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Nuclear import of RPA in *Xenopus* egg extracts requires a novel protein XRIPα but not importin α

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Replication protein A (RPA) is a eukaryotic single-stranded (ss) DNA-binding protein that is essential for general DNA metabolism. RPA consists of three subunits (70, 33 and 14 kDa). We have identified by two-hybrid screening a novel *Xenopus* protein called XRIPα that interacts with the ssDNA-binding domain of the largest subunit of RPA. XRIPα homologues are found in human and in *Drosophila* but not in yeast. XRIPα is complexed with RPA in *Xenopus* egg extracts together with another 90 kDa protein that was identified as importin β. We have demonstrated that XRIPα, but not importin α, is required for nuclear import of RPA. Immunodepletion of XRIPα from the egg extracts blocks nuclear import of RPA but not that of nucleoplasmin, a classical import substrate. RPA import can be restored by addition of recombinant XRIPα. Conversely, depletion of importin α blocks import of nucleoplasmin but not that of RPA. GST–XRIPα pull-down assay shows that XRIPα interacts directly with recombinant importin β as well as with RPA *in vitro*. Finally, RPA import can be reconstituted from the recombinant proteins. We propose that XRIPα plays the role of importin α in the RPA import scheme: XRIPα serves as an adaptor to link RPA to importin β.
Keywords: importin/nuclear transport/Ran/RPA/XRIPα

Introduction

Replication protein A (RPA) is a eukaryotic single-stranded (ss) DNA-binding protein (SSB) composed of three subunits of 70, 33 and 14 kDa (reviewed by Wold, 1997). It was identified originally as a cellular factor essential for SV40 DNA replication *in vitro* (Fairman and Stillman, 1988; Wold and Kelly, 1988; Kim *et al*., 1992). Subsequently, RPA was shown to be required for chromosomal DNA replication (Brill and Stillman, 1991; Fang and Newport, 1993; Adachi and Laemmli, 1994), repair and recombination (Heyer *et al*., 1990; Coverley *et al*., 1991; Moore *et al*., 1991; Longhese *et al*., 1994; New *et al*., 1998; Shinohara et al., 1998). The primary structure of RPA is evolutionarily well conserved among eukaryotes. The major ssDNA-binding activity resides in the largest subunit, $p70^{RPA}$ (Wold *et al*., 1989; Kenny *et al*., 1990); however, the smaller subunits are essential to support DNA replication (Kenny *et al*., 1990; Erdile *et al*., 1991; Santocanale *et al*., 1995; Maniar *et al*., 1997). $p70^{RPA}$ can be subdivided into four domains (Gomes and Wold, 1995; Gomes *et al*., 1996; Lin *et al*., 1996). The N-terminal domain (1–170) is known to interact with DNA polymerase α, large T antigen and p53 (Dornreiter *et al*., 1992; Dutta *et al*., 1993; He *et al*., 1993; Li *et al*., 1993). The central domain (170–442) binds ssDNA, and the crystal structure of this complex has been reported recently (Gomes *et al*., 1996; Bochkarev *et al*., 1997). The C-terminal domain (507–616) is required for complex formation with the smaller subunits (Gomes and Wold, 1995; Lin *et al*., 1996). $p70^{RPA}$ also has a putative C4-type zinc finger motif between the central and the C-terminal domains. The zinc finger motif is required for DNA replication and mismatch repair but not for excision repair (Lin *et al*., 1998). RPA has also been shown to bind excision repair proteins, XPA and XPG (He *et al*., 1995; Matsuda *et al*., 1995).

Despite these extensive functional domain studies, little is known about the nuclear transport of RPA. The classical nuclear import pathway of macromolecules is dependent on a family of short basic amino acid motifs called NLS (the nuclear localization signal; reviewed by Dingwall and Laskey, 1991). The classical NLS import pathway is mediated by the heterodimer composed of importin (also called karyopherin) α and β (reviewed by Görlich and Mattaj, 1996). NLS motifs of cargoes are recognized by the C-terminal domain of importin α consisting of 10 armadillo motifs (Conti *et al*., 1998). The N-terminal domain of importin α, in turn, binds importin β which mediates the docking of the cargo–importin α/β complex with nuclear pores (the N-terminal domain of importin α is called the importin β-binding or IBB domain; Görlich *et al*., 1996a; Weis *et al*., 1996). Docking is followed by an energy-dependent translocation through the nuclear pore. This process requires the small GTPase Ran and NTF2/p10/pp15 (Melchior *et al*., 1993; Moore and Blobel, 1993, 1994). Binding of Ran-GTP to importin β inside the nucleus induces dissociation of the cargo–importin α/β complex (reviewed in Melchior and Gerace, 1998). Thus importin α functions as an adaptor to link NLS cargoes to importin β.

Recent experimental evidence has revealed alternative nuclear import pathways that are distinct from the classical one described above (reviewed in Weis, 1998; Wozniak *et al*., 1998). Transportin, a member of the family of importin β-related proteins (Fornerod *et al*., 1997; Görlich *et al*., 1997), binds directly to the glycine-rich M9 domain of the hnRNP A1 protein to mediate its transport into the nucleus (Pollard *et al*., 1996). In yeast, other importin β-related proteins, Kap123/Yrb4p and Pse1p, also appear to bind directly to some ribosomal proteins to mediate

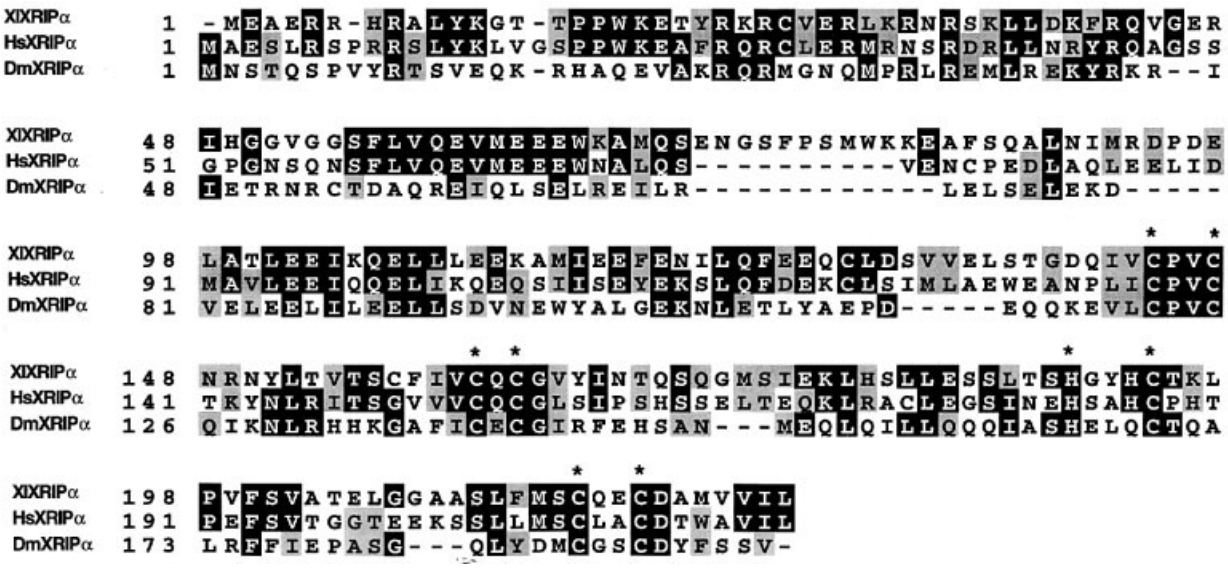

Fig. 1. Amino acid sequences of *Xenopus* XRIPα (XlXRIPα), its human (HsXRIPα) and *Drosophila* (DmXRIPα) EST homologues. *Xenopus* XRIPα was isolated as a protein interacting with the ssDNA-binding domain of the largest subunit of RPA by yeast two-hybrid screening. The sequence of *Xenopus* XRIPα is available from DDBJ/EMBL/GenBank under the accession No. AJ243177. The XRIPα sequences are aligned using the Clustal W program (Thompson *et al*., 1994). Identical amino acids are boxed in black and similar residues are boxed in grey. The Zn finger-like motif is indicated by asterisks.

their nuclear import (Rout *et al*., 1997; Schlenstedt *et al*., 1997). In mammalian cells, importin β itself and importin β-related proteins, transportin, RanBP5 (homologous to Pse1p) and RanBP7, have also been shown to bind directly to ribosomal proteins for the same purpose (Jäkel and Görlich, 1998). Recently, a 45 kDa protein has been identified that interacts specifically with the 5′-trimethylguanosine (m_3G) cap structure of the U snRNA to facilitate the m_3G cap-dependent nuclear import of U snRNPs (Huber *et al*., 1998; see also Palacios *et al*., 1997). Interestingly, this protein called snurportin1 has an IBB domain at its N-terminus and its C-terminal cap-binding domain does not show any structural similarity to the armadillo repeat of importin α. The common feature of these alternative pathways is that their transport does not require importin α, the receptor for the NLS cargo.

In this report, we show that RPA is transported into the nucleus through an alternative pathway that is independent of importin α. We have identified a novel protein of 33 kDa, called XRIPα, that interacts with RPA and is required for the nuclear import of RPA in *Xenopus* egg extracts. We propose that XRIPα works as an adaptor to connect RPA to importin β.

Results

Identification of the RPA-interacting protein XRIPα by two-hybrid screening

Since we were interested in proteins interacting with RPA in *Xenopus* egg extracts (Adachi and Laemmli, 1994), we used two strategies to isolate interacting proteins. One was yeast two-hybrid screening (Fields and Song, 1989) and the other was co-immunoprecipitation with RPA. We prepared different bait constructs by fusing three domains of the $p70^{RPA}$ subunit to the Gal4 DNA-binding domain (GBD). These constructs encompass the N-terminal (N: 1–192), middle (M: 181–422) and C-terminal (C: 391–609) regions of $p70^{RPA}$. A *Xenopus* oocyte cDNA library fused to the Gal4 activation domain was used for screening. Two of the bait constructs, containing the N- and C-terminal region of $p70^{RPA}$, were inappropriate for screening due to a low transformation efficiency of the cells harbouring either plasmid. In contrast, we obtained seven histidine-prototrophic colonies from 2.2×10^6 transformants with the M domain as a bait. Sequence analyses of the seven recovered plasmids showed that they contained the same cDNA.

This cDNA was used for screening the oocyte cDNA library to obtain the full-length cDNA. The longest cDNA contained an open reading frame (ORF) encoding 226 amino acid residues with a calculated mol. wt of 25.8 kDa. This protein is called XRIPα (*Xenopus* RPA-interacting protein α). Database searches showed that XRIPα is not homologous to any proteins of known function. However, we found several homologues in human and *Drosophila* expressed sequence tag (EST) databases (Figure 1). XRIPα and its EST homologues display a similar domain structure. While the N-terminal domain (1–45) is rich in basic residues, the middle (46–140) is acidic and the C-terminal (141–226) domain contains a Zn finger-like motif (C2-C2-CH-C2). The XRIPα cDNA isolated by two-hybrid screening lacked the first 45 amino acids of the basic N-terminal domain, indicating that they are not required for interaction with $p70^{RPA}$.

The interaction of XRIPα with the M domain of $p70^{RPA}$ in yeast was evaluated more quantitatively with a two-hybrid complementation experiment using a *lacZ* reporter gene (Table I). Yeast cells co-transformed with a bait containing the M domain (M-$p70^{RPA}$) and the XRIPα activator fusion protein resulted in a high level of β-galactosidase activity. This level of activity was similar to that obtained with the interacting proteins, p53 and SV40 large T antigen, which were included as controls (Table I). The N- and C-terminal domains of $p70^{RPA}$ gave only a background level of β-galactosidase activities with

Table I. β-galactosidase activity

DNA-binding domain hybrid	Activation-domain hybrid		
	Vector	XRIPα	Large T
Vector	0.16 ± 0.07	0.18 ± 0.01	ND
N-p70^{RPA}	ND	0.19 ± 0.03	ND
M-p70^{RPA}	0.19 ± 0.04	44.2 ± 11.7	ND
C-p70^{RPA}	ND	0.17 ± 0.07	ND
p70^{RPA}	ND	1.87 ± 0.30	ND
p53	ND	ND	44.1 ± 4.60

Activities are indicated in Miller units (Miller, 1972).

XRIPα. It was noteworthy that full-length p70^{RPA} and XRIPα yielded significantly lower β-galactosidase activity. The presumptive weaker interaction between these proteins might arise from improper folding of the full-length p70^{RPA} in the absence of its smaller subunits (p33 and p14; Gomes and Wold, 1995). The interaction between XRIPα and the M domain of p70^{RPA} was confirmed by a GST pull-down assay. Affinity beads loaded with GST–XRIPα protein, in contrast to the GST mock controls, efficiently bound the M domain translated *in vitro* (data not shown).

XRIPα is complexed to RPA in Xenopus egg extracts

Anti-RPA precipitates two other proteins in addition to RPA (Figure 2). One has the mobility of 90 kDa and will be discussed in the next section. The other associated protein migrates as a 33/34 kDa doublet and was identified as XRIPα since antibodies raised against bacterially expressed XRIPα recognized this doublet (Figure 2, lane 4). Its identity as XRIPα was confirmed by *in vitro* translation of the longest XRIPα cDNA in the reticulocyte lysate, which yielded proteins of very similar mobilities in SDS–PAGE (data not shown). Moreover, anti-XRIPα immunoprecipitated RPA as is expected for an interacting protein (Figure 2, lane 6). We conclude the 33/34 kDa doublet represents XRIPα, which was identified above by two-hybrid screening.

A complication of interpreting the above stems from the observation that the affinity-purified anti-XRIPα recognized two additional bands in *Xenopus* egg extracts of 55 and 45 kDa (Figure 2, lane 1; indicated by open triangles) which were not detected by the pre-immune serum (not shown). These proteins did not co-immunoprecipitate with RPA (lane 4) and may simply share epitopes with XRIPα. This question was not pursued further.

Association of the importin β with the RPA–XRIPα complex in Xenopus egg extracts

Figure 2, lane 8 shows the stained gel pattern of proteins precipitated with anti-RPA beads from the high-speed supernatant (HSS) extracts of *Xenopus* eggs. In addition to RPA, a 90 kDa protein (p90) was found reproducibly as a major band. p90 was also detected in precipitates obtained with anti-XRIPα (Figure 2, lane 6 for blotting and lane 9 for stain). Note that the stoichiometry of p90 to p70^{RPA} in the anti-XRIPα precipitate was ~1:1 (Figure 2, lane 9). Proteins were quantified by gel scanning. For quantification of XRIPα, a larger scale of immunoprecipitation was performed to visualize the band more clearly.

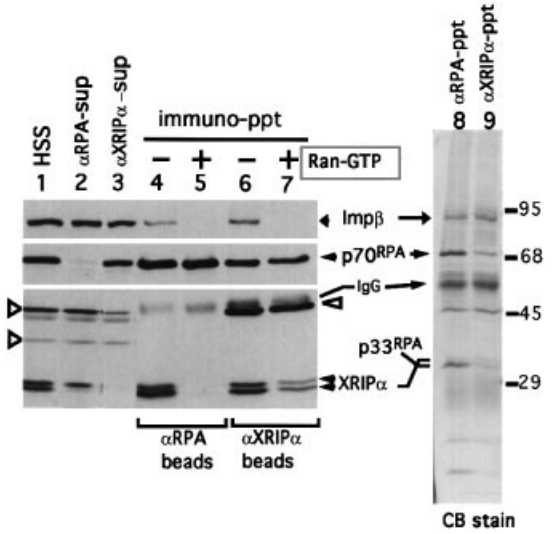

Fig. 2. Complex formation of RPA, XRIPα and p90 importin β in *Xenopus* egg extracts and its regulation by Ran. High speed supernatant (HSS) extracts were subjected to immunoprecipitation using anti-RPA (αRPA) or anti-XRIPα (αXRIPα) antibodies in the presence (+) or absence (–) of RanQ69L-GTP. Supernatant (sup) and pellet fractions (immuno-ppt) were gel electrophoresed and transferred onto a nitrocellulose filter for immunoblotting (lanes 1–7). The filter was cut longitudinally into pieces and probed, from top to bottom, with anti-human importin β, anti-p70^{RPA} and with affinity-purified anti-XRIPα antibodies, respectively. The positions of the various antigens are indicated. Anti-RPA or anti-XRIPα precipitated p90 importin β, RPA and XRIPα (lanes 4 and 6) from HSS extracts. Addition of Ran-GTP dissociated p90 importin β as observed in either immunoprecipitate (lanes 5 and 7). Note that in the presence of Ran-GTP, XRIPα dissociated as well as p90 importin β if anti-RPA is used for immunoprecipitation (lane 5). This is not observed in the case of precipitation with anti-XRIPα (lane 7). XRIPα signals were reproducibly weaker in anti-XRIPα precipitates in the presence of Ran-GTP for unknown reasons. RPA and XRIPα were completely depleted from the supernatant fractions by their respective antibodies under the experimental conditions used (lanes 2 and 3). Lanes 8 and 9 are the Coomassie Blue-stained pattern of proteins immunoprecipitated with anti-RPA (lane 8) or with anti-XRIPα (lane 9) antibodies. The positions of molecular weight markers are indicated in kDa on the right.

Since XRIPα migrates as a doublet and the lower band had the same mobility as p33^{RPA}, the upper band might look fainter than expected in this figure. Note that smaller proteins bind less dye that is approximately proportional to their size.

Tryptic peptide sequence determination of p90 was carried out. A database search with the tryptic peptide sequences suggested that p90 is very closely related to importin β, since the two peptide sequences matched exactly with the human importin β amino acid sequence (TLATWATK and LLETTDRPDGHQNNLR). Moreover, p90 cross-reacted with antibodies against human importin β (Figure 2, lane 4; Görlich *et al*., 1995). We also carefully compared the reactivity of antibodies raised against *Xenopus* importin β with p90 associated with RPA and with *Xenopus* importin β that was partially purified by a zzRanQ69L affinity column. In a quantitative immunoblotting, the same amount of p90 and importin β showed exactly the same extent of reactivity to the anti-*Xenopus* importin β antibodies (data not shown).

Importin β-related proteins form a protein family. A common functional feature of this family is their inter-

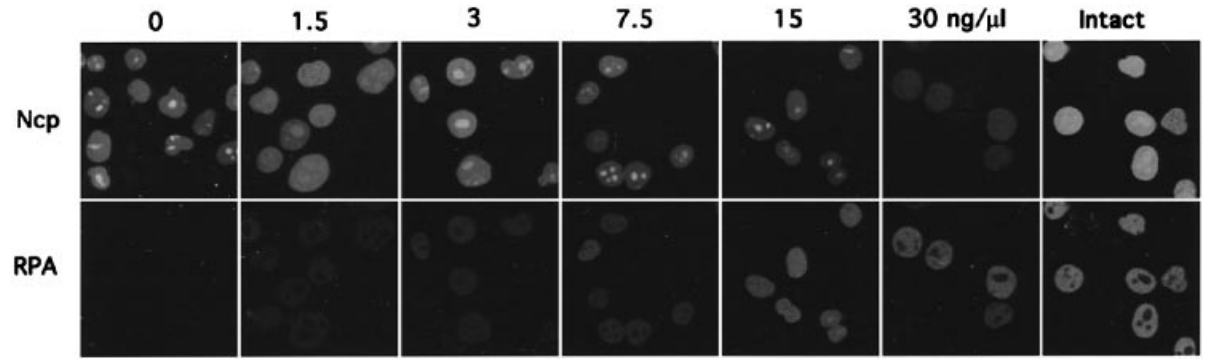

Fig. 3. Nuclear import of RPA requires XRIPα. HSS egg extracts were immunodepleted for XRIPα and the depleted extracts were assayed for their ability to support the import of fluorescein-labelled nucleoplasmin (Ncp) and rhodamine-labelled RPA into the nuclei of permeabilized HeLa cells. Nucleoplasmin and RPA were imported efficiently in untreated control extracts (intact). However, depletion of XRIPα specifically blocked the import of RPA but not that of nucleoplasmin (see panels below 0, Ncp and RPA, respectively). Addition of increasing amounts of recombinant His-XRIPα restored the RPA import activity. The efficiency of the complementation was proportional to the amount of added His-XRIPα (the final concentration of His-XRIPα is indicated above the panels in ng/μl; 30 ng/μl of XRIPα corresponds to 900 nM).

action with Ran-GTP. We examined the interaction of p90 with Ran-GTP by an overlay assay (Coutavas *et al*., 1993; Görlich *et al*., 1997; reviewed in Wozniak *et al*., 1998). Proteins co-immunoprecipitated either with RPA or with XRIPα were separated by SDS–PAGE, transferred to a nitrocellulose filter, renatured and the filter was probed with Ran-[γ-^{32}P]GTP. p90 co-immunoprecipitated with anti-RPA or with anti-XRIPα beads bound to Ran-GTP (data not shown). From these results, we conclude that p90 is *Xenopus* importin β itself or a very closely related protein at least. This was confirmed further by *in vitro* interaction of recombinant importin β with XRIPα as shown in a later section. Hereafter we refer to p90 as importin β.

Dissociation of importin β from RPA and XRIPα by Ran-GTP

Binding of Ran-GTP to importin β dissociates importin β from the importin α–cargo complex (Rexach and Blobel, 1995; Görlich *et al*., 1996b; Chi *et al*., 1996; Izaurralde *et al*., 1997; Jäkel and Görlich, 1998). We examined the effect of Ran-GTP on the co-immunoprecipitation of importin β and RPA with the help of the GTPase-deficient RanQ69L mutant (Klebe *et al*., 1995). GTP complexed to RanQ69L is expected to remain unhydrolysed despite the presence of GTPase-activating proteins in the cytoplasmic *Xenopus* egg extracts.

As shown above, anti-RPA or anti-XRIPα precipitated a complex consisting of RPA, XRIPα and importin β (Figure 2, lanes 4 and 6). We found that addition of Ran-GTP to the extracts dissociated importin β from the above complex (lanes 5 and 7). Unexpectedly, we noted that XRIPα also dissociated from RPA if anti-RPA (but not anti-XRIPα) was used in the immunoprecipitation (lanes 3 and 7). These results indicated that the association of importin β with XRIPα and RPA is controlled by Ran-GTPase.

XRIPα is required for the nuclear import of RPA in Xenopus egg extracts

The dissociation of XRIPα from RPA in the presence of RanQ69L-GTP suggested a possible role for this protein in nuclear import of RPA. We examined this possibility using the *in vitro* import assay system based on permeabilized cultured cells (Adam *et al*., 1990). Human RPA was purified from *Escherichia coli* cells expressing all three subunits (Henricksen *et al*., 1994) and fluorescently labelled with rhodamine. We confirmed the interaction of the purified human RPA with *Xenopus* XRIPα by a GST–XRIPα pull-down assay (see below; Figure 6A, lane 2). To assay for nuclear transport, HeLa cell membrane was permeabilized with a low concentration of digitonin and then the cells were incubated with rhodamine-labelled RPA in the HSS extracts. Nuclear transport was examined by fluorescence confocal microscopy. Fluorescein-labelled nucleoplasmin, a classical NLS-containing substrate, was used as a control. Under standard conditions, RPA and nucleoplasmin were imported into nuclei efficiently. RPA import was blocked by incubation at 4°C, by addition of wheat germ agglutinin, by depletion of ATP through apyrase treatment, and by RanQ69L (3 μM), as shown previously for NLS-containing substrates (data not shown).

The HSS extracts were immunodepleted for XRIPα (see Figure 2, lane 3). The depletion efficiency was estimated to be >90% (data not shown). Nuclear transport of RPA was strongly blocked in the extracts depleted for XRIPα (Figure 3, panel 0, RPA; compare with Intact). Mock-treated extracts transported RPA as efficiently as the intact HSS extracts (data not shown). Significantly, nuclear import of nucleoplasmin added to the same reaction mixture remained unaffected by depletion of XRIPα (panel 0, Ncp; the slight reduction compared with the intact extract was due to an ~2-fold dilution of the extract by the depletion procedure).

Since anti-XRIPα beads precipitated not only XRIPα but also other proteins (e.g. Figure 2, lane 9), it was possible that the block of the RPA import was due to loss of other essential factors in the extracts. To exclude this possibility, we examined whether addition of purified XRIPα would restore RPA import. His-tagged XRIPα was expressed in *E.coli*, partially purified and tested by adding to the XRIPα-depleted extracts (Figure 3). We observed that XRIPα at a concentration of 15 ng/μl (450 nM) restored the import level to that of the intact extracts (panel 15, RPA). This concentration approximately matched the XRIPα concentration of intact extracts (data not shown). Interestingly, we noted some reduction of

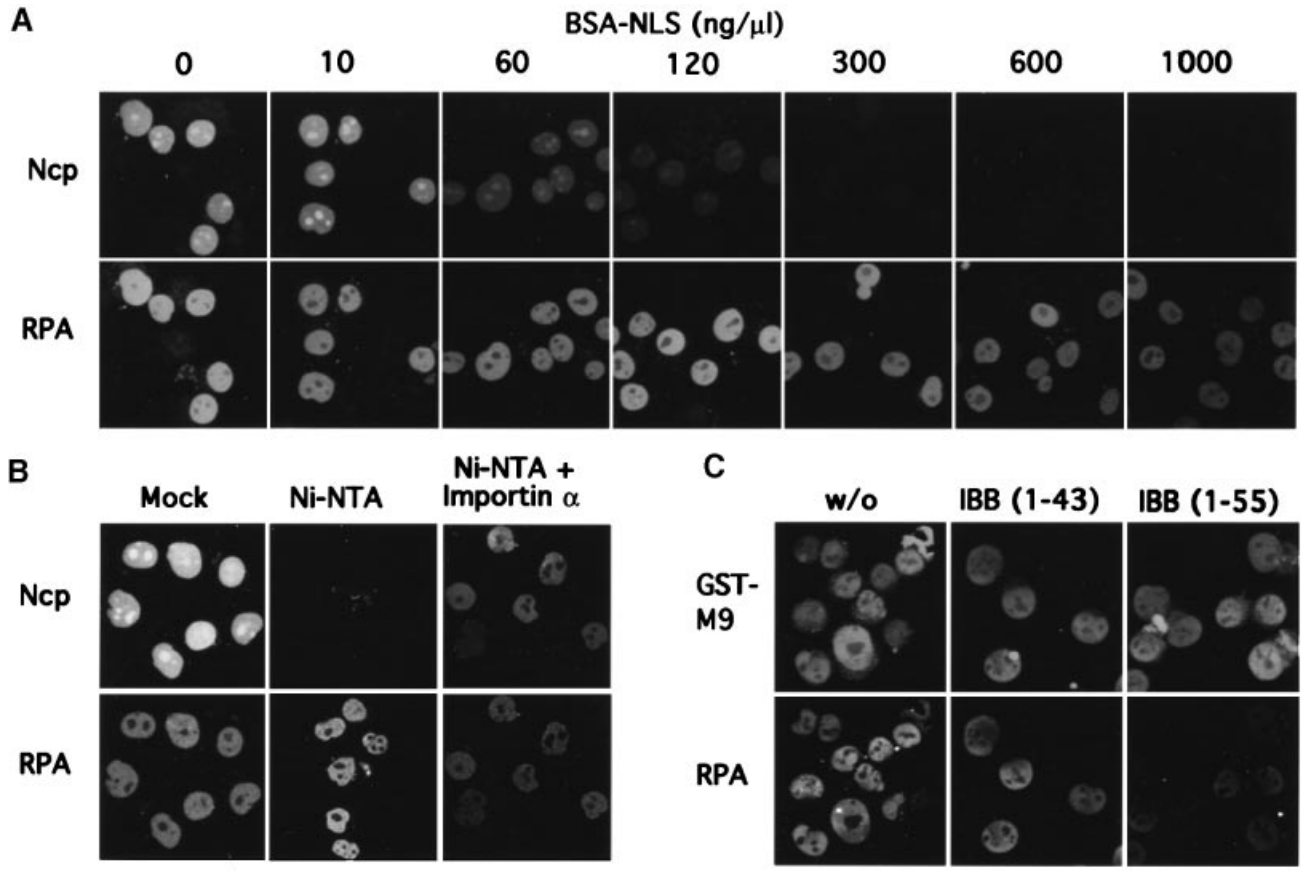

Fig. 4. Nuclear import of RPA does not require importin α and is independent of the classical NLS. (**A**) Increasing amounts of BSA conjugated with the NLS peptides of SV40 large T antigen (BSA-NLS) were added to HSS extracts that were then assayed for import of fluorescein-labelled nucleoplasmin (Ncp) or rhodamine-labelled RPA into the HeLa cell nuclei. The amounts of BSA-NLS are indicated as the final concentration in ng/μl. Import of nucleoplasmin (Ncp) was suppressed gradually by addition of increasing amounts of BSA-NLS. In contrast, RPA import was significantly more resistant to the competitor. (**B**) Importin α is known to be depleted from the extracts by Ni-NTA bead treatment. The HSS extracts treated with Ni-NTA beads were unable to support nuclear import of nucleoplasmin. Addition of recombinant importin α restored the import of nucleoplasmin significantly. Again RPA import was not reduced in Ni-NTA-treated extracts, indicating that RPA import does not require importin α. Control extracts (Mock) were treated with Sepharose beads. (**C**) The IBB domain (1–55), its deletion derivative (1–43) or buffer alone (w/o) were added to HSS extracts that were assayed for import of rhodamine–RPA or fluorescein-labelled GST–M9 fusion protein. IBB (1–55) specifically blocked import of RPA but not that of GST–M9 that is mediated by another transporter transportin.

nucleoplasmin import in the extracts supplemented with excess His-XRIPα (panel 30 ng/μl, Ncp).

Nuclear import of RPA is independent of importin α but requires XRIPα and importin β

The results described above showed that nuclear import of RPA requires XRIPα. Although the N-terminal domain of XRIPα was basic, it did not appear to contain a clear classical NLS motif (Figure 1). We examined whether nuclear import of RPA occurred through the classical NLS using two approaches: competition with an NLS-containing protein and depletion of importin α. If RPA was imported by interaction with another NLS-containing protein, its import should be reduced by addition of an excess of this competitor (Pollard *et al*., 1996). The experiment demonstrated that this was not the case, i.e. import of RPA was not blocked even at the highest competitor dose of 1000 ng/μl bovine serum albumin (BSA)-NLS (Figure 4A, RPA; 300, 600 and 1000 BSA-NLS). In contrast, import of nucleoplasmin (Ncp) was significantly reduced or nearly completely blocked at BSA-NLS doses of 300 and 600 ng/μl, respectively (Figure 4A, Ncp; 300 and 600 BSA-NLS).

These competition experiments suggested that nuclear import of RPA is independent of the classical NLS. This was confirmed by depletion of importin α. Görlich *et al*. (1994) had shown that Ni-NTA–Sepharose treatment of HSS extracts blocked the classical import pathway due to depletion of importin α. Indeed, little nucleoplasmin was imported into the nucleus in these depleted extracts, and addition of recombinant importin α significantly restored import of nucleoplasmin (Figure 4B, Ncp; Ni-NTA and Ni-NTA + importin α, respectively). In contrast, RPA was imported into the nucleus efficiently in the extract depleted for importin α (Figure 4B, RPA; Ni-NTA). Addition of importin α to the extract treated with Ni-NTA led to a slight reduction of RPA import (Figure 4B, RPA; Ni-NTA + importin α). Therefore, nuclear import of RPA does not require either a classical NLS motif or importin α.

Association of importin β with RPA–XRIPα complex suggested that it is required for RPA nuclear import. We examined this further by competition experiment with the IBB domain of importin α (Figure 4C); this domain was used previously as a competitive inhibitor of nuclear import mediated by importin β (Görlich *et al*., 1996a; Weis *et al*., 1996). Addition of the IBB domain to the

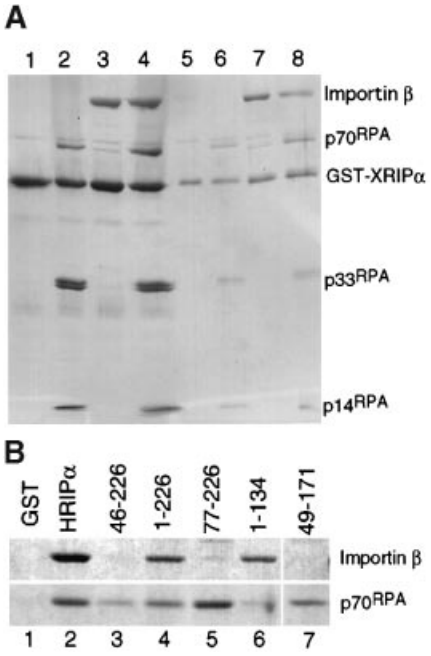

Fig. 5. Pull-down assay with GST–XRIPα and its deletion constructs. (**A**) Purified recombinant trimeric RPA (lanes 2, 4, 6 and 8) and importin β (lanes 3, 4, 7 and 8) were incubated with GST–XRIPα. GST–XRIPα and the proteins bound to it were recovered by glutathione–beads, separated on an SDS–gel and stained with Coomassie Blue. Lanes 1–4 are proteins recovered with the beads, and lanes 5–8 are unbound supernatant fractions. Lanes 1 and 5 are GST–XRIPα alone. RPA and importin β bound to GST–XRIPα and were recovered with the beads. (**B**) Deletion derivatives of XRIPα were fused to GST and their interactions with importin β and RPA were examined by pull-down assay. The proteins recovered with the GST fusions are shown. GST did not show any affinity for importin β and RPA (lane 1). Full-length XRIPα (lane 4) and its human homologue (HRIPα; lane 2) bound both RPA and importin β. XRIPα deletions lacking the N-terminal basic domain failed to bind importin β (46–226, lane 3; 77–226, lane 5; 49–171, lane 7). For the interaction with RPA, region 49–171 of XRIPα was sufficient (lane 7). The 1–134 derivative showed a significantly weaker interaction with RPA (lane 6).

HSS extracts (20 μM) significantly interfered with RPA import (Figure 4C, RPA, IBB 1–55). Import of nucleoplasmin which is mediated by importin α/β complex was also inhibited by the IBB domain (Weis *et al*., 1996; data not shown). A deletion mutant of the IBB domain (1–43) that is incapable of interacting with importin β failed to block RPA import (Görlich *et al*., 1996a; Figure 4C, RPA, IBB 1–43). GST fused to the M9 domain of hnRNP A1 served as a control, demonstrating the specificity of the inhibition. Since nuclear import of GST–M9 is mediated by transportin but not by importin β (Pollard *et al*., 1996), its import was not affected by the IBB domain under the same experimental conditions (Figure 4C, GST–M9).

XRIPα interacts directly with importin β as well as with RPA in vitro

Complex formation of XRIPα, RPA and importin β was examined further *in vitro* by pull-down assay with GST–XRIPα fusion protein and its derivatives. GST–XRIPα efficiently bound to purified human RPA (Figure 5A, lane 2), confirming its direct interaction with RPA. Next we examined whether XRIPα interacts with importin β directly or through RPA. GST–XRIPα could co-precipitate recombinant human importin β in the absence of RPA, indicating that XRIPα interacts with importin β directly (Figure 5A, lane 3). When importin β and RPA were added together, nearly stoichiometric amount of the proteins were recovered with GST–XRIPα (lane 4), suggesting formation of a trimeric complex. Next we examined whether XRIPα co-precipitates with importin β from HSS extracts in the absence of RPA as follows: RPA was depleted quantitatively from the HSS extracts with anti-RPA beads and then the RPA-depleted extracts were incubated with anti-XRIPα beads. We detected a significant amount of importin β in the immunoprecipitates obtained with anti-XRIPα despite the depletion of RPA (data not shown).

Since the amino acid sequence of XRIPα appears to be separated into three domains, we were interested in examining domains required for the interactions with importin β or with RPA. GST fusions were constructed with deletion mutants of XRIPα and these derivatives were used for pull-down assay with recombinant importin β and RPA (Figure 5B). Deletion mutants of XRIPα lacking the first 45 residues failed to bind importin β (Figure 5B, lanes 3, 5 and 7), indicating that the N-terminal basic domain is required for the interaction. Amino acid residues 1–134 were sufficient to pull-down importin β (lane 6). In contrast, the N-terminal 76 residues were dispensable for the interaction with RPA (lane 5, 77–226). Deletion of residues 135–226 significantly reduced the affinity for RPA (lane 6, 1–134). The construct (49–171) which contains the central acidic domain and a part of Zn finger-like motif was sufficient for efficient retention of RPA (lane 7).

Reconstitution of RPA import from recombinant proteins

Above we established by depletion experiments that import of RPA is dependent on XRIPα but not on importin α. We demonstrated next that this process could be reconstituted with purified recombinant proteins. In the experiment shown (Figure 6), fixed amounts of fluorescently labelled RPA were mixed with different concentrations of XRIPα and importin β, and the efficiency of its import into the nuclei of permeabilized HeLa cells was compared with that mediated by an HSS extract (HSS; Figure 6J); a negative control is also shown (No factors; Figure 6K). No significant RPA import was observed with importin α and β alone over a variety of concentrations (data not shown). In contrast, optimal import was achieved, matching that obtained with HSS, with XRIPα and importin β at concentrations of 300 and 50 nM, respectively (compare Figure 6B and J). Stimulation by importin β appeared to have a narrow concentration optimum since we noticed some reduction of RPA import if this protein was added at higher levels (150 nM, Figure 6C). This reduction may arise by the inhibitory effect of breakdown products of importin β on nuclear import through pores (Kutay *et al*., 1997).

The efficiency of RPA import was dependent at a lower concentration of XRIPα (300 nM) on addition of importin β (Figure 6A–C). In contrast, at a higher concentration of XRIPα (700 nM), RPA import became largely independent of importin β addition (Figure 6D–F). Despite this observation, one cannot conclude that importin β is dispensable. This independence is probably

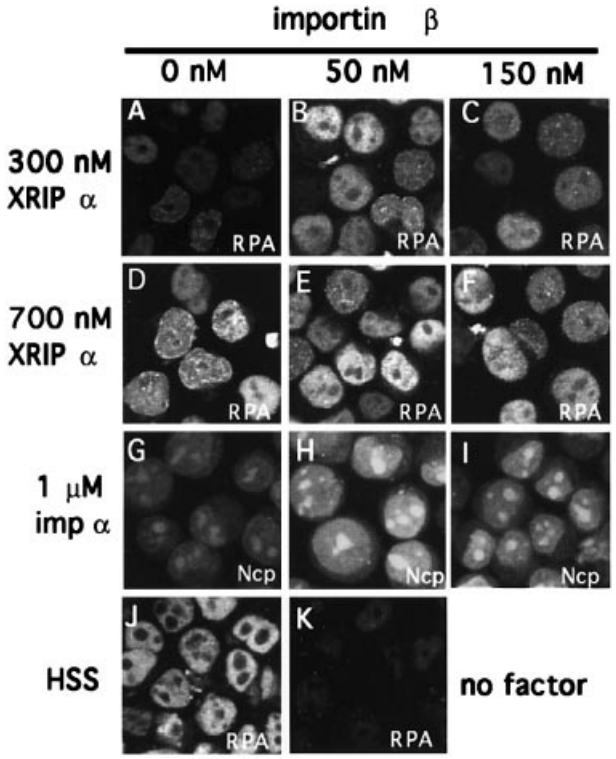

Fig. 6. Reconstitution of RPA import using recombinant proteins. Nuclear import of rhodamine-labelled RPA (400 nM) into permeabilized HeLa nuclei was studied using different concentrations of purified recombinant XRIPα and importin β. (**A**–**C**) Import of RPA using a fixed concentration of XRIPα (300 nM) and different concentrations (0, 50 and 150 nM) of importin β. (**D**–**F**) Import of RPA using a fixed concentration of XRIPα (700 nM) and different concentrations (0, 50 and 150 nM) of importin β. (**G**–**I**) Import of fluorescein-labelled nucleoplasmin (400 nM) using a fixed concentration of importin α (1 mM) and different concentrations (0, 50 and 150 nM) of importin β. (**J**) RPA import mediated by the HSS extract. (**K**) Control for RPA import with no factors added.

due to the endogenous importin β content of the permeabilized HeLa cells detected by immunoblotting (data not shown). This phenomenon was also noted previously (Görlich *et al*., 1994, 1995).

Included in Figure 6 are import controls with nucleoplasmin. Addition of importin α alone resulted in basal level nuclear import of nucleoplasmin (Figure 6G). Simultaneous addition of importin β greatly stimulated the transport of nucleoplasmin, as had been observed previously (Figure 6H and I; Görlich *et al*., 1995).

Discussion

Nuclear import of proteins containing a classical NLS motif involves complex formation with importin α. Here we have demonstrated that nuclear transport of RPA is independent of importin α but instead requires a novel protein, XRIPα.

We have isolated XRIPα in a two-hybrid screen using the middle region of the largest subunit of RPA (p70), which harbours the ssDNA-binding domain of RPA, as a bait. The N-terminal basic domain of XRIPα is not required for the interaction with RPA, since this domain was absent in the originally isolated two-hybrid clone which expressed only the acidic middle and the C-terminal Zn finger domains. Purified RPA is also retained on a GST–XRIPα affinity column, indicating that it directly interacts with XRIPα (Figure 5). XRIPα migrates as a 33/34 kDa doublet in SDS–PAGE. XRIPα expressed in bacteria also migrates as a doublet. It would be interesting to study the nature and function of the presumed protein modification that is responsible for this appearance.

Complex formation between RPA and XRIPα in *Xenopus* egg extracts was revealed by immunoprecipitation. Interestingly, ssDNA appears to dissociate the XRIPα–RPA complex. This notion stems from the following observation: if RPA is purified by DNA–Sepharose chromatography, it is devoid of XRIPα and all of it is found in the flowthrough fraction (data not shown). This mutually exclusive interaction of XRIPα and ssDNA with RPA may facilitate rapid dissociation of the XRIPα–RPA complex inside the nucleus when RPA needs to execute its function as a ssDNA-binding protein.

Besides XRIPα, RPA is also complexed with a 90 kDa protein (p90) in egg extracts, which we identified as *Xenopus* importin β. Complex formation between nearly stoichiometric amounts of RPA, XRIPα and importin β in cytoplasmic extracts of *Xenopus* eggs is quite reminiscent of previously observed complex formation of karyophilic proteins with the classical NLS and importin α/β (Imamoto *et al*., 1995; Radu *et al*., 1995).

Importin β is very likely to play a role in the RPA import scheme analogous to that played by this protein in the classical NLS transport pathway together with importin α (Figure 7). This is strongly supported by our observation that the interaction of importin β with the XRIPα–RPA complex is under the regulation of Ran. Addition of RanQ69L-GTP to egg extracts led to the dissociation of importin β from the XRIPα–RPA complex. Curiously, XRIPα is also dissociated from RPA in the presence of RanQ69L-GTP, if examined by immunoprecipitation with anti-RPA but not with anti-XRIPα (Figure 2, lanes 4 and 5). This unexpected observation may not be physiological and could be due to a weakening of the XRIPα–RPA interaction in the presence of anti-RPA antibodies and Ran-GTP.

We provided direct evidence that nuclear import of RPA requires XRIPα but not importin α. The egg extracts depleted for XRIPα failed to transport RPA, although they were competent to transport nucleoplasmin and RPA import could be restored efficiently by adding back recombinant XRIPα. In contrast, the extracts depleted for importin α, although competent to import RPA, were defective for import of nucleoplasmin that could be complemented by adding back importin α. Interestingly, we reproducibly observed a reduction in nuclear import of RPA in extracts supplemented with exogenous importin α (see Figure 4B). Conversely, addition of increasing amount of XRIPα led to a reduction of nucleoplasmin import (Figure 3). These interference effects are likely to be due to a competitive and mutually exclusive interaction of XRIPα or importin α with the common component, importin β, of either import pathway. These observations further support the proposed scheme depicted in Figure 7. This was established more directly by reconstitution of RPA nuclear import into permeabilized cells using purified recombinant components (Figure 6). We reproducibly observed significant stimulation of RPA

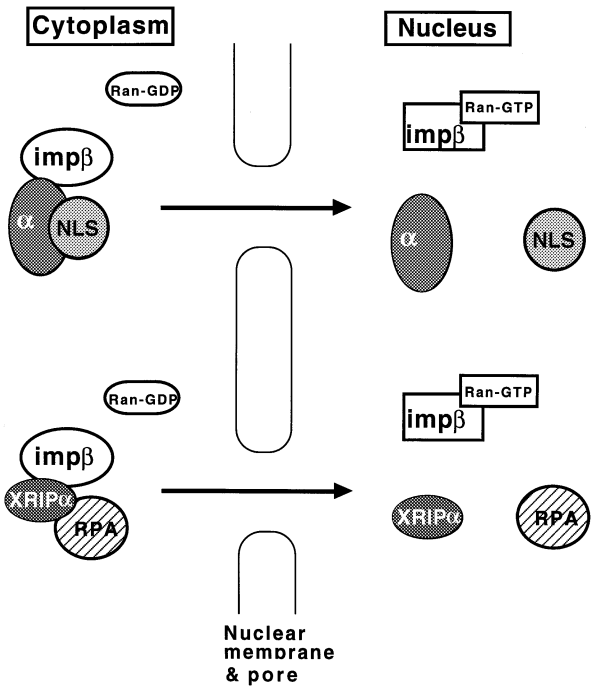

Fig. 7. Nuclear import of RPA and XRIPα. The upper part of the Figure shows the classical NLS pathway mediated by the importin α/β heteromeric complex. In the cytoplasm, Ran is in the GDP-bound form because of the biased localization of RanGAP in the cytoplasm. Importin β complexes with importin α serve as an adaptor molecule connecting the NLS-containing protein and importin β. In the nucleus, Ran is in the GTP-bound form due to the biased localization of GTP-exchange factor or RCC1, and the binding of Ran-GTP to importin β induces the release of importin α and the NLS protein. The lower part shows the nuclear import of RPA in *Xenopus* egg extracts. In the cytoplasm, RPA forms a complex with XRIPα. XRIPα interacts with importin β that mediates the import of the complex. Inside the nucleus, binding of Ran-GTP to importin β induces release of the RPA–XRIPα complex. XRIPα is dissociated from RPA upon RPA binding to ssDNA since they are sharing the common binding site on RPA.

import by recombinant XRIPα. At an appropriate concentration of XRIPα, co-addition of importin β strongly enhanced the transport efficiency of RPA, as was observed for NLS proteins and importin α/β (Görlich *et al*., 1994, 1995). XRIPα alone, if added at a higher concentration, supported RPA import efficiently (Figure 6D–F). It is likely that the significant endogenous content of importin β of permeabilized cells is sufficient to promote this import. Titration experiments of importin β with the IBB domain have provided the means to address this notion further (Görlich *et al*., 1996a). We found that import of RPA was blocked specifically by the IBB domain competitor (Figure 4C), demonstrating that importin β is a mediator of RPA import.

Expression of XRIPα is not restricted to *Xenopus* eggs, since we noted homologous human ESTs derived from uterus, germ cell tumour, heart, liver–spleen and neurons (data not shown). XRIPα is also conserved in *Drosophila* (Figure 1). This conservation suggests that RPA import could also be mediated by XRIPα in these systems. Interestingly, we did not detect any obvious homologues in budding yeast, *Saccharomyces cerevisiae*. Perhaps, in yeast, a different pathway imports RPA.

GST–XRIPα pull-down experiments using recombinant importin β established that interaction of XRIPα with importin β does not require RPA (Figure 5). This indicates that XRIPα can function as an adaptor to link RPA and importin β (Figure 7). Recently emerging evidence indicates that nuclear import pathways of karyophilic proteins could be divided into two categories. One is the direct interaction of import cargoes with the importin β-related proteins (transporter) and the other is the indirect interaction of cargoes with the transporters through adaptor molecules. A direct interaction was first demonstrated for the hnRNP A1 protein and transportin (Pollard *et al*., 1996). A direct interaction of the ribosomal proteins L23a, S7 and L5 with importin β, transportin, RanBP5 and RanBP7 was established (Jäkel and Görlich, 1998). Also, the yeast poly(A)$^+$ RNA-binding protein, Nab2p, cyclin B1, HTLV Rex and HIV Rev proteins were shown to bind directly to importin β (Henderson and Percipalle, 1997; Truant *et al*., 1998; Moore *et al*., 1999; Palmeri and Malim, 1999; Truant and Cullen, 1999). Importin α is the first adaptor molecule that represents the latter category of indirect interaction between cargoes and transporters. Several additional adaptor molecules for the classical NLS have been identified (Miyamoto *et al*., 1997). Recently identified snurportin 1 also belongs to this category and links m_3G cap and importin β. XRIPα could be classified as a novel adaptor molecule of this latter category.

Deletion analysis showed that the N-terminal domain of XRIPα (1–45) is required for its interaction with importin β. The N-terminal domain is basic (isoelectric point 11.4) and arginine rich (9/45). However, these arginines do not form continuous stretches of basic residues that are, in contrast, found in the IBB domain of importin α and in arginine-rich domains of HTLV Tat and HIV Rev which bind directly to importin β. Comparison of import signals of the ribosomal proteins that bind directly to β-like transporters has indicated that they are very basic but cannot be narrowed down to a discrete amino acid stretch (Jäkel and Görlich, 1998). Recently cyclin B1 (121–397) has been shown to interact directly with importin β (Moore *et al*., 1999). The N-terminal domain of XRIPα does not show any significant sequence similarities either to the import signals of ribosomal proteins or to cyclin B1 (121–397), suggesting that the interaction of XRIPα with importin β might also be structurally complex.

XRIPα, RPA and importin β are the major proteins recovered in the immunoprecipitates with anti-XRIPα antibodies, and the molar ratio between the proteins is ~1:1 (Figure 2, lane 9). RPA is found to be the main interaction partner for XRIPα since immunodepletion of RPA also removes most of XRIPα from the extracts (Figure 2, lane 2). Thus, XRIPα appears to be an RPA-dedicated nuclear import protein. Consequently, it will be of interest to ask whether XRIPα shuttles between the nucleus and the cytoplasm.

Materials and methods

Cloning and sequencing of XRIPα

The cDNA sequences corresponding to the three domains (1–192, 181–422 and 391–609) of the largest 70 kDa subunit of *Xenopus* RPA were amplified by PCR using *Pwo* polymerase and appropriate primer pairs that introduced a 5′ *Eco*RI and a 3′ *Bam*HI site. The amplified fragments were cloned into the *Eco*RI–*Bam*HI sites of pAS2-1 (*TRP1*) so as to make an in-frame fusion with the GAL4 DNA-binding domain (from the N-terminus, pEA1, 2 and 3, respectively). Yeast cells (Y190, *his3*-

200, leu2-3, trp1-901; Harper *et al*., 1993) harbouring each of these bait plasmids were transformed with a *Xenopus* oocyte cDNA library in pGAD10 (*LEU2*; Clontech). Yeast transformation was performed using the lithium acetate procedure (Ito *et al*., 1983). Seven His prototrophic colonies were obtained in the presence of 25 mM 3-amino-1,2,4-triazole from 2.2×10^6 *LEU2* transformants of Y190 harbouring pEA2 containing the middle ssDNA-binding domain of $p70^{RPA}$. The Y190 cells harbouring either pEA1 or pEA3 showed significantly lower transformation efficiency ($2–3\times10^3$/μg DNA) and were not used for screening. Plasmids (i.e. pEA19 consisting of pGAD10 and an *Eco*RI fragment of cDNA encoding the part of XRIPα after the 46th E or Glu) were recovered from yeast cells by complementation of the *leuB* mutation of *E.coli* (M1066) using electroporation. The *Eco*RI insert of the cDNA was excised, labelled with [α-^{32}P]dCTP by random priming and used as a probe for screening an oocyte cDNA library in λgt10 (6×10^5 plaques) to obtain a full-length cDNA (Rebagliati *et al*., 1985). The longest cDNA encoding XRIPα (0.83 kb) was subcloned into the *Eco*RI site of pBluescript SK– (pEA30) and sequenced using a dye terminator cycle sequencing kit and a sequencer (Applied Biosystem). Database searches were carried out using the TBLASTN program (Altschul *et al*., 1997).

Antibody preparation, immunoprecipitation and Western blotting

cDNA encoding *Xenopus* importin β was cloned by PCR using degenerated oligonucleotides designed according to the peptide sequence information of p90 co-immunoprecipitated with RPA. The PCR fragment was used to screen a *Xenopus* oocyte cDNA library (Rebagliati *et al*., 1985). The longest cDNA had an ORF of 774 amino acid residues lacking the N-terminal end (unpublished results). The 1.4 kb *Bgl*II–*Eco*RI fragment including the ORF was cloned in pGEX-3X to yield a GST fusion protein that was used for antisera production. The 0.7 kb *Eco*RI fragment of the two-hybrid clone or pEA19 was cloned in pRSETb (pEA26), yielding 180 His-tagged C-terminal amino acids of XRIPα. The protein was expressed in BL21(DE3) with pLysS and found to be mostly insoluble. The proteins were eluted from the SDS–gel by diffusion and used as an antigen to immunize rabbits following the standard protocol except using poly(A)·poly(U) as an adjuvant (Adachi and Laemmli, 1992; Harlow and Lane, 1988; Hovanessian *et al*., 1988).

Immunoprecipitation/depletion was performed essentially as described previously (Adachi and Laemmli, 1994). Protein A–Sepharose CL4-B (Pharmacia Biotech) was swollen and washed three times with phosphate-buffered saline (PBS). The resin was divided into aliquots and mixed with an equal volume of antiserum against *Xenopus* RPA, XRIPα or pre-immune serum in Eppendorf tubes. The mixtures were incubated at room temperature for 1 h on a rotator. The resins were washed three times with 30 vols of PBS and the antibodies were cross-linked with dimethylpimelimidate as described (Harlow and Lane, 1988). Antibody beads were washed twice with 30 vols of 10 mM HEPES–KOH, pH 7.5, 2.5 mM magnesium acetate, 50 mM potassium acetate, 250 mM sucrose 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (IXB buffer). One (for anti-XRIPα) or three (for anti-RPA) volumes of the HSS egg extracts supplemented with an ATP-regenerating system were added to the protein A–Sepharose coupled as described above and incubated at 4°C for 1 h on a rotator. The Sepharose was pelleted in an Eppendorf microfuge for 5 s and the supernatant was collected. Antibody beads were washed five times with 30 vols of IXB buffer with 0.02% NP-40. The proteins were eluted from the beads either with 10 mM glycine-HCl, pH 2.3, 500 mM NaCl or with SDS–PAGE sample buffer.

SDS–PAGE was carried out as described (Laemmli, 1970). Western blotting was performed as described (Towbin *et al*., 1979) using a 1:250 dilution of rabbit anti-human importin β antibodies (generous gift from Dr D.Görlich), a 1:250 dilution of rabbit anti-$p70^{RPA}$ antibodies or a 1:250 dilution of rabbit anti-XRIPα antibodies. For the identification of XRIPα in the crude egg extracts, anti-XRIPα antibodies were affinity purified with a strip of nitrocellulose on which 100 μg of the recombinant protein had been immobilized (Smith and Fisher, 1984; Adachi and Yanagida, 1989). Peroxidase-conjugated anti-rabbit IgG antibodies (Sigma) were used as a secondary antibody. Enhanced chemiluminescence (ECL; Amersham) was used for detection. Protein concentration was estimated by the method of Bradford (1976) or by scanning the SDS–gel stained with Coomassie Blue. BSA was used as a standard.

Expression and purification of recombinant proteins

The *Xenopus* XRIPα coding sequence was amplified by PCR using *Pwo* proofreading DNA polymerase and primers that introduced an *Xho*I site at the 5′ end and an *Eco*RI site after the stop codon. The fragment was cloned into the *Xho*I–*Eco*RI sites of pRSET-b (Invitrogen), allowing the production of His-tagged XRIPα in *E.coli*. The *E.coli* cells were grown at 37°C to an OD_{600} of 0.6 and were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h. The cells were pelleted, resuspended in 20 mM HEPES–KOH, pH 7.5, 300 mM NaCl, 10 mM imidazole, 2 mM PMSF, 2 mM β-mercaptoethanol, and disrupted by sonication. His-XRIPα was purified from the clarified lysate on Ni-NTA–agarose. Peak fractions were pooled, dialysed against nuclear import buffer (20 mM HEPES–KOH, pH 7.5, 110 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose) and stored at –70°C. Recombinant human RPA was prepared as described for p11d-tRPA by co-expression of the three subunits of RPA in *E.coli* (Henricksen *et al*., 1994). RanQ69L was expressed with an N-terminal His tag, and purified in HEPES buffer on Ni-NTA–agarose (Görlich *et al*., 1994). The bound nucleotide was exchanged for GDP using the EDTA method and the final purification was achieved on SP-Sepharose FF (Pharmacia; Klebe *et al*., 1993). RanQ69L was labelled with [γ-^{32}P]GTP and was used for overlay blot assay according to Görlich *et al*. (1997). The expression and purification of His-tagged nucleoplasmin, IBB domain, human importin β, *Xenopus* importin α and the GST–M9 fusion were essentially as described (Görlich *et al*., 1994, 1995, 1996a,b; Pollard *et al*., 1996).

Preparation of protein conjugates

Nucleoplasmin and GST–M9 were fluorescently labelled with fluorescein isothiocyanate (stock, 1 mg/ml in DMSO) in 0.1 M sodium borate, pH 9. RPA was conjugated with carboxytetramethyl-rhodamine succinimidyl ester (Molecular probe; stock solution, 1 mM in DMF). The molar ratio was ~1:1 and incubations were performed for 45 min at room temperature. Free fluorophore was removed by gel filtration on Bio-Gel P-6 DG (Bio-Rad) equilibrated with 10 mM HEPES–KOH, pH 7.5, 50 mM potassium acetate, 100 mM sucrose. SV40 NLS peptide (cgggPKKKRKVED) was conjugated to SMCC-activated BSA as described (Melchior *et al*., 1993). The molar ratio of coupling was 20–30 NLS peptides per molecule as estimated from the electrophoretic mobility.

Nuclear import assay with permeabilized HeLa cells

The basic methods for preparation of permeabilized HeLa cells and for import reactions were as described (Adam *et al*, 1990; Görlich *et al*., 1994). Superovulation of *Xenopus* and the egg extract preparation were performed as described (Finlay and Forbes, 1990; Murray, 1991; Adachi and Laemmli, 1994). The HSS egg extracts were used as a cytosol source. XRIPα depletion was performed by incubating the HSS egg extracts with anti-XRIPα beads for 1 h at 4°C as described above. Importin α was depleted from egg extract by incubation of the HSS extracts with Ni-NTA–agarose in 10 mM HEPES–KOH, pH 7.5, 50 mM potassium acetate, 2.5 mM magnesium acetate, 250 mM sucrose with a ratio of 200 μl of egg extract per 100 μl of Ni-NTA resin, for 2 h at 4°C on a rotating wheel. Unless indicated otherwise, transport substrates were added at a final concentration of 15 μg/ml for nucleoplasmin–fluorescein, 30 μg/ml for GST–M9–fluorescein and 12 μg/ml for RPA–rhodamine; import reactions with cytosolic extracts were supplemented with an energy-regenerating system (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine kinase) and performed at 23°C for 45 min. His-tagged IBB domain (1–55) or its deletion derivative (1–43) were added to 20 μM in competition experiments. For reconstitution of the import reaction using purified components, permeabilized cells were pre-incubated with Ran-GDP for 10 min, then added with an equal volume of import substrates/transporter mixture and incubated for another 15 min at 23°C. Samples were fixed with 4% paraformaldehyde for 5 min on ice and spun through a 30% sucrose cushion in tubes containing polylysine-coated coverslips at the bottom (Görlich *et al*., 1994). After centrifugation (1000 *g* for 10 min), the coverslips were rinsed briefly with water and mounted on Vectashield mounting medium containing DAPI (Vector). The pictures were taken using a Leica TCS NT confocal microscope, capture settings being identical for all images.

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