in an increase in the amount of CBC/RNA and p55/CBC/RNA complexes. Thus, we can conclude that dephosphorylation of p55 in the U snRNA complex leads to its disassembly, even in the absence of GTP hydrolysis by Ran.

Discussion

A novel component required for CRM1/Xpo1-mediated U snRNA export from the nucleus is described. The protein has an apparent molecular weight of 55 kDa and is a phosphoprotein. In vitro experiments established that this protein bridges the previously identified components required for U snRNA export, CBC-bound RNA on the one hand and RanGTP together with the export receptor CRM1/Xpo1 on the other (Figure 7D). To perform this function, p55 must be phosphorylated, so we propose to name the protein PHAX (phosphorylated adaptor for RNA export).

All the components of the export complex shown in Figure 7D are required for complex formation and for U snRNA export, namely an m^7G-capped RNA (Hamm and Mattaj, 1990; Jarmolowski et al., 1994), CBC (Izaurralde et al., 1995a), CRM1/Xpo1 and RanGTP (Fornerod et al., 1997; Izaurralde et al., 1997) and phosphorylated PHAX (this paper). Note however that we cannot exclude the possibility that there are additional components associated with the export complex in vivo. We have previously provided evidence that importin α, through a stable interaction with the NLS of CBP80, may be part of the complex (Görlich et al., 1996). Importin α is, however, unlikely to be an essential component since a mutant of CBP80 that lacks the NLS and no longer binds importin α is capable of participating in U snRNA export (unpublished data).

Mechanisms to Reduce Competition between PHAX and Other Export Substrates

An interesting aspect of U snRNA complex formation is the prevalence of cooperative binding steps. Previous studies of export complex formation have established that RanGTP and cargo molecules interact cooperatively with several export receptors (Fornerod et al., 1997; Kutay et al., 1997, 1998; Arts et al., 1998; Kaffman et al., 1998). The existence of both CBC and PHAX as adaptors between the export receptor and the U snRNA cargo allows for additional cooperative interactions. Neither CBP80 nor CBP20 alone can interact with capped RNA; they must first heterodimerize (Izaurralde et al., 1994; 1995a). PHAX binding to CBC is greatly increased by the presence of capped RNA (Figure 4A, compare lanes 11 and 12 with 15 and 16), perhaps because PHAX itself binds nonspecifically to RNA (unpublished data). Phosphorylated PHAX can bind to RanGTP/Xpo1 (Figure 4, lane 1) but does so more strongly in the presence of CBC and even more in the presence of both CBC and RNA (Figure 4A, lanes 3 and 7). Thus, every step in U snRNA export complex formation involves some degree of cooperativity. The consequence of this in vivo is that neither CBC nor p55 detectably leaves the nucleus in the absence of U snRNA, but both do so in its presence (Figures 1 and 6). Note that since both components are actively reimported into the nucleus, this indicates an increase in their export rate in the presence of the RNA rather than an absolute lack of export activity in its absence. Nevertheless, the cooperative assembly of the complex results in a reduction of the rate of export of PHAX and CBC in the absence of RNA substrates, thus preventing futile shuttling of these components and reducing competition between PHAX and other substrates of CRM1/Xpo1 for export.

Phosphorylation of PHAX might also function to reduce competition between U snRNA export and NES protein export. CRM1/Xpo1 and Importin β currently seem unusual among nuclear transport receptors in the diversity of their substrates. Importin β imports a large variety of NLS-containing proteins in combination with a family of Importin α adaptor proteins (Kohler et al., 1999 and references therein). It also mediates U snRNP import via the specialized adaptor Snurportin-1 (Palacios et al., 1997; Huber et al., 1998). Importin β functions without an adaptor in the import of some ribosomal proteins (Jäkel and Görlich, 1998), the HIV-1 Rev and Tat proteins (Truant and Cullen, 1999) and probably Cyclin B1 (Takizawa et al., 1999). Finally, Importin β, in concert with a second import receptor, Importin 7, is involved in histone H1 import (Jäkel et al., 1999).

CRM1/Xpo1 exports a corresponding variety of export substrates. Apart from its direct interaction with and export of many NES-containing proteins, CRM1/Xpo1 exports Snurportin-1 via a different sort of direct binding interaction (Paraskeva et al., 1999). It exports U snRNAs in conjunction with the adaptors discussed above. Finally, CRM1/Xpo1 may have a role in mRNA export,

particularly in yeast, although this is controversial (Fischer et al., 1995; Fornerod et al., 1997; Pasquinelli et al., 1997; Stade et al., 1997; Wolff et al., 1997; Bogerd et al., 1998; Neville and Rosbash, 1999).

It is conceivable that the plethora of transport substrates carried by these receptors might lead to situations where either Importin β or CRM1/Xpo1 become limiting. In such a situation, a mechanism to specifically prevent competition could be useful. Phosphorylation of PHAX could provide such a mechanism since preventing PHAX phosphorylation would specifically preclude U snRNA export complex formation while allowing the export of other CRM1/Xpo1 cargoes. We are currently investigating the possibility that PHAX phosphorylation is regulated in this way.

PHAX Dephosphorylation and Export

Since PHAX must be phosphorylated to be functional in export, it was unexpected to find that it is subject to dephosphorylation in the cytoplasm. The likely significance of cytoplasmic dephosphorylation was illustrated by demonstrating that U snRNA export complexes disassemble on dephosphorylation of PHAX. In general, GTP hydrolysis by Ran, mediated by RanBP1/2 and RanGAP, is believed to trigger export complex dissociation. Conversely, RanGTP binding to import receptors on their entry into the nucleus can trigger import complex disassembly (see the Introduction). The asymmetric distribution of RanGTP across the nuclear envelope is therefore a critical aspect in conferring directionality on transport events.

In some cases, however, the RanGTP/GDP switch is insufficient to allow efficient import or export complex disassembly. One example in the case of import involves the yeast RNA-binding protein Npl3, whose import receptor is Mtr10. In this case, efficient dissociation of Npl3 from Mtr10 requires not only RanGTP, but also RNA (Senger et al., 1998). Similarly, efficient dissociation of Importin β–RanGTP complexes, the form in which Importin β is thought to be recycled to the cytoplasm (Izaurralde et al., 1997), requires not only RanBP1 and RanGAP but also Importin α (Bischoff and Görlich, 1997; Floer et al., 1997). Thus, binding partners can influence transport complex disassembly reactions.

In the case of U snRNA export complexes, in vitro experiments show that disassembly can be triggered by RanBP1 and RanGAP or even by an excess of RanBP1 alone (unpublished data). However, when the nonhydrolyzing RanQ69L mutant was used to form a stable export complex, it was possible to demonstrate that PHAX dephosphorylation can also cause export complex disassembly. Thus, aside from the Ran switch, the phosphorylation/dephosphorylation cycle involving PHAX can also contribute to the assembly and disassembly of U snRNA export complexes and thus to the directionality of U snRNA export. Although phosphorylation and dephosphorylation of substrate molecules has previously been shown to affect their nucleocytoplasmic transport (Kaffman and O'Shea, 1999), the example of PHAX is different in two respects. In this case, the mechanism applies to a transport mediator rather than to a substrate. Further, in the PHAX example, the phosphorylation/dephosphorylation cycle is an integral part of a constitutive nucleocytoplasmic transport cycle rather than serving primarily as a means of regulation of specific transport events. Further study will be required to assess the relative importance of GTP hydrolysis by Ran and PHAX dephosphorylation in export complex disassembly. Presumably both are needed for optimal efficiency. In addition, while it is known that interaction between CBC and the Importin α/β heterodimer can release capped RNA from CBC (Görlich et al., 1996), further study of the fate of the PHAX-CBC-RNA complex will be required to establish if and how PHAX is removed from this complex before reentry into the nucleus.

U snRNP Assembly and Evolution

In metazoa, U snRNAs are transcribed in the nucleus and exported to the cytoplasm. There, they bind to the core U snRNP proteins, undergo cap hypermethylation, and are then reimported to the nucleus (see the Introduction). Export involves the conserved components CBC, CRM1/Xpo1, and RanGTP and, as shown here, PHAX. PHAX homologs are identifiable in the databases in *D. melanogaster*, *C. elegans*, zebrafish, newts, humans, mice, and rats, but no obvious homolog is present in the complete *S. cerevisiae* genome. U snRNP import in vertebrates involves Importin β (Palacios et al., 1997) and a U snRNP-specific import mediator, Snurportin-1, that binds to the mature trimethylated U snRNA cap structure (Huber et al., 1998). As in the case of PHAX, there is no obvious Snurportin-1 homolog in the *S. cerevisiae* genome. It therefore seems probable that U snRNP assembly may be a nuclear, rather than a cytoplasmic, event in *S. cerevisiae*. It will be interesting to determine at what point in evolution cytoplasmic U snRNP assembly was gained and/or lost.

Experimental Procedures

Purification of p55

All the procedures were performed at 4°C. Rabbit reticulocyte lysate (10 ml, Promega) that had been treated with micrococcal nuclease was fractionated by ammonium sulfate precipitation, and the pellet from 20 to 36% saturation was collected. The pellet was washed with 40% ammonium sulfate solution to remove most of the hemoglobin. The pellet was dissolved in buffer A (20 mM Tris-HCl, [pH 7.5], 0.1 mM EDTA, 6 M Urea, 5% glycerol, 1 mM DTT, and 0.2 mM PMSF) and applied to a mono Q (FPLC, Pharmacia) column equilibrated with the same buffer. The bound material was step-eluted with 0.2, 0.5, and 1 M NaCl in buffer A. The active 0.5 M eluate was dialyzed against buffer B (buffer A without Urea) containing 0.1 M NaCl and 22% glycerol and applied to a Heparin-Sepharose column (1 ml, Pharmacia) equilibrated with buffer B containing 0.1 M NaCl. The bound protein was step-eluted with 0.3, 0.5, and 1 M NaCl in buffer B. The active 0.5 M eluate was dialyzed against buffer B containing 0.1 M NaCl and applied to a mini Q column (SMART system, Pharmacia) equilibrated with the same buffer. The bound protein was eluted with a linear gradient of 0.1 to 0.5 M NaCl in buffer B. The activity was eluted around 270 mM NaCl.

Protein Sequencing

The protein was in-gel digested with trypsin in a buffer containing 33% ^{18}O water to partially label the C termini of the peptides. The peptides were extracted and desalted on a 100 nl Poros T2 column assembled in a pulled glass capillary (Shevchenko et al., 1996). The total peptide mixture was eluted in 2 μl 60% methanol and 5% formic acid into a gold-coated nanoelectrospray glass capillary and investigated on an API III triple quadrupole mass spectrometer (PE-Sciex, Ontario, Canada) (Wilm and Mann, 1996). For every peptide,

two tandem MS spectra were acquired, the first selecting the entire $^{16}O/^{18}O$ isotopic envelope of the peptide for fragmentation (spectrum I) and a second selecting only the ^{18}O isotope (spectrum II). Sequences were determined by identifying the C-terminal y-ions throughout the fragment spectrum via their $^{16}O/^{18}O$ isotopic pattern and their different isotopic representation in spectrum I and II. The EMBL DNA Sequence database accession number for the PHAX sequence is AJ276504.

Recombinant Proteins

Recombinant CRM1 (Xpo1), RanQ69L, RanT24N, and CBP20 were expressed and purified as described (Izaurralde et al., 1995a; Askjaer et al., 1999). CBP80 was expressed from pQE30 vector (Qiagen) and purified by its his-tag. Recombinant CBC was formed by mixing CBP80 and CBP20 at 4°C overnight. Full-length mouse p55 cDNA was amplified by PCR from a mouse EST clone (IMAGE: 988750) and cloned into the pQE30 vector. Recombinant p55 was expressed in *E. coli* and purified according to the standard protocol from Qiagen. For in vitro translation, the p55 cDNA was cloned into pT77-TT vector and translated either in the TNT T7 quick transcription/translation system or in *E. coli* S30 extract (Promega). In vitro translated Rev protein was prepared as described by Fornerod et al. (1997).

In Vitro Phosphorylation of p55

Recombinant p55 was immobilized on Ni-NTA agarose and incubated with a 0%–66% ammonium sulfate fraction from a rabbit reticulocyte lysate in PBSGM (PBS containing 8.7% glycerol and 5 mM $MgCl_2$) containing 10 mM β-mercaptoethanol, 0.01% NP40, 15 mM imidazole, 0.4 mM PMSF, and 1 mM ATP at 30°C for 1 hr on a rotary shaker. The resin was washed extensively with the same buffer containing 8 M Urea and 0.5 M NaCl, and the bound protein was eluted with the same buffer containing 0.5 M imidazole. The eluted p55 was dialyzed against buffer A (see above) and further purified by elution from a monoQ column with a linear gradient of 0 to 0.5 M NaCl in buffer A. Phosphorylated active p55 fractions were pooled and dialyzed against buffer B (see above) containing 0.1 M NaCl and 22% glycerol and then the same buffer containing 8.7% glycerol. In some cases, purified recombinant Casein Kinase II from *E. coli* (5.3 units/μg p55) (Boehringer Mannheim) was used to phosphorylate recombinant p55 instead of the reticulocyte lysate fraction.

Band-Shift Assay

^{35}S-labeled protein probe or ^{32}P-labeled RNA probe was mixed with the other recombinant components (1 μM) in TB (Transport Buffer; 40 mM Hepes-KOH [pH 7.3], 110 mM KOAc, 6 mM Mg(OAc)2, and 250 mM Sucrose) containing 0.8 mg/ml *E. coli* tRNA (Boerhinger Mannheim) in a volume of 5 μl and incubated for 40 min at 25°C. The mixture was fractionated 6% native polyacrylamide gel in 0.5× TBE run at 8.5 V/cm. The gel was fixed in 40% methanol, 20% acetic acid at room temperature for 30 min. The gel was dried immediately in the case of the ^{32}P-labeled probe or after treatment with Amplify reagent (Amersham) in the case of ^{35}S-labeled probe and autoradiographed. For the experiment in Figure 7C, 1 mg/ml ApppG-capped U1ΔSmRNA, 1 mM $MnCl_2$, and 1 mM DTT were also added, and incubation was at 30°C. ^{35}S-labeled CBC was prepared by incubating CBP80 NLS-2 (Izaurralde et al., 1995b) translated in vitro in rabbit reticulocyte lysate with recombinant CBP20 (40 ng/μl) at 4°C overnight.

UV Cross-Linking

A short m^7G-capped RNA probe (25 nt long) was synthesized from two annealed DNA oligos, 5′-TAATACGACTCACTATA-3′ and 5′-CCT TCGCGCCCCTCTACACAGACCCTATAGTGAGTCGTATTA-3′, with T7 RNA polymerase in the presence of ^{32}P-GTP and 4-thio-UTP. The probe was mixed with the purified p55 fraction (1 μl) and/or various recombinant components (1 μM) in TB plus 0.8 mg/ml *E. coli* tRNA in a total volume of 5 μl. UV cross-linking was performed as described previously (Sontheimer, 1994).

Oocyte Injection

Xenopus oocyte microinjection was performed as previously described (Jarmolowski et al., 1994).

Preparation of Cytoplasmic Extract of *Xenopus* Oocytes

A cytoplasmic fraction was prepared from 66 oocytes in 250 μl TB containing protease inhibitor cocktail (Boehringer Mannheim), 1 mM DTT, 0.4 mM PMSF, 1 μg/μl *E. coli* tRNA, and 2 U/μl RNasin, and homogenized by repeated pipeting. The homogenate was spun in a microfuge at 4°C for 20 min, and the supernatant was recovered and frozen in aliquotes.

Acknowledgments

We thank Minoru Yoshida for leptomycin B and Angel Nebreda for microcystin and staurosporine. The members of our laboratories, particularly Maarten Fornerod, as well as Elisa Izaurralde and Bertrand Séraphin, are thanked for criticism of the manuscript and discussion of the project.

Received December 6, 1999; revised February 2, 2000.

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GenBank Accession Number

The EMBL DNA Sequence Database accession number for the PHAX sequence is AJ276504.