The Nuclear Import of RCC1 Requires a Specific Nuclear Localization Sequence Receptor, Karyopherin α3/Qip*

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RCC1 is the only known guanine nucleotide exchange factor for the small GTPase Ran and is normally found inside the nucleus bound to chromatin. In order to analyze in more detail the nuclear import of RCC1, we created a fusion construct in which four IgG binding domains of protein A were fused to the amino terminus of human RCC1 (pA-RCC1). Surprisingly, we found that neither Xenopus ovarian cytosol nor a mixture of recombinant import factors (karyopherin α2, karyopherin β1, Ran, and p10/NTF2) were able to support the import of pA-RCC1 into the nuclei of digitonin-permeabilized cells. Both, in contrast, were capable of supporting the import of a construct containing another classical nuclear localization sequence (NLS), glutathione S-transferase-green fluorescent protein-NLS. Subsequently, we found that only one of the NLS receptors, karyopherin α3 (Kapα3/Qip), would support significant nuclear import of pA-RCC1 in permeabilized cells, while members of the other two main classes, Kap1 and Kap2/β2, would not. Accordingly, in vitro binding studies revealed that only Kapα3 showed significant binding to RCC1 (unlike Kapα1 and Kapα2) and that this binding was dependent on the basic amino acids present in the RCC1 NLS. In addition to Kapα3, we found that the nuclear import of pA-RCC1 also required both karyopherin β1 and Ran.

RCC1 (regulator of chromosome condensation) plays a critical role in the eukaryotic cell, as it is the only known guanine nucleotide exchange factor for the small GTPase Ran. Ran plays a key role in many diverse nuclear transport pathways (1), and both Ran and RCC1 have also recently been implicated in mitotic spindle formation (2–6). RCC1 is located inside the nucleus bound to chromatin, except at mitosis when RCC1 dissociates from the chromatin (7, 8). The majority of RCC1 consists of seven internal amino acid repeats, and its crystal structure has revealed that these repeats form a seven-bladed propeller (9).

RCC1 catalyzes guanine nucleotide exchange on Ran, but unlike many guanine nucleotide exchange factors it shows no preference for catalyzing exchange on RanGDP versus RanGTP in vitro (10). The concentration of GTP in the cell is approximately 30 times higher than the GDP concentration, and this is thought to result in the preferential production of RanGTP inside the nucleus (1). In contrast to the nuclear production of RanGTP, the only known Ran GTPase-stimulating protein (GAP1) is located in the cytoplasm (11–13). Thus, RanGAP1 (assisted by another cytoplasmic protein, RanBP1) will rapidly convert any RanGTP that enters the cytoplasm to RanGDP. RanGTP appears to be required inside the nucleus for the disassembly of import complexes, while export complexes need RanGTP to assemble, and disassemble upon the hydrolysis of GTP by Ran in the cytoplasm (14–16).

In combination with Ran, two other soluble factors are absolutely required to mediate the nuclear import of a protein containing a classical or basic nuclear localization sequence (NLS) into the nucleus. The first is karyopherin/importin α (Kapα), which binds a classical NLS and then forms a complex with karyopherin/importin β1 (Kapβ1) (17–22). Kapβ1 interacts with nuclear pore complex (NPC) proteins and appears to mediate movement of the import complex through the NPC via these interactions (14, 20, 23, 24). Another protein, p10/NTF2, has also been implicated in nuclear import, but its function may only be to take Ran into the nucleus, where it is subsequently needed to disassemble an incoming import complex (25–28).

Although there is only one Kapα homologue in yeast (Srp1 or Kap60), vertebrate cells contain a number of proteins that can bind a classical NLS and share sequence homology (see Ref. 29, and references therein). These proteins have been given a variety of names but can be grouped into three major families. The Kapα1 family contains the human protein NPI-1/importin α1/karyopherin α1/Rch2/hsRSP1 and a second related protein importin α6, in addition to the mouse S2 protein (19, 30–33). The second family, Kapα2, contains human Rch1/hsRSP1α/importin α2/karyopherin α2 and the mouse protein pendulin/PTAC 58 (18, 34–37). The third family, Kapα3, consists of the two human proteins, QIP-1/importin α3 and KPN3a/hSPR1y/hsRSP4, and the mouse proteins Q1 and Q2 (29, 32, 33, 38–40). Each of these classes share about 50% homology with each other and to the yeast Srp1, and each of these mammalian proteins has been shown to be capable of mediating the import of one or more classical NLS-containing proteins (29, 41–43).

It is believed that this diversity of receptors for a classical NLS in vertebrate cells indicates that an individual NLS-containing protein may preferentially use one or another NLS receptor for its nuclear import, but to date, very few NLS-containing proteins have been tested for a specific binding preference to a single Kapα. Sekimoto et al. (41) however, showed that Kapα1, but not Kapα2, mediates the nuclear import of activated Stat1. Miyamoto et al. (40) also showed that, to support the nuclear import of the helicase Q1, Kapα3 requires an additional sequence upstream of the Q1 NLS that is

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The abbreviations used are: GAP, GTPase-stimulating protein; NLS, nuclear localization sequence; NEM, N-ethylmaleimide; NPC, nuclear pore complex; GFP, green fluorescent protein; GST, glutathione S-transferase; wt, wild type; DTT, dithiothreitol.
not required by the other two Kapao.
Although RCC1 has been shown to contain an NLS that appears similar in sequence to a classical bipartite NLS, many questions remain about its nuclear trafficking. For example, does RCC1 need Ran for its import and which, if any, of the known Kapao mediate RCC1’s nuclear import? Here we show that, when members of the three classes of NLS receptors were tested, RCC1 preferentially bound Kapao3 and was only imported into the nuclei of permeabilized cells when this protein was present. Kapao2 or Kapao1 were unable to substitute for Kapao3 in supporting nuclear import of RCC1, providing additional evidence that the different classes of nuclear localization sequence receptors may serve very different roles in vivo.

EXPERIMENTAL PROCEDURES

Construction of Proa-RCC1 Fusions—The pCMVPA2-protein A fusion vector containing four IgG binding domains, as described in Ref. 44, was obtained from R. Zeller. Human RCC1 was obtained by polymerase chain reaction from an RCC1 expression vector provided by T. Nishimoto (7). XhoI and XbaI sites were added to enable direct in-frame ligation into pCMVPA2 vector. For bacterial expression the protein A-RCC1 DNA was cloned into the plSETB Vector (Invitrogen).

Construction of mNLS pA-RCC1—The QuickChange site-directed mutagenesis kit (Stratagene) was used to mutate all arginine and lysine residues to threonine (RCC1 mNLS) within the RCC1 NLS region. Briefly, large primers were generated with 14 base pairs of overlap on either side of the target region. Fpu polymerase was used in a polymerase chain reaction-based procedure to create unmethylated copies of the entire plasmid incorporating the mutant primers. The resulting DNA was cut with Dpn1 and transformed into DH5α. Selected clones were sequenced to ensure accuracy of mutagenesis.

Production of Recombinant Proteins—The wild type RCC1 was expressed and purified as described (45). Recombinant RCC1 fusion proteins were produced by incubating 4 × 500 ml of LB-Kan/Amp each with a single colony of plSETB-RCC1 in BL21(DE3) E. coli bacterium and incubating at 37 °C shaking overnight. 14 h later the temperature was lowered to 28 °C, and the cultures were induced with 1 mM isopropyl-β-d-thiogalactopyranoside for 4 h. Cultures were pelleted at 3000 × g for 10 min in GSA rotor (Beckman) and resuspended in lysis buffer containing 20 mM HEPES-KOH, pH 7.3, 500 mM NaCl, 10 mM magnesium chloride, 20 mM imidazole, and 20 mM 2-mercaptoethanol with 1 mM phenylmethylsulfonyl fluoride. Cells were lysed with a French press and quantified using a Zeiss Axioskop microscope equipped with a MicroMAX CCD camera (Princeton Instruments, Inc.) and the MetaMorph version 4.01 software program (Universal Imaging) with a custom macro written by Scott Monroe of ASI (Chalmette, LA). The recombinant import mix contained 0.3 mM Kapβ1, 1.3–2.0 mM Ran, 0.2 mM p10, and 1 mM GTP in transport buffer. The import substrates were 1.0 mM GST-GFP-NLS or 10 mM NLS-treated pA-RCC1. The GST-Kapαs were used at 0.4 μM. Assays were also performed using Xenopus cytosol, prepared as in Ref. 47, at 5 mg/ml with 1 mM GTP. All assays were performed for 15 min at room temperature, samples were rinsed three times in cold transport buffer, and fixed for 25 min on ice in 3% paraformaldehyde in TB. For localization of pA-RCC1 or pA-RCC1 mNLS after the import assay, rabbit Cy3-IgG (Jackson Immunoresearch Laboratories, Inc.) was used after fixation.

RESULTS

Because RCC1 catalyzes guanine nucleotide exchange on Ran, we questioned if the nuclear import of RCC1 would follow the standard classical NLS-mediated import pathway (i.e. using Ran) or if a unique pathway exists for the import of RCC1. One complication involved when studying the nuclear transport of RCC1 is its size. RCC1 is 45 kDa, which puts it at the borderline of the so-called diffusion limit of the NPC. Thus, a protein of this size can potentially enter (or exit) the nucleus by diffusion rather than active transport. Seino et al. (48) identified the region of RCC1 that appears to function as its NLS in vivo. They also showed, however, that a construct lacking this region is not totally excluded from the nucleus. This indicates that possibly some RCC1 (with its NLS deleted) can enter the nucleus by diffusion rather than NLS-mediated transport. To prevent unwanted diffusion, four of the IgG binding domains (3xK), of the Staphylococcus aureus protein A were fused to the amino terminus of RCC1 (Fig. 1A). The protein A tag raised the total size of the construct (pA-RCC1) to 75 kDa and allowed us to follow its diffusion using an anti-RCC1 antibody (see Supporting Information).

We then examined the ability of pA-RCC1 and pA-RCC1 mNLS to be imported into the nuclei of digitonin-permeabilized cells. We had found previously that RCC1 active for nucleotide exchange will inhibit import of an NLS-bovine serum albumin...
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FIG. 1. Protein A-RCC1 constructs. A, diagrams of the constructs. Four IgG binding domains of protein A were fused to the amino terminus of RCC1 (pA-RCC1). For pA-RCC1 mNLS the fusion was further modified in the NLS region (amino acids 8–29) by mutation of the basic amino acids (lysine and arginine) to threonine. On the right is the sequence of the NLS regions as they are found in each construct. * represents basic amino acids in the wild type RCC1, which were mutated to threonines in the mNLS mutant.

FIG. 2. Effect of cytoplasmically added RCC1. Nuclear import in digitonin-permeabilized HeLa cells was carried out for 15 min at room temperature prior to washing and fixation. In addition to the indicated amount of RCC1, each sample contained 0.5 μM Kapa2, 0.3 μM Kapb1, 1.3 μM Ran, 0.2 μM p10, and 1 mM GTP in transport buffer. In addition, the samples contained the indicated amount of either pA-RCC1 (striped bars) or pA-RCC1 mNLS (solid bars). No RCC1 added was set as 100% import, while a sample containing 7 μM wheat germ agglutinin, (Roche Molecular Biochemicals catalog no. 166,006) in addition to the import mix was used to represent 0% import.

causing the release of Kapb1 from the NPC (14, 15). We also found previously that the exchange activity of RCC1, and its inhibitory effects on nuclear import, could be abolished by pretreatment of RCC1 with the alkylating reagent NEM (45). Consistent with their ability to stimulate nucleotide exchange on Ran in a filter assay, we found that both the pA-RCC1 and pA-RCC1 mNLS constructs would inhibit the in vitro nuclear import of GST-GFP-NLS that was supported with a complete import mix consisting of Kapa2, Kapb1, Ran, p10, and GTP (Fig. 2). Therefore, in the subsequent experiments to examine the nuclear import of RCC1, we used NEM-treated RCC1 to avoid the possibility of the exchange activity of RCC1 inhibiting its own import.

Surprisingly, when we tested the import of these RCC1 constructs using Xenopus ovarian cytosol as a source of transport factors, we found that, although GST-GFP-NLS (which contains the NLS PKKKRKRV) was imported very well, pA-RCC1 was not (Fig. 3). Identical results were obtained when both GST-GFP-NLS and pA-RCC1 were included in the same reaction, with the GST-GFP-NLS being imported very well but the pA-RCC1 remaining excluded from the nuclei (data not shown). As we had already observed that microinjected pA-RCC1 was imported in HeLa cells very well (data not shown), these results indicated that either a factor was missing from the Xenopus cytosol needed for RCC1 nuclear import or possibly the human RCC1 NLS was poorly recognized by the available Xenopus transport factors. RCC1 has what appears to be a bipartite NLS, which RCC1 appears to contain, is usually recognized by one or more members of the Kap family (17–20, 37, 50). We decided to test a member from each of the three known mammalian classes of Kapas in the in vitro import assay (together with recombinant Kapb1, Ran, and p10) to see if one of them was capable of supporting RCC1 import. Kap1 (human NPI-1), Kap2 (mouse PTAC 58), and Kap3 (human Qip) were expressed and purified as GST fusions (36, 39, 40). We found that Kap2 fused to GST was similar in import activity to untagged Kap2, indicating that the GST tag was not interfering with a Kapα’s ability to support import (data not shown).
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First, each of the three Kapαs was tested for its ability to support nuclear import of 1.0 μM GST-GFP-NLS when added with Kapβ1, Ran, p10, and GTP (Fig. 4A). We found that all three would support the import of GST-GFP-NLS, albeit with different efficiencies. When added at the same concentrations (0.4 μM), GST-Kapα2 was the best at supporting import of GST-GFP-NLS, while GST-Kapα3 was the worst. When 0.01 μM NEM-treated RCC1 was used as the import substrate, however, only GST-Kapα3 stimulated a significant amount of pA-RCC1 import (Fig. 4B). The addition of GST-Kapα2 resulted in a small amount of import; however, the import was much less than that achieved with GST-Kapα3. While we found that NEM treatment abolishes most of the guanine nucleotide exchange activity toward Ran, at the same concentration (1.0 μM) we commonly use GST-GFP-NLS in the assay, NEM-treated pA-RCC1 still showed some inhibition of GST-GFP-NLS import. This indicates that a small proportion of the RCC1 was still active after the NEM treatment conditions used (10 mM NEM for 15 min on ice) (data not shown). Because the system we use to detect pA-RCC1 (Cy3-IgG) is very sensitive, we used the lowest concentration of pA-RCC1 as an import substrate that allowed for good detection in the assay (0.01 μM).

To directly test their binding affinity for RCC1, the three GST-Kapαs were immobilized on glutathione-Sepharose beads and incubated with untreated wt RCC1 or the untreated pA-RCC1 (Fig. 5). After washing, bound RCC1 was detected by Western blotting with an anti-RCC1 antibody. As shown in Fig. 5A, Kapα3 bound significantly more wt RCC1 than either Kapα1 or Kapα2. That this binding is NLS-dependent is shown in Fig. 5B. In this experiment, either untreated pA-RCC1 or untreated pA-RCC1 mNLS were tested for binding to either Kapα2 or Kapα3. As before, only Kapα3 bound a significant fraction of pA-RCC1 and this binding was abolished by substitution of the pA-RCC1 mNLS for the pA-RCC1. This indicated that the binding of Kapα3 to RCC1 is dependent on at least some of the basic amino acids in the NLS region of RCC1.

Now that we were able to achieve import of pA-RCC1 in the permeabilized cells with a mixture of Kapα3, Kapβ1, Ran, p10, and GTP, we wanted to determine which components were most important for RCC1 import to occur. In particular, we were interested to see if the import of RCC1 was Ran-dependent. Shown in Fig. 6A (top panel) is the import of NEM-treated pA-RCC1 achieved with the complete mix. The quantitation of this experiment is shown in Fig. 6B. Leaving out the GTP resulted in bright nuclear envelope staining of the pA-RCC1 but no import. We found that the import of RCC1 was Ran-dependent, as omitting Ran from the mixture abolished import (Fig. 6, A and B). In the bottom panel, NEM-treated pA-RCC1 mNLS was added; as can be seen, no import or nuclear envelope binding was seen with this construct. This result is consistent with Kapα3’s much lower binding affinity for RCC1 (Fig. 5) when the basic amino acids in RCC1’s NLS region are mutated to threonines.

**DISCUSSION**

We have shown here that the import of human RCC1 in vitro is mediated most effectively by a member of the Kapα3 class of NLS receptors (Fig. 4B). We found that import of pA-RCC1 into the nuclei of digitonin-permeabilized HeLa cells was minimal when a member of either the Kapα1 or Kapα2 class was used instead. In column binding experiments, untreated wild type and pA-RCC1 were specifically bound by Kapα3 and this binding was dependent on the basic amino acids present in the known NLS region of RCC1 (Fig. 5, A and B).

Our current understanding of the functional differences and substrate specificities between the various members of these three classes of Kapαs is still very limited. Each class of Kapα appears to have a different, tissue-specific expression pattern (29, 33, 42, 51, 52), and Nadler et al. (42) showed that some degree of preference could be seen between the binding of Kapα1 and Kapα2 to several different NLS-containing proteins. Our data confirm that each class of Kapα has a different capacity to mediate import of a T-antigen type NLS substrate (Fig. 4A). In the case of RCC1 import, much larger differences were observed. Using pA-RCC1 as an import substrate, Kapα3 showed about 5 times more import activity than the second best Kapα, Kapα2 (Fig. 4B). At the time this work was performed,
this was the first time Kapα3 had been shown to preferentially mediate the import of an NLS-bearing protein. While this paper was under review, a separate study was published by Kohler et al. (53), which also found that RCC1 was preferentially bound by the Kapα3 class of nuclear import receptors. Additionally, our binding studies indicated that this specificity of binding is dependent on at least some of the basic amino acids present within the NLS region of RCC1 (Fig. 5-B).

In a recent paper Sekimoto et al. (41) showed that Stat-1 import is mediated by Kapα1/NPI-1 but not Kapα2/Rch1. However, activated Stat-1 appears to bind to a COOH-terminal region of Kapα1 that is distinct from the NLS binding Armadillo repeats. The differences in binding of the different Kapαs to RCC1 we have observed here appear to be solely to the NLS on RCC1 and therefore probably due to the NLS binding region of Kapα3. Kamei et al. (52) showed that, in mice, the Kapα3 homologue is expressed in many tissues and theorized that Kapα3 may play a role in importing “a limited number of unique karyophilic proteins, such as helicase Q1.” Our results suggest that RCC1 should be included in the group of proteins that use Kapα3 to mediate their nuclear import.

We also found that the import of RCC1 is Ran-dependent. How then does newly synthesized RCC1 get imported if it is capable of generating RanGTP in the cytosol that in turn can inhibit its own import? We explored the possibility that the presence of the import factors might inhibit RCC1’s exchange activity. However, after testing various combinations of Kapβ1, Kapα3, Ran, p10, and RanBP1, we were never able to demonstrate an inhibition of RCC1’s ability to stimulate GDP-GTP exchange on Ran in the filter binding assay (data not shown).

In vivo the amount of RCC1 which is synthesized at any one time is probably low enough that it does not create a significant amount of Ran-GTP in the cytoplasm before it is transported into the nucleus, and any that it does produce is probably quickly stimulated to hydrolyze its bound GTP by RanGAP1.

Surprisingly, Xenopus cytosol was unable to mediate import of pA-RCC1 (Fig. 3) but was able to support the import of another substrate, GST-GFP-NLS, as were Kapαs of all three classes (Figs. 3 and 4A). Possibly, the human and Xenopus RCC1 s differ enough in their NLS region such that they are not imported efficiently by Kapαs from the other species.
ably supporting this hypothesis was the demonstration by Nishimoto (49) that the *Xenopus* RCC1 was not very effective at complementing the hamster RCC1 mutant in tsBN2 cells. The major area of sequence divergence between human and *Xenopus* RCC1 is at the amino terminus which includes the NLS region (Fig. 7) (49). Because the crucial amino acids of the RCC1 NLS (human or *Xenopus*) have not yet been determined, we can only speculate as to which NLS receptor in vivo mediates the nuclear import of RCC1 RCC1, and no member of the Kap3 family has been identified in *Xenopus* to date. Further studies are required to answer these questions.

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