RNA (2001), *7*:351–360. Cambridge University Press. Printed in the USA.
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The evolutionarily conserved region of the U snRNA export mediator PHAX is a novel RNA-binding domain that is essential for U snRNA export

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ABSTRACT

In metazoa, a subset of spliceosomal U snRNAs are exported from the nucleus after transcription. This export occurs in a large complex containing a U snRNA, the nuclear cap binding complex (CBC), the leucine-rich nuclear export signal receptor CRM1/Xpo1, RanGTP, and the recently identified phosphoprotein PHAX (phosphorylated adaptor for RNA export). Previous results indicated that PHAX made direct contact with RNA, CBC, and Xpo1 in the U snRNA export complex. We have now performed a systematic characterization of the functional domains of PHAX. The most evolutionarily conserved region of PHAX is shown to be a novel RNA-binding domain that is essential for U snRNA export. In addition, PHAX contains two major nuclear localization signals (NLSs) that are required for its recycling to the nucleus after export. The interaction domain of PHAX with CBC is at least partly distinct from the RNA-binding domain and the NLSs. Thus, the different interaction domains of PHAX allow it to act as a scaffold for the assembly of U snRNA export complexes.

Keywords: cap; nuclear transport; phosphorylation; RNA export

INTRODUCTION

The existence of the nucleus in eukaryotic cells necessitates efficient mechanisms to exchange macromolecules between the nucleus and the cytoplasm. Many proteins and RNAs are transported across the nuclear envelope through the nuclear pore complexes (NPCs). 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 simple but robust models of import and export processes (reviewed in Cole & Hammel, 1998; Dahlberg & Lund, 1998; Izaurralde & Adam, 1998; Mattaj & Englmeier, 1998; Ohno et al., 1998; Görlich & Kutay, 1999; Hood & Silver, 1999). In these models, an import receptor binds to a cargo and carries it into the nucleus. The receptor then binds to RanGTP (the nuclear form of Ran), resulting in release of the cargo. In the case of export, an export receptor binds to a cargo in the nucleus cooperatively with RanGTP, forming a trimeric export complex. This complex moves to the cytoplasm and disassembles due to GTP hydrolysis triggered by Ran's GTPase activating factors in the cytoplasm.

Export of RNA cargoes usually requires more complex mechanisms. One example of this is U snRNA export. Major spliceosomal U snRNAs such as U1, U2, and U5 are transcribed in the nucleus by RNA polymerase II and acquire a monomethylated cap structure. In metazoa, these U snRNAs are initially exported from the nucleus. They bind in the cytoplasm to a group of proteins termed the Sm proteins. Subsequently the cap structure is hypermethylated, and the RNA–protein complexes (snRNPs) are imported back to the nucleus, where they take part in pre-mRNA splicing reactions (Mattaj, 1988; Lührmann et al., 1990). It has been shown that the monomethyl cap structure is the only essential signal for U snRNA export and that the leucine-rich nuclear export signal (NES) receptor CRM1/Exportin 1 (Xpo1) mediates this RNA export (Hamm & Mattaj, 1990; Jarmolowski et al., 1994; Fischer et al., 1995; Fornerod et al., 1997).

The interaction between Xpo1 and U snRNA is mediated by two adaptors. The first one is the nuclear cap

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binding complex (CBC), a heterodimeric protein complex composed of an 80 kDa subunit, CBP80, and a 20 kDa subunit, CBP20 (Izaurralde et al., 1994, 1995a; Kataoka et al., 1995). CBC binds specifically to the monomethyl cap structure of nascent RNA polymerase II transcripts (Visa et al., 1996) and promotes U snRNA export as well as pre-mRNA processing (Izaurralde et al., 1994, 1995a; Flaherty et al., 1997).

The second adaptor required for U snRNA export is the recently identified 55-kDa phosphoprotein termed PHAX (phosphorylated adaptor for RNA export; Ohno et al., 2000). PHAX binds to both CBC and U snRNA forming a trimeric complex called the precomplex. The precomplex can efficiently interact with Xpo1 in a RanGTP-dependent manner, forming a higher order complex, the U snRNA export complex (Ohno et al., 2000). Although the NES of PHAX is essential for the precomplex to interact with Xpo1, this binding might be more complicated than simple NES-Xpo1 interactions, because phosphorylation of PHAX is essential for the formation of the export complex. In contrast, the phosphorylation of PHAX is not required for precomplex formation (Ohno et al., 2000). After translocating to the cytoplasm through an NPC, the U snRNA export complex disassembles in a manner that involves both GTP hydrolysis by Ran and dephosphorylation of PHAX (Ohno et al., 2000). After disassembly, all the protein components of the export complex, including PHAX, must recycle back to the nucleus for the next round of U snRNA export.

Although some aspects of PHAX/Xpo1 interaction were investigated in our previous study, how PHAX interacts with the other components of the export complex and how PHAX recycles to the nucleus after export were not addressed. In this study, we have systematically mapped PHAX functional domains. The evolutionarily most highly conserved region of PHAX is found to be a novel RNA-binding domain that is essential for PHAX function in U snRNA export. More generally, different regions of PHAX are found to be required for different aspects of PHAX function as a scaffold on which U snRNA export complexes assemble.

RESULTS

PHAX is imported into the nucleus via the classical NLS pathway

PHAX has to recycle to the nucleus to mediate multiple rounds of U snRNA export. We have previously shown that PHAX moves into the nucleus upon injection into the cytoplasm of *Xenopus* oocytes, whereas it is not apparently exported upon injection into the nucleus in the absence of U snRNAs (Ohno et al., 2000; see also below). This means that although PHAX can shuttle between the nucleus and the cytoplasm, its steady-state localization in *Xenopus* oocytes is nuclear. To investigate if this is also the case in mammalian cells, we performed indirect immunofluorescence staining of HeLa cells with affinity purified anti-PHAX antibody. As shown in Figure 1A, strong PHAX signal was detected in the nucleoplasm (excluding nucleoli) with little if any cytoplasmic signal, indicating that nuclear localization of PHAX at steady state is not a *Xenopus* oocyte-specific phenomenon. Although the nucleoplasmic staining was not entirely uniform, and several brighter spots were often seen, further analysis of PHAX distribution has not been performed.

Next, attempts were made to characterize the nuclear localization signal (NLS) of PHAX in *Xenopus* oocyte injection experiments. When ^{35}S-methionine labeled PHAX was injected into the cytoplasm together with two control proteins, PHAX, as well as the positive control CBP80, moved to the nucleus, whereas the negative control (GSTM10) stayed in the cytoplasm (Fig. 1B, lanes 1–4). Nuclear migration of PHAX was severely inhibited by coinjection of either the importin β binding domain of importin α (IBB; Görlich et al., 1996a; Weis et al., 1996) or BSA-conjugated with NLS peptides derived from SV40 T antigen (BSA-NLS; Goldfarb et al., 1986), but not by truncated IBB or reverse NLS (SLN) controls (Fig. 1B, lanes 5–12). The behavior of PHAX was similar to that of CBP80, which contains a classical bipartite NLS (Izaurralde et al., 1995b). These results indicate that PHAX is imported via the classical NLS pathway, that is, via the importin α/β heterodimer.

To roughly map the location of the PHAX NLS, the protein was divided into three parts (N: amino-acid residues 1–133, M: 134–262, C: 263–385), and each part was fused to GST to make the proteins too large to diffuse through the NPCs. When these GST fusion proteins were injected into the cytoplasm, all of them moved to the nucleus, whereas GSTM10 stayed in the cytoplasm (Fig. 1C). Nuclear import of the three proteins was severely inhibited by injection of IBB (data not shown). These results suggest that there might be at least three classical NLSs distributed throughout the PHAX primary sequence.

PHAX has two major NLSs

Because classical NLS sequences are expected to contain basic residues (Dingwall & Laskey, 1991), we inspected the PHAX amino-acid sequence and found four regions that contain more than three consecutive basic amino-acid residues (Fig. 2A, NLS 1–4). When these four candidate NLS sequences were mutated in the context of GST-N, M, and C, import of GST-N (containing NLS1) and GST-M (containing NLS2) was abolished by the corresponding mutations, whereas import of GST-C (containing NLS 3 and 4) was slightly reduced by the NLS3 mutation and more strongly inhibited by the NLS4 mutation (data not shown).

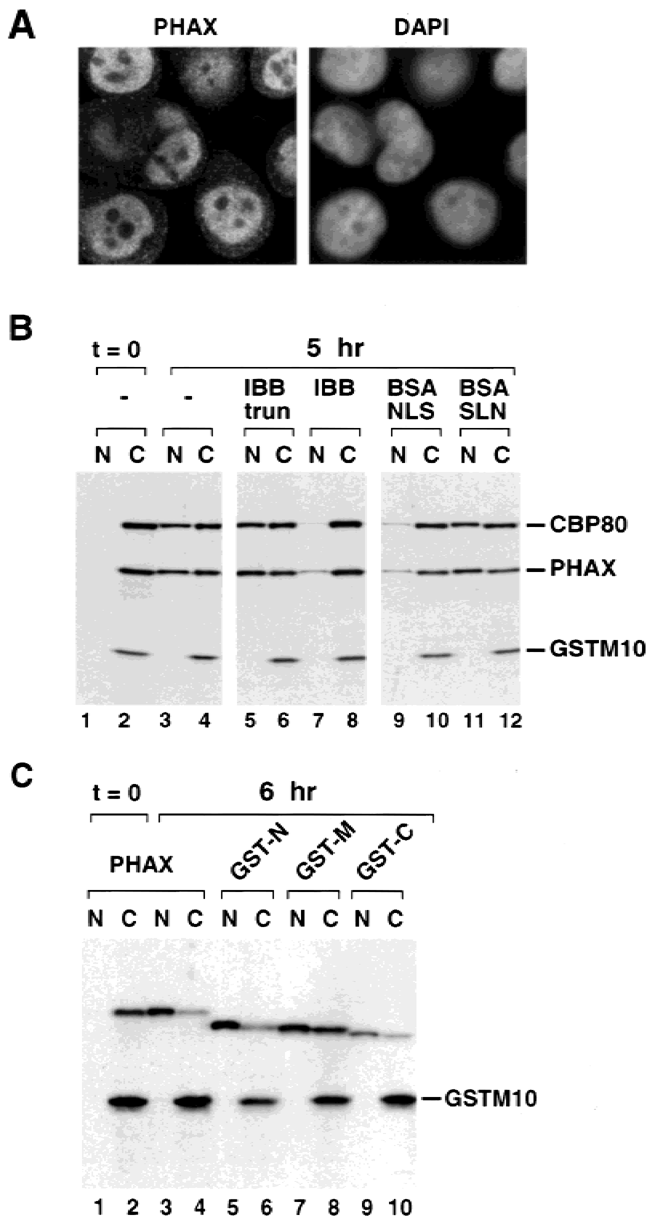

FIGURE 1. Localization of PHAX. **A**: Immunolocalization of PHAX in HeLa cells. HeLa S3 cells were immunostained with affinity-purified anti-PHAX antibody (left panel). Nuclei were visualized by DAPI staining of DNA (right panel). **B**: A mixture of ^{35}S-methionine labeled PHAX, CBP80, and GSTM10 was injected into the cytoplasm of *Xenopus* oocytes and protein was extracted from nuclear (N) and cytoplasmic (C) fractions either immediately (lanes 1 and 2) or 5 h (lanes 3–12) after injection. For lanes 5–8, either functional IBB (importin β binding domain, lanes 7 and 8) or its inactive truncated form (lanes 5 and 6) was preinjected at a concentration of 0.9 μg per oocyte into the cytoplasm 1 h prior to the injection of the labeled proteins. For lanes 9–12, BSA-NLS conjugate (lanes 9 and 10) or a control conjugate (lanes 11 and 12, BSA-SLN) was coinjected with the labeled proteins at a concentration of 1.1 μg per oocyte. The protein samples were analyzed by SDS/PAGE followed by fluorography. **C**: ^{35}S-methionine-labeled full-length PHAX or truncated PHAX-GST fusion proteins were injected together with GSTM10 into the cytoplasm and protein was extracted either immediately (lanes 1 and 2) or 6 h (lane 3–10) after injection, and analyzed as in **B**. PHAX: full-length PHAX protein; GST-N: GST fused to an N-terminal PHAX fragment containing amino acids 1–133; GST-M: GST fused to a central PHAX fragment containing amino acids 134–262; GST-C: GST fused to a C-terminal PHAX fragment containing amino acids 263–385.

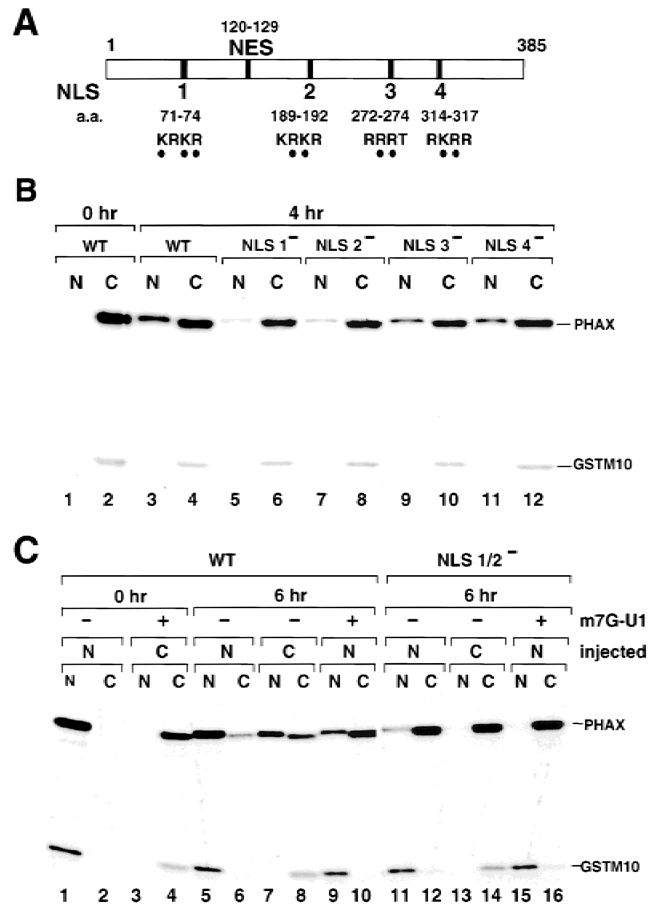

FIGURE 2. PHAX nuclear import. **A**: Schematic representation of PHAX nuclear transport signals. The positions of four putative NLSs and the NES (Ohno et al., 2000) are shown. The amino-acid residues that were substituted by alanines are indicated by closed circles. **B**: Nuclear import of NLS mutants. ^{35}S-methionine-labeled wild-type PHAX (WT, lanes 1–4) or the four NLS single mutants (NLS1$^-$ through NLS4$^-$, lanes 5–12) were injected together with GSTM10 into the cytoplasm. Protein was extracted from N and C fractions immediately (lanes 1 and 2) or 4 h (lanes 3–12) after injection and analyzed as in Figure 1B. **C**: A mixture of wild-type PHAX or the NLS1/2$^-$ double mutant with GSTM10 was injected into the nucleus (lanes 1, 2, 5, 6, 9–12, 15, and 16) or the cytoplasm (lanes 3, 4, 7, 8, 13, and 14), either alone (lanes 1, 2, 5–8, and 11–14) or together with unlabeled m^{7}G-capped U1 ΔSm RNA (500 fmol per oocyte, lanes 3, 4, 9, 10, 15, and 16). Protein was extracted immediately (lanes 1–4) or 6 h (lanes 5–16) after injection and analyzed by SDS-PAGE.

To investigate which is the major NLS in the context of full-length PHAX, we mutated the NLSs separately and injected the corresponding full-length proteins into the cytoplasm of oocytes. As shown in Figure 2B, mutations in NLS1 and NLS2 greatly reduced import activity whereas the other two mutations had a more minor effect. When the mutations in NLS1 and NLS2 were combined (NLS1/2$^-$), the protein lost its ability to localize to the nucleus (Fig. 2C, lanes 13 and 14). Please note however that this does not mean an absolute lack of import activity of the NLS1/2$^-$ protein because the protein is continuously exported from the nucleus (see below). Nevertheless, these results indicate that NLSs 1 and 2 are the major import signals of PHAX.

We have previously shown that although the steady-state localization of PHAX is nuclear, injection of a saturating amount of m^{7}G-capped U1 snRNA results in net movement of PHAX to the cytoplasm, by enhancing its export rate (Ohno et al., 2000). When the NLS1/2$^-$ mutant protein was injected into the nucleus, it accumulated in the cytoplasm even without coinjection of U1 snRNA, whereas the wild-type protein mainly localized in the nucleus (Fig. 2C, compare lanes 11 and 12 with 5 and 6), indicating that PHAX is continuously exporting from but recycling back to the nucleus in the natural situation. The NLS1/2$^-$ mutant protein appears to be functional in U snRNA export as coinjection of U1 snRNA stimulated export of the mutant protein (Fig. 2C, compare lanes 15 and 16 with 11 and 12) demonstrating that it could participate in U snRNA export complexes. In addition, injection of recombinant NLS1/2$^-$ protein stimulated U snRNA export to the same level as the wild-type protein (data not shown). The NLS1/2$^-$ protein can also efficiently form both the pre- and export complexes in vitro (data not shown). These results indicate that the NLS1/2$^-$ protein retains full activity to interact with RNA, CBC, and Xpo1, but is severely impaired in nuclear import.

Identification of an evolutionarily conserved RNA-binding domain in PHAX

We have previously shown, by UV crosslinking, that PHAX interacts with RNA (Ohno et al., 2000). Because PHAX is a U snRNA export mediator, it was possible that PHAX preferentially binds U snRNA sequences or structures. To investigate this, purified recombinant PHAX was examined in a band-shift assay with various ^{32}P-labeled RNA probes (Fig. 3A). PHAX could bind efficiently to all the RNAs tested with the exception of the shortest RNA probe (25 nt). At higher concentrations, PHAX gave rise to multiple shifted bands, indicating that multiple PHAX molecules could bind to a single RNA molecule. Because the recombinant PHAX from *Escherichia coli* is unphosphorylated, and thus in the state that can form the precomplex but not the export complex, the PHAX protein was phosphorylated with recombinant Casein Kinase II in vitro (Ohno et al., 2000), and its RNA-binding activity tested. The phosphorylation state of PHAX did not significantly affect its RNA-binding activity (Fig. 3B and data not shown). These results indicate that PHAX exhibits RNA-binding activity regardless of its phosphorylation

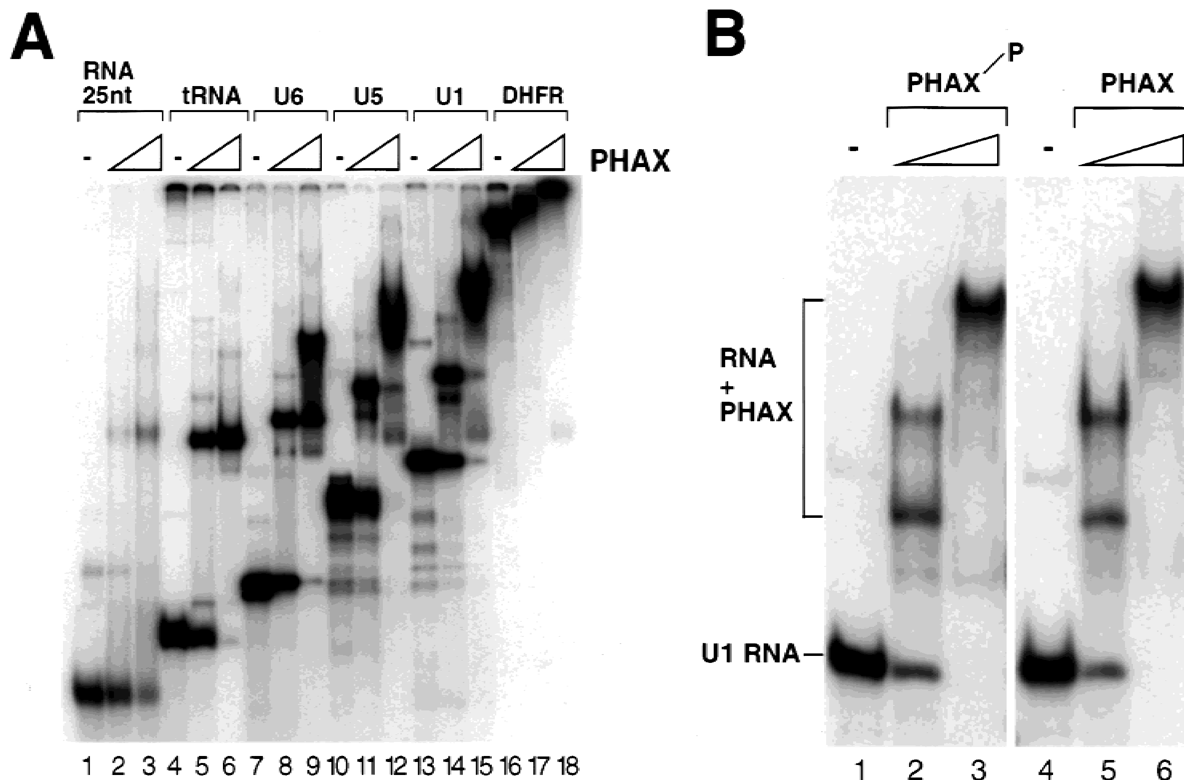

FIGURE 3. Electrophoretic mobility shift assays. **A**: RNA band-shift assay with recombinant PHAX protein. ^{32}P-labeled 25 nt RNA (Ohno et al., 2000), human initiator methionyl tRNA, U6Δss, m^{7}G-capped U5ΔSmRNA, m^{7}G-capped U1ΔSm RNA, or m^{7}G-capped DHFR mRNA were incubated with recombinant PHAX protein (0.2 or 0.4 μM) on ice for 20 min. The RNA probes and RNA–protein complexes were analyzed by native 6% PAGE and autoradiography. **B**: Effect of PHAX phosphorylation on RNA binding. Recombinant PHAX protein was phosphorylated in vitro (Ohno et al., 2000). Binding of phosphorylated PHAX (lanes 1–3, PHAX-P) and unphosphorylated PHAX (lanes 4–6, PHAX) to m^{7}G-capped U1ΔSm RNA probe was compared as in **A**.

state, without any obvious sequence or structure specificity.

The fact that PHAX does not contain any known RNA-binding motifs (Denti et al., 2000; Ohno et al., 2000) prompted us to map its RNA-binding domain. A series of truncated PHAX mutants were made, some of which are illustrated in Figure 4A. The corresponding proteins were synthesized in vitro in rabbit reticulocyte lysate and examined for their ability to bind to poly U Sepharose. The binding activity of the mutants was quantified (Fig. 4B), and the results are summarized in Figure 4A. A typical result is shown in Figure 4C. Wild-type PHAX bound efficiently to poly U, whereas a control protein (GSTM10) did not. C-terminal truncation of PHAX up to amino acid 320 (C5) did not affect RNA binding. Removal of an additional 18 amino acids, however, led to a decrease in RNA binding (C4), and larger deletions abolished activity (C1 and C3). When 140 N-terminal residues were deleted (N4), the mutant protein still bound RNA efficiently, and this was also the case when the N4 and C5 truncations were combined (N4/C5). A fragment spanning residues 219–319 (N6/C5) retained strong RNA-binding activity, but further deletion abolished the activity (Fig. 4A, N7/C5, and data not shown), consistent with the lack of RNA binding of the internal deletion mutant M3 (Fig. 4A,B). In addition, a small in-frame deletion of 9 amino-acid residues in the center of the N6/C5 region in the full-length context (Fig. 4A, CD) resulted in defective RNA binding (Fig. 4B,C).

Because we used rabbit reticulocyte lysate to synthesize the mutant proteins, it was possible that PHAX bound to RNA via one or more other factors in the lysate. To clarify this point, we employed an *E. coli* in vitro translation system, where the involvement of other eukaryotic factors could be excluded. As shown in Figure 4D, N6/C5 from the *E. coli* system bound as well as wild-type PHAX to RNA. In addition, binding of both proteins to poly U-Sepharose was abolished by pretreatment of the resin with RNAse A, confirming that the proteins bound directly to the polyribonucleotide.

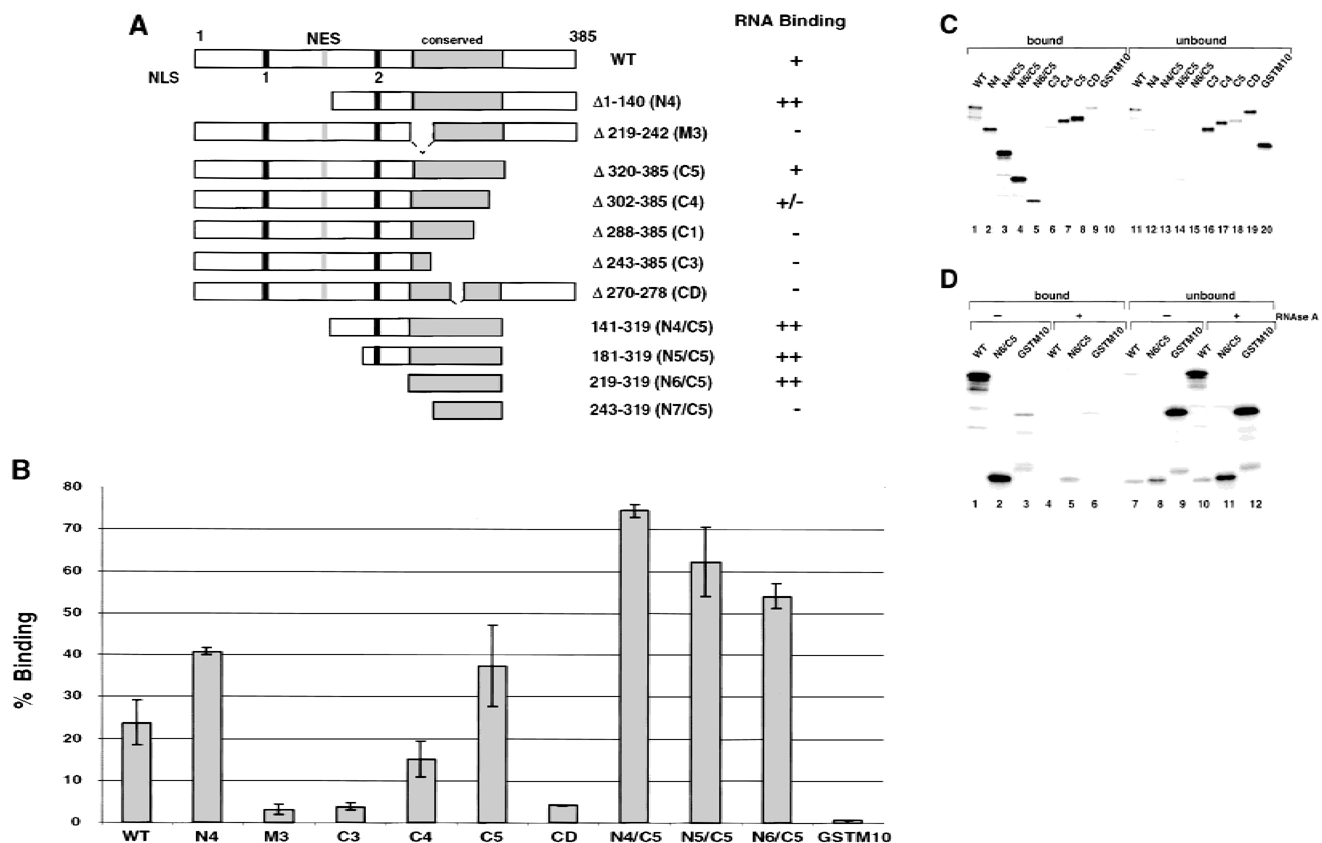

FIGURE 4. The RNA binding domain of PHAX. **A**: Diagram of PHAX deletion mutants. RNA-binding activity of the mutants is summarized on the right. **B**: Quantitation of poly U Sepharose binding experiments. Average binding and standard deviation were calculated from five independent experiments (WT, GSTM10), three independent experiments (N4, C3, C5) or two independent experiments (M3, C4, CD, N4/C5, N5/C5, N6/C5). **C**: Poly U Sepharose binding assay with PHAX mutants. ^{35}S-methionine-labeled PHAX wild-type and mutant proteins or a negative control protein (GSTM10) were translated in rabbit reticulocyte lysate and incubated with poly U Sepharose. Fifty percent of the bound fraction (lanes 1–10) and 10% of the supernatant (lanes 11–20) was analyzed by SDS/PAGE and fluorography. **D**: ^{35}S-methionine-labeled PHAX proteins were translated in *E. coli* S30 extract and their poly U Sepharose binding activity tested as in **C** either in the presence (lanes 4–6 and 10–12) or absence (lanes 1–3 and 7–9) of pretreatment of the resin with RNAse A (10 μg/mL) at 25 °C for 40 min.

The CD and C3 mutant proteins were chosen to test the significance of PHAX RNA-binding activity in U snRNA export in *Xenopus* oocyte injection experiments. These two mutants are completely defective in the RNA-binding assay (Fig. 4) but still bind to CBC (see below). As seen in Figure 5A, coinjection of a saturating amount of m^7G-capped U1 RNA could not induce export of the CD or C3 mutants, whereas the same amount of U1 RNA efficiently induced export of wild-type PHAX. In contrast, the import activity of the mutants was similar to wild type (Fig. 5A, lanes 3, 4, 9, 10, 15, and 16). We also investigated the effect of injection of an excess of the recombinant version of the mutant protein on RNA export. When a mixture of ^{32}P-labeled U1, U5, U6, and tRNA was injected into the nucleus, a fraction of the RNAs was transported to the cytoplasm within 1 h whereas the negative control U6 RNA stayed in the nucleus (Fig. 5B, lanes 1–4). When wild-type PHAX was coinjected with the RNAs, export of U1 and U5 RNAs was stimulated (Fig. 5B, lanes 5 and 6). However, the CD and C3 mutant proteins failed to stimulate U snRNA export (Fig. 5B, lanes 7–10), suggesting that these mutants are nonfunctional in mediating U snRNA export. These results strongly suggest that the RNA binding domain of PHAX is essential for mediating U snRNA export.

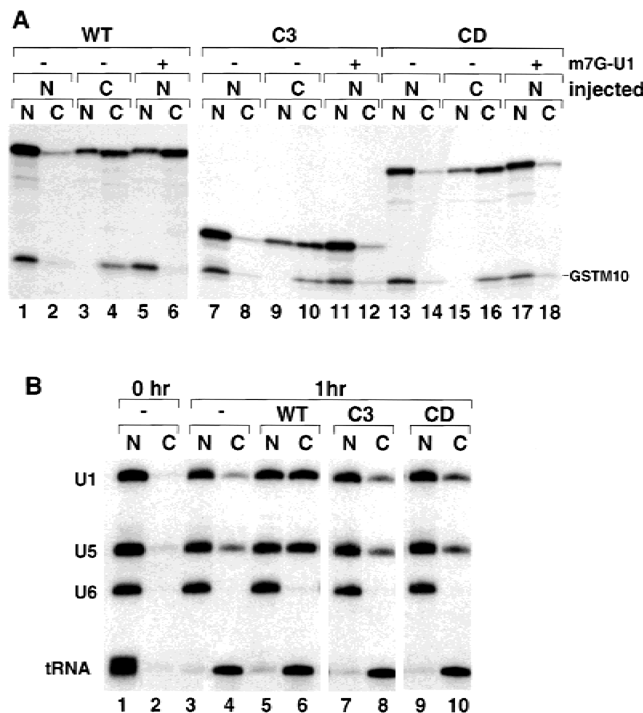

FIGURE 5. RNA binding mutants are defective in export. **A**: The C3 and CD mutants are defective in U1 RNA-induced export. ^{35}S-methionine-labeled wild-type PHAX or the C3 or CD mutants were injected together with GSTM10 into the nucleus (lanes 1, 2, 5–8, 11–14, 17, and 18) or into the cytoplasm (lanes 3, 4, 9, 10, 15, and 16) of *Xenopus* oocytes, either alone (lanes 1–4, 7–10, and 13–16) or together with m^7G-capped U1ΔSm RNA (500 fmol per oocyte, lanes 5, 6, 11, 12, 17, and 18). Protein was extracted 5 h after injection and analyzed by SDS-PAGE and fluorography. **B**: A mixture of ^{32}P-labeled m^7G-capped U1ΔSm RNA, m^7G-capped U5ΔSm RNA, U6Δss RNA, and initiator methionyl tRNA was injected into the nucleus of *Xenopus* oocytes either alone (lanes 1–4) or together with recombinant PHAX wild-type protein (4.4 μM in the injection mixture, lanes 5 and 6) or the same amount of the C3 mutant PHAX protein (lanes 7 and 8) or the CD mutant (lanes 9 and 10). RNA was extracted from nuclear (N) and cytoplasmic (C) fractions immediately (lanes 1 and 2) or 1 h (lanes 3–10) after injection, and analyzed by SDS-PAGE followed by autoradiography.

Interestingly, the minimal RNA-binding fragment (N6/C5, Fig. 4A) corresponds to the most conserved region among the PHAX homologs in the databases (Fig. 6A,B). In fact, this is the only region of the protein that is highly conserved between distant species. When alanine mutations were introduced into the most conserved region in the full-length PHAX context (Fig. 6B), RNA binding activity was affected variably. The CM3 and CM5 mutations inhibited RNA binding severely; CM2, CM4, and CM6 partially; and CM1 and CM7 not at all (Fig. 6C shows a representative experiment and Fig. 6D a summary of the results of several experiments). These data indicate that many, but not all, of the conserved residues are important for RNA-binding activity. The alanine-substitution mutants also showed variable activity in the RNA-induced protein export assay. The defects of different mutants in RNA binding correlated well with their export defects (data not shown). Thus, the evolutionarily conserved region of PHAX corresponds to its RNA-binding domain, and RNA binding is required for the activity of PHAX in U snRNA export. Although the RNA-binding domain is found in all identifiable PHAX homologs, no significant homology to other proteins in the databases was observed.

Interaction of PHAX with CBC

Efforts to map the interaction domain of PHAX with CBC have also been undertaken. We labeled some of the PHAX mutants shown in Figure 4A by in vitro translation and tested their ability to bind CBC by assaying their coimmunoprecipitability with anti-CBP80 antibody (Izaurralde et al., 1994; Fig. 7). This antibody did not inhibit U snRNA export complex formation in vitro (data not shown). Wild-type PHAX and the C5 mutant (that efficiently supports U snRNA export in *Xenopus* oocytes; data not shown) bound similarly to CBC. However, the CD mutant coprecipitated less efficiently, and N4 did not bind above the background level of the negative control Rev protein. Because in vitro-translated PHAX proteins were used in this assay, it was possible that capped RNAs present in the reticulocyte lysate were affecting the results. The same experiment was therefore performed in the presence of RNAse A. In this condition, the coprecipitation with CBC of both wild-type and C5 was reduced to a level similar to the CD mutant. These results suggest that capped RNAs in the lysate stabilize PHAX-CBC interaction by allowing as-

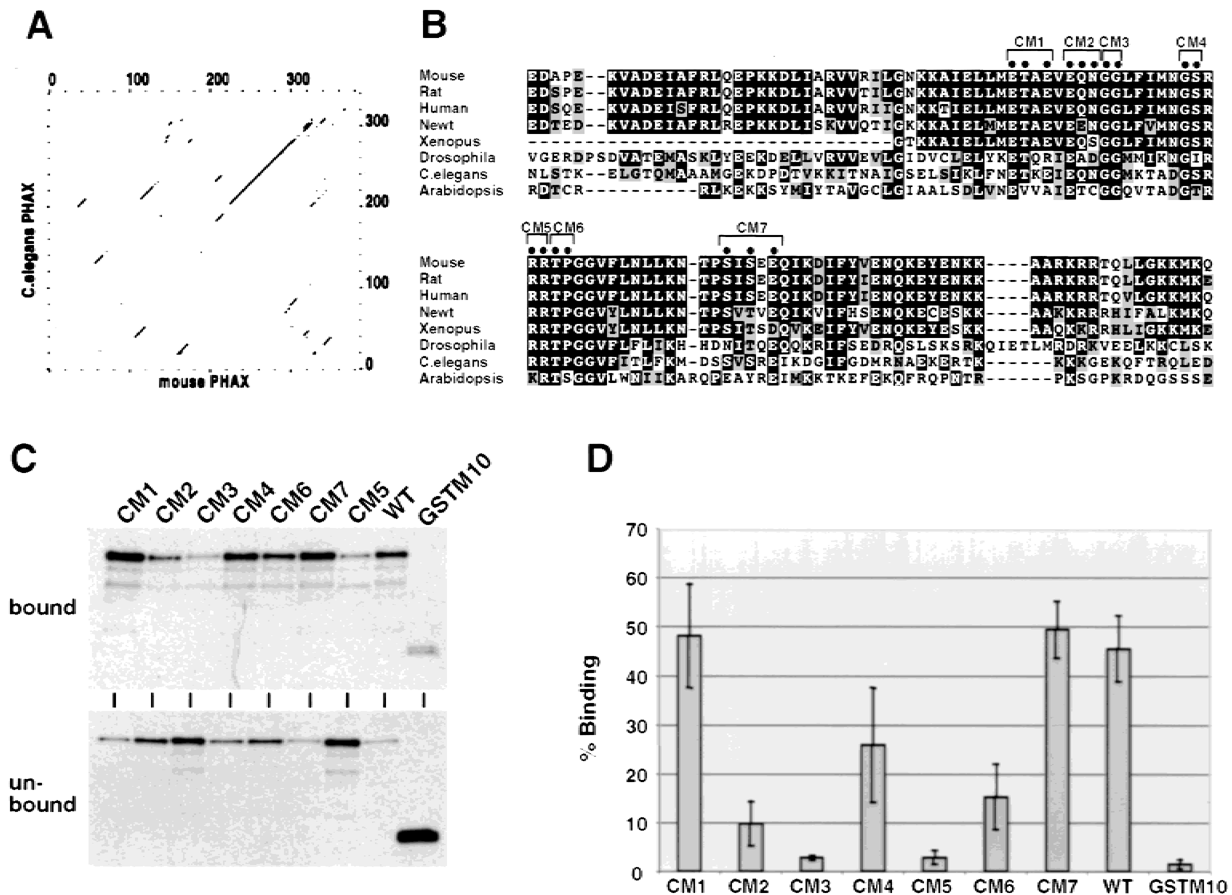

FIGURE 6. The PHAX RNA-binding domain is conserved. **A**: Similarity plot analysis of mouse PHAX (EMBL DNA database accession no. CAB87994) versus *Caenorhabditis elegans* PHAX (AAF36030). Sequences were compiled and analyzed with DotPlot computer software (Maizel & Lenk, 1981) from the Wisconsin Package, version 10.1, Genetics Computer Group (GCG), with the settings of stringency 13 and window 35. **B**: Sequence alignment of mouse PHAX (amino-acid residues 215–327) with the corresponding regions from rat (X67877), human (AW607076), newt (CAB54141), *X. laevis* (AW147615, AW148211), *Drosophila melanogaster* (AAF58985), *C. elegans* (AAF36030) and *Arabidopsis thaliana* (BAB02826). Sequences were compiled and analyzed using BLAST (Altschul et al., 1997) and Clustal_X (Thompson et al., 1998), and displayed using the BOXSHADE 3.21 program (http://www.ch.embnet.org/software/BOX_form.html). The residues indicated by closed circles in the mouse sequence were replaced by alanines in the CM1–7 mutants. **C**: Poly U Sepharose binding assay with the alanine mutants. The experiment was performed as in Figure 4. **D**: Quantitation of RNA-binding activity of the alanine mutants from three experiments similar to **C**.

sembly of the precomplex consisting of RNA, CBC, and PHAX. Intrinsic, RNA-independent, CBC-binding activity of the PHAX variants was observed in the absence of capped RNAs, that is, in the presence of RNAse. The N4 mutant binds RNA efficiently (Fig. 4), but was completely defective in CBC binding. The CD mutant, on the other hand, is defective in RNA binding (Fig. 4) but could bind CBC similarly to the wild-type protein in the absence of RNA. This suggests that at least part of the CBC-binding domain of PHAX is located more N-terminally than the RNA-binding domain. However, we were unsuccessful in precisely mapping the minimal CBC-binding domain because the affinity of PHAX for CBC in the absence of RNA is very low and many PHAX truncations, like C5 (Fig. 7), partially affected the interaction, reducing it to a level that prevented accurate quantitation.

DISCUSSION

In this study, we have performed a systematic analysis of PHAX, and revealed three important interaction domains within the protein that are all required for U snRNA export. First, we have mapped NLSs of PHAX that are required for recycling of PHAX to the nucleus. Second, we have found that the evolutionarily conserved region of PHAX is a novel RNA-binding domain required for mediating U snRNA export. Third, the interaction domain of PHAX with CBC was shown to be at least partly distinct from the NLSs and the RNA-binding domain. Our previous work had shown that the NES-containing region of PHAX, together with PHAX phosphorylation, is required for interaction between PHAX and CRM1/Exportin1 (Ohno et al., 2000). Thus, mutant forms of PHAX that allow the effects of its various in-

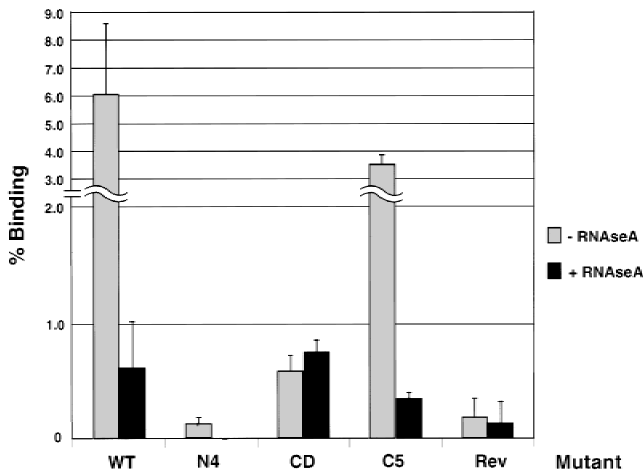

FIGURE 7. Quantitation of PHAX binding to recombinant CBC. ^{35}S-methionine-labeled PHAX wild-type and mutant proteins together with the negative control REV protein were translated in rabbit reticulocyte lysate and incubated with 1 μg of recombinant CBC pre-bound to an anti-CBP80 antibody bound to Protein-A Sepharose. Binding was performed for 1 h at 25 °C either without (left panels) or with (right panels) RNAse A (10 μg/mL). Average binding and standard deviations were calculated from six (REV), five (WT) or two (N4, CD, C5) independent trials in the absence of RNAse A and three (REV) or two (WT, N4, CD, C5) independent experiments in the presence of RNAse A.

teractions on function in U snRNA export to be tested have now been generated and analyzed.

The nuclear import signals of PHAX

We have found that PHAX is imported to the nucleus via two major nuclear localization signals (NLS1 and -2), both of which interact with the importin α/β heterodimeric import receptor. Mutation of both signals abolished the ability of PHAX to migrate into the nucleus. Classical NLS signals often contain two clusters of basic amino-acid residues separated by ~10 amino-acid spacer (bipartite NLS; Dingwall & Laskey, 1991). Because two consecutive lysines are found 14 amino acids upstream of NLS2, it is possible that NLS2 is part of a bipartite signal. No second cluster of basic residues for NLS1 was found.

We have also identified two additional minor NLSs. Interestingly, one of them, NLS3, is located in the center of the RNA-binding domain. In fact, the NLS3$^-$ mutation is identical to the CM5 mutation that greatly reduces RNA binding activity (Fig. 6C,D). It is possible that there is some coordinated regulation between RNA binding and import. In the case of the HIV-1 Rev protein, it has been reported that the RNA-binding domain overlaps with the NLS, and that binding of importin β to the Rev NLS competes with RNA binding (Truant & Cullen, 1999). Incompatibility between RNA binding and import receptor binding was also demonstrated in the case of CBC, another adaptor for U snRNA export (Görlich et al., 1996b). It will be of interest to test whether binding of importin α/β to NLS3, or to the other PHAX NLSs, plays a role in releasing RNA from PHAX in the cytoplasm.

Because PHAX can interact with CBC, it might have been imagined that PHAX might piggy-back into the nucleus together with CBC, as CBP80, the larger subunit of CBC, has a bipartite NLS (Izaurralde et al., 1995b). In that case, the major NLSs identified in this study would therefore be interaction domains with CBC. However, this possibility is very unlikely for a number of reasons. First, the affinity of PHAX for CBC is very weak in the absence of other components of the export complex (Ohno et al., 2000; Fig. 7). Second, even when PHAX protein was divided into three parts, efficient import of each fragment was observed (Fig. 1C). Third, the import defective mutant NLS1/2$^-$ is able to interact with CBC because the mutant can efficiently mediate U snRNA export (data not shown). It is therefore very likely that PHAX and CBC recycle to the nucleus independently.

The RNA-binding domain of PHAX is essential for U snRNA export

We previously showed by UV crosslinking that PHAX makes direct contact with RNA, although PHAX did not contain any known RNA-binding motifs (Ohno et al., 2000). In this study, we have shown that PHAX can bind RNA in a sequence-unspecific and a phosphorylation-independent manner. The newt homolog of PHAX was recently identified as a hammerhead ribozyme-binding protein (Denti et al., 2000), underlining the ability of PHAX to bind RNA and raising the possibility that the newt ribozyme might be exported to the cytoplasm by the same mechanism as U snRNAs. The RNA-binding activity of the newt homolog was not confined to the ribozyme (Denti et al., 2000). The apparent lack of RNA-binding specificity found in the studies to date does not, of course, necessarily mean that high-affinity PHAX-binding RNA sequences do not exist. PHAX might bind tightly to specific RNA sequences or structures, as is the case, for example, with another RNA export mediator, TAP, which shows apparently nonspecific general RNA-binding activity as well as much higher affinity binding to a viral RNA export element, the CTE (Grüter et al., 1998).

We could successfully narrow down the RNA-binding domain of PHAX to a 101 amino acid fragment by using a poly U Sepharose binding assay. This region corresponds closely to the most conserved region of PHAX (Fig. 6A,B). Because there is no similarity with other known RNA-binding motifs on the level of primary sequence, it will be of interest to investigate how this novel domain interacts structurally with RNA.

The CD mutant and other mutants in this domain whose RNA binding was impaired failed to mediate U

snRNA export, indicating that this RNA-binding domain is essential for U snRNA export. This suggests that PHAX interaction with CBC, in the absence of RNA binding, is not sufficiently strong to result in the formation of the export complex. We also did not observe a dominant negative effect of the CD and C3 mutants on U snRNA export in these conditions (Fig. 5B). These results probably reflect the fact that export complex assembly is highly cooperative, with RNA greatly stabilizing the interactions between the components (Ohno et al., 2000). Therefore, the PHAX mutant that cannot bind RNA cannot interact with any of the other components of the export complex strongly enough to act as a dominant inhibitor of the export process.

Our previous study showed that PHAX is UV crosslinked much more strongly to RNA in the context of the U snRNA export complex (Ohno et al., 2000). Because export complex assembly is highly cooperative, it is possible that the RNA-binding activity of PHAX is increased by its association with the other components of the complex. This kind of mechanism would contribute to strong interaction with the RNA substrate in the complex during export and its efficient release when the export complex is disassembled in the cytoplasm. Another interesting aspect of PHAX is that, because the m^7G-cap structure is the essential signal for U snRNA export (Hamm & Mattaj, 1990; Jarmolowski et al., 1994) PHAX should preferentially associate with capped RNAs in the presence of uncapped RNAs. The protein–protein interaction between PHAX and CBC probably plays an essential role in this discrimination (Ohno et al., 2000). CBC binds to the cap structure, and is likely to recruit PHAX to the capped RNA. In relation to this, it remains to be investigated whether PHAX also associates with mRNAs, the major class of m^7G-capped RNAs, in vivo.

MATERIALS AND METHODS

Plasmids, recombinant protein expression, and phosphorylation

For construction of GST-PHAX-N, -M, and -C, the relevant PHAX PCR fragments and a GST cDNA fragment were cloned into the *Bam*HI and *Hin*dIII sites of the pT7-7 vector (Ohno et al., 2000). For the PHAX deletion mutants, the corresponding regions of the pT7-7-PHAX plasmid were PCR amplified, and the amplified fragments were recircularized. Alanine mutagenesis of PHAX was performed in the context of the pT7-7 vector with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. In vitro translation in rabbit reticulocyte lysate and in *E. coli* S30 was as described previously (Ohno et al. 2000). For recombinant protein expression in *E. coli*, the corresponding fragments were subcloned into the *Bam*HI and *Hin*dIII sites of the pQE30 vector (Qiagen), and the proteins were expressed and purified on Ni-NTA resin according to the manufacturer's instructions. In vitro phosphorylation of recombinant PHAX with recombinant Casein kinase II was as described (Ohno et al., 2000).

Immunofluorescence in HeLa cells

HeLa S3 cells were grown to ~50% confluence, washed twice with PBS containing 5 mM $MgCl_2$, and fixed in 4% paraformaldehyde in PBS containing 5 mM $MgCl_2$ at room temperature (RT) for 15 min. The fixed cells were permeabilized in 0.1% Triton X-100/PBS plus $MgCl_2$ at RT for 15 min and washed twice with PBS plus $MgCl_2$. The cells were blocked with 2% BSA in PBS plus $MgCl_2$ at RT for 30 min, incubated with anti-PHAX affinity purified antibody at 1:1,000 dilution (Ohno et al. 2000) at RT for 30 min, and washed three times with PBS plus $MgCl_2$. Subsequently, the cells were treated by Cy3-labeled anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, Inc.) at 1:1,000 dilution. After the secondary antibody treatment, the cells were washed, treated with DAPI (3 μM), and examined by confocal microscopy.

Oocyte injection

Xenopus oocyte microinjection was performed as previously described (Jarmolowski et al., 1994). BSA-NLS and BSA-SLN conjugates and full-length and truncated IBB fragments were prepared and used as described (Görlich et al., 1996a; Palacios et al., 1996).

Band-shift assay

^{32}P-labeled RNA probes were mixed with recombinant PHAX (0.2–0.4 μM) in Transport Buffer 2 (40 mM HEPES-KOH, pH 7.5, 110 mM KOAc, 6 mM $Mg(OAc)_2$, 5% Glycerol) in a volume of 5 μL, and incubated on ice for 20 min. The samples were fractionated on a 6% native polyacrylamide gel run in 0.5$\times$ TBE buffer at 8.5 V/cm. The gel was dried and autoradiographed.

Poly U Sepharose binding assay

Poly U Sepharose 4B (20 μL; Amersham Pharmacia Biotech) that had been equilibrated in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 4 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, and 1 mg/mL BSA was incubated in the same buffer with 4 μL of ^{35}S-methionine-labeled proteins in a total volume of 100 μL at 25 °C for 1 h with continuous shaking. After the incubation, the mixture was briefly spun in a microfuge, and the supernatant was immediately mixed with an equal amount of SDS-PAGE sample buffer. The pellet was washed three times with the binding buffer and the bound proteins were eluted with SDS sample buffer. Both fractions were subjected to SDS polyacrylamide electrophoresis, and the gel was fixed in 40% methanol, 20% acetic acid, treated with Amplify reagent (Amersham Pharmacia Biotech), dried, and autoradiographed.

CBC binding assay

Anti-human CBP80 antiserum was prebound to Protein-A Sepharose 4B (Amersham Pharmacia: 20 μL antibody to

20 μL beads) in 60 μL IPPc buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 10% glycerol) for 1 h at 25 °C, then washed three times in IPPc buffer. One microgram of recombinant CBC was added per reaction in 200 μL IPPc buffer and incubated for 1 h at 25 °C in a rotating shaker, washed three times, and 4 μL of ^{35}S-methionine-labeled protein was added either alone or with 10 μg/mL of RNAse A and continuously shaken for 1 h at 25 °C. After the incubation, the mixture was briefly spun, and the supernatant was mixed with an equal volume of SDS-PAGE sample buffer. The pellet was washed three times with IPPc buffer. Bound proteins were eluted with SDS-PAGE sample buffer. Both bound and unbound fractions were subjected to SDS-PAGE and analyzed by autoradiography.

ACKNOWLEDGMENTS

We thank the members of our laboratory, particularly Maarten Fornerod and Scott Kuersten, for ongoing discussion of the project and criticism of the manuscript. We also thank Thomas Schell for providing HeLa S3 cells, and Tobias Walther for assistance in computer analysis. A.S. and M.O. contributed equally to this work.

Received November 10, 2000; returned for revision December 7, 2000; revised manuscript received December 18, 2000

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