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The nuclear import of RNA helicase A is mediated by importin-α3

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Abstract

RNA helicase A (RHA), an ATPase/helicase, regulates the gene expression at various steps including transcriptional activation and RNA processing. RHA is known to shuttle between the nucleus and cytoplasm. We identified the nuclear localization signal (NLS) of RHA and analyzed the nuclear import mechanisms. The NLS of RHA (RHA-NLS) consisting of 19 amino acid residues is highly conserved through species and does not have the consensus classical NLS. In vitro nuclear import assays revealed that the nuclear import of RHA was Ran-dependent and mediated with the classical importin-α/β-dependent pathway. The binding assay indicated that the basic residues in RHA-NLS were used for interaction with importin-α. Furthermore, the nuclear import of RHA-NLS was supported by importin-α1 and preferentially importin-α3. Our results indicate that the nuclear import of RHA is mediated by the importin-α3/importin-β-dependent pathway and suggest that the specificity for importin may regulate the functions of cargo proteins.

Keywords: RNA helicase A; Nuclear localization signal; Importin

RNA helicase A/nuclear DNA helicase II (RHA) was isolated as a human homologue of *Drosophila* maleless (MLE) involved in sex-specific gene dosage compensation of fruit fly [1–3]. It belongs to the DExH family of ATPase/helicase and unwinds both double-stranded DNA (dsDNA) and RNA (dsRNA) [4,5]. RHA contains several functional domains. The amino terminus has two double-stranded RNA-binding domains (dsRBD1 and dsRBD2). The catalytic core domain, containing seven well-conserved motifs and ATP binding site, is located within the central region. The minimal transactivation domain (MTAD) is next to the ATP binding site and the carboxyl terminus contains glycine-rich single-stranded nucleic acid-binding domain (RGG) [6,7].

RHA displays various functions at several stages of gene expression. For example, it is involved in transactivation in an ATP-dependent manner and/or functions as a bridging factor [8]. We showed previously that RHA mediates the recruitment of RNA polymerase II (Pol II) through MTAD to cAMP-responsive element binding protein (CREB)-binding protein (CBP) and enhancement of cAMP-mediated transcriptional activation [6]. RHA also mediates various transactivation, such as BRCA1 [9], the activation function 1 domain of mineralocorticoid receptor (MR) in a ligand-selective manner [10], and nuclear factor κB (NF-κB) [11]. In addition, recent studies showed that RHA directly binds to promoters such as the *cis*-acting transactivation response element (TAR) of HIV-1 [12],

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the *p16INK4a* promoter [13], and multidrug resistance gene (MDR1) via MEF1 [14]. These studies indicated that RHA has important roles in the transcription of several genes.

RHA localizes predominantly in the nucleus. It is also known that RHA is a shuttling protein and regulates the gene expression in the cytoplasm. In the nucleus, RHA plays roles in post-transcriptional regulation. It is included in spliceosome and involved in the processing of transcripts [15]. Furthermore, it promotes the viral gene expression and the export of the constitutive cytoplasmic transport element (CTE) containing viral mRNA. Thus, the translocation of RHA between the nucleus and cytoplasm seems to be regulated in a transcription-dependent manner [16,17]. In the cytoplasm, RHA is reported to relate with translation. These studies indicate that RHA plays a common role in the expression of a wide variety of genes at various steps.

The transport of proteins between the cytoplasm and the nucleus occurs through nuclear pore complexes (NPCs) in the nuclear envelope. These proteins contain specific sequences, the nuclear localization signal (NLS), required for the nuclear import. SV40 large T-antigen (T-NLS) and nucleoplasmin have the classical NLSs and are well analyzed. They contain one or more clusters of basic amino acids, particularly lysine, which are important for their activity. Other types of NLSs have also been identified such as M9 sequence of heterogeneous nuclear ribonucleoprotein (hnRNP) A1, which bears no sequence similarity to classical NLSs [18,19].

The nuclear import of classical NLSs is mediated by a heterodimeric receptor complex composed of importin-α and -β. Importin-α is responsible for binding to the NLS, while importin-β mediates binding of the transport complex to the NPC. Importin-α interacts with importin-β through its N-terminal importin-β-binding (IBB) domain, is rich in basic amino acids, and functions as an adapter molecule [20,21]. In addition to the import receptors, a small GTPase Ran is required for nuclear import pathway. In the cytoplasm, the GTPase-activating protein (RanGAP) hydrolyzes GTP bound with Ran to GDP and Ran-GDP rapidly is imported into the nucleus. The nuclear Ran is created by asymmetric distribution of regulatory factors for Ran. The nuclear exchange factor RCC1 (Ran GEF) promotes the exchange of Ran-GDP to Ran-GTP. Ran-GTP binds to the import receptor at the nuclear side of NPC and causes the dissociation of the NLS-import receptor complex [22–24].

Only one gene coding for importin-β has been identified. In contrast, six isoforms of importin-α have been identified in human cells, whereas there is one importin-α gene in *Saccharomyces cerevisiae*. The importin-α isoforms are classified into three groups based on their sequence homology. The first group contains importin-α1/Rch1. Although importin-α2 has similarity with importin-α1, it is found in *Xenopus laevis* and other vertebrates but not in mammals. The second group has importin-α3/Qip1 and α4/hSRP1γ, and they have 85% sequence identity. The third group consists of importin-α5/NPI-1, α6, and α7, which has 80% homology. Although there are differences in their expression levels, almost all importins are expressed ubiquitously. Previous studies showed that members of the importin-α family have different substrate specificities [25–27].

Previous study reported that the transport of RHA is mediated by the domain of 110 amino acids at its C-terminus (termed NTD for nuclear transport domain), and that the import and export activities of this domain can be separated. It is also suggested that the nuclear import of NTD is importin-α/β-dependent [16]. Moreover, it is reported that methylation of NTD by protein arginine methyltransferase 1 (RPMT1) regulates the import of RHA [28]. However, the involvement of importin-α and -β has neither been demonstrated in binding studies nor in vitro nuclear import assays. For further understanding of the nuclear import of RHA, we identified in the present study the NLS of RHA and characterized its nuclear import mechanisms.

Materials and methods

Transfection. For in vivo expression experiments, HeLa cells were seeded onto 14-mm^2 square coverslips and incubated for 24 h before transfection. Transfections were performed by using FuGENE 6 reagent (Roche Diagnostics) with 0.5 μg of each construct, according to the protocol provided by the manufacturer. Cells were incubated for 24 h after transfection, washed three times with PBS, and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. After removal of formaldehyde, cells were washed with PBS and water, then the coverslips were mounted and analyzed by TCS4D confocal laser microscopy system (Leica).

Construction of plasmids. The plasmids encoding the enhanced green fluorescent protein fusion protein (pEGFP-RHA) were constructed by inserting the RHA fragments into pEGFP-C2 (Clontech Laboratories). The fragments of RHA1, 2, 3, and 4 regions were obtained from pGEX RHA1, 2, 3, and 4 [8], respectively, and inserted into pEGFP-C2. To create the expression plasmid encoding the EGFP-pyruvate kinase (PK) fusion protein, the fragment corresponding to the PK sequence was generated by PCR. The pyruvate kinase fragment was inserted into pEGFP-C2 and named pEGFP-PK. To generate the deletion mutants, the fragments were generated by PCR and termed RHA 4b, RHA C4, RHA N2, RHA NC2, and RHA NC4, respectively. These fragments were subcloned into pEGFP-PK. For mutational analysis, substitutions of arginine or lysine with alanine were introduced into the RHA4b region by sequential PCR steps. The plasmids encoding GFP-RHA R1160A, K1163A, and R1166A were constructed by inserting the fragments of RHA derived from pEGFP-RHA and the corresponding mutated RHA4b fragments into pEGFP-C2.

For glutathione *S*-transferase pull-down assay, the fragments of deletion mutants and point mutated RHA were inserted into the bacterial expression vector pGEX-5X-1 (Amersham Biosciences). The control plasmids GST-M9-GFP and GST-T-NLS-GFP were described previously [29]. To generate pGEX-5X-1-GFP for import assay, the coding sequence of EGFP was amplified by PCR and inserted into pGEX-5X-1. For GST-RHA-NLS-GFP, a PCR fragment corresponding to RHA-NLS was inserted into pGEX-5X-1-GFP and pGEX-5X-1. To express the biotinated importin proteins as probes, importin-α1 and importin-β fragments were obtained from pGEX-2T-PTAC58 [30] and pGEX-2T-PTAC 97 [31], respectively. Fragments of other importin families were generated by RT-PCR. These fragments were subcloned into PinPoint-Xa-3 (Promega).

Expression and purification of recombinant proteins. GST fusion proteins were expressed in *Escherichia coli* (*E. coli*) strain BL21 (DE3) and purified with glutathione–Sepharose beads (Amersham Biosciences), using the instructions provided by the manufacturer. To prepare import substrates for the in vitro import assays, purified GST proteins were eluted from beads and concentrated. The eluted proteins were purified with Sephadex 75 (Amersham Biosciences). For in vitro import assay,

expression and purification of recombinant importin-αs and importin-β proteins were performed as described previously [32]. The recombinant wild type and G19V Ran were expressed, purified, and charged with GDP and GTP, respectively, as described previously [29]. As probes for the binding assay, importin-αs and -β were expressed and labeled with biotin in *E. coli*. The cells were suspended into lysis buffer (20 mM Hepes, pH 7.3, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 μg/ml of aprotinin, leupeptin, and pepstatin A). The lysates were centrifuged to remove cell debris and used for probes.

In vitro import assay. Nuclear import assays were performed essentially as described previously [33,34]. Total cytosol from Ehrlich ascites tumor cells was prepared as described previously. For the competition experiments, biotinylated BSA, which was chemically coupled to a synthetic peptide containing the T-NLS (T-BSA) [34] and recombinant IBB [35], was prepared as described previously and added to the reaction mixtures as an unlabeled competitor.

Pull-down assay. The bacterial lysates containing biotin-tagged importin-α and -β were diluted fivefold with Probe dilution buffer (20 mM Hepes, pH 7.3, 1 mM EDTA, 1 mM DTT, 0.0625% Tween 20, 6.25% glycerol, and protease inhibitors). The diluted bacterial lysates were precleaned by incubation with GST bound to glutathione–Sepharose beads in binding buffer (20 mM Hepes, pH 7.3, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Tween 20, 5% glycerol, and protease inhibitors) for 1 h at 4 °C. After centrifugation, supernatants were incubated with 2 μg of GST fusion proteins immobilized on glutathione–Sepharose beads for 4 h at 4 °C. At the end of incubations the beads were washed four times with binding buffer and bound fractions were separated by SDS–PAGE. Recovered importin-αs and -β were detected by using streptavidin conjugated with horseradish peroxidase (HRP) (Amersham Biosciences).

Result

NLS of RHA consists of 19 amino acids

It is reported that RHA contains the bidirectional nuclear transport domain in its C terminal region. The region includes 110 amino acids mapped to 1150–1259 [16]. To understand further the nuclear import mechanism of RHA, we identified the minimal sequence necessary for the NLS activity. A series of deletion mutants were constructed and expressed as GFP fusion proteins in HeLa cells (Fig. 1A). The C terminal region of RHA which comprises 1138–1270 amino acids and contains NTD (RHA 4b) was localized in the nucleus, while the mutant which has the region between 1155 and 1172 deleted (RHAΔ4b) was localized in the cytoplasm (Fig. 1B). As indicated in previous studies, this region has nuclear import activity.

To identify the minimal region required for nuclear import of RHA, we generated another series of deletion mutants of RHA 4b. The mutants were termed RHA C2 (aa 1138–1173), N2 (1173–1270), NC2 (1155–1222), and NC4 (1155–1173) (Fig. 1C). To prevent the nuclear entry by passive diffusion, mutants were expressed as a fusion protein with GFP-pyruvate kinase. PK has been used as a reporter protein for NLS identification because it is normally located in the cytoplasm and can localize to the nucleus when attached to a functional NLS. As shown as Fig. 1D, GFP-PK was localized in the cytoplasm. Three mutants containing the NC4 region accumulated in sufficient amounts in the nucleus, while RHA N2 was localized in the cytoplasm. To test the importance of the NC4 region in the context of full-length RHA, NC4 region was deleted and monitored for the effect on GFP-RHA localization. This mutant deleted the NC4 region from RHA full length (RHA ΔNLS) as well as RHA Δ4b showed cytoplasmic localization (Fig. 1B). These results indicate that the region extending from amino acid 1155 to 1173 is required for

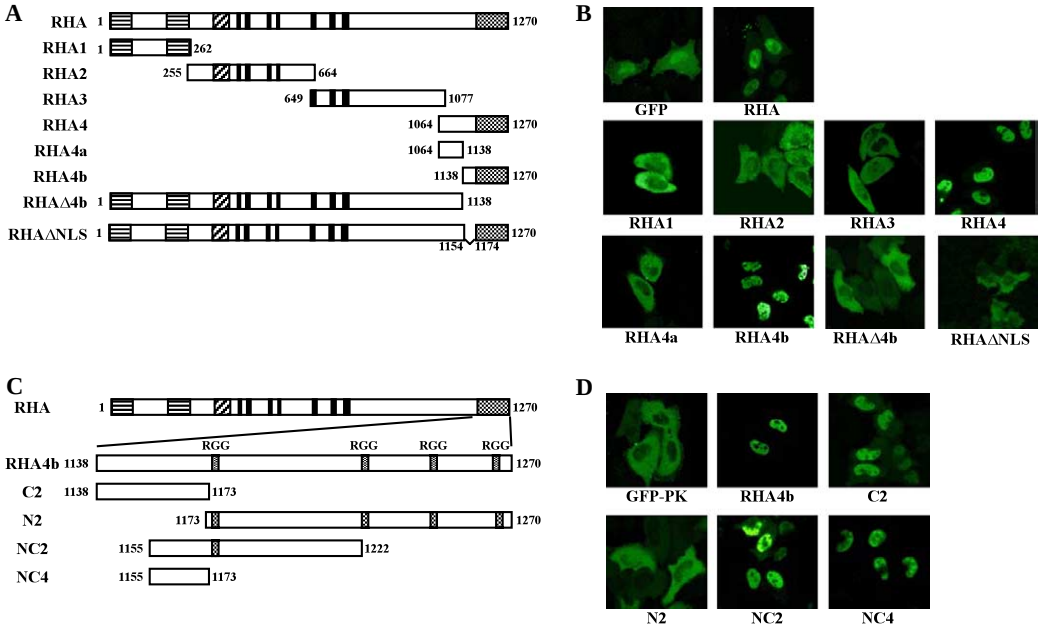

Fig. 1. NLS of RHA (RHA-NLS) consists of 19 amino acid residues. (A,C) Schematic representation of RHA and deletion mutants fused to the C-terminus of GFP. Relative position of individual domains of RHA. Solid boxes indicate the functional domain, the N-terminal double-stranded RNA binding domain (dsRBD), transactivation domain, central core helicase domain (I–VII), and C-terminal RGG box (B,D). Fluorescent microscopy of wild type (wt) and deletion mutants of RHA expressed in HeLa cells as GFP-fusion proteins.

human	1150	YGDGPRPPKMARYDNGSGY
bovin	1147	YGDGPRPPKMARYDNGSGY
mouse	1151	YGDGPRPPKMARYDNGSGY
fruit fly	1165	FSDGGGPPKRGRFETGRFT
SV40		PKKKRKV
nucleoplasmin		KRPAAIKKAGQAKKKKLD
c-Myc		PAAKRVKLD
RanBP3		PPVKRERTS

Fig. 2. RHA-NLS is highly conserved through species. The amino acid sequences of the RHA-NLS region in RHA homologues.

nuclear localization of RHA. The NC4 region consists of 19 amino acids and does not include RGG motifs. It has no significant similarity to other known NLSs such as the classical monopartite NLS, bipartite NLS or M9 of hnRNP A1 (Fig. 2).

Nuclear import of RHA is mediated by importin-α/β complex

It is reported that the nuclear import of RHA competes with NLS peptide, suggesting that the nuclear import of RHA is mediated by importin α/β pathway [16]. To characterize the nuclear import mechanism of RHA-NLS through importins, we performed the nuclear import assays in digitonin-permeabilized HeLa cells. As a transport substrate, we constructed and purified a fusion protein that comprised RHA-NLS between GST and GFP. To compare the nuclear import pathway of RHA-NLS with those of other known NLSs, T-NLS, and M9 fused to GST-GFP were used as controls. The nuclear import of T-NLS is mediated by importin-α/β and that of M9 is mediated by transportin. As shown in Fig. 3A, RHA-NLS, T-NLS, and M9 substrates all failed to effectively enter the nuclei when incubated in buffer alone (panels a, f, and k). The addition of cytosol as a source of the soluble import factors resulted in efficient nuclear entry of all three substrates (panels b, g, and l). Next, to test the involvement of Ran in the nuclear import of RHA-NLS, we used Ran mutant (G19V Ran) which lacks GTPase hydrolysis activity and inhibits several nuclear import pathways mediated by importin-β family. Addition of G19V Ran-GTP markedly inhibited the nuclear accumulation of all three substrates (panels c, h, and m). These results indicate that the nuclear import of RHA-NLS utilizes Ran-dependent pathway and requires soluble factors. To determine the contribution of nuclear import receptors for the import of RHA, two factors known to block importin-α- and -β-mediated nuclear import pathways were used in the import assays. A synthetic T-NLS peptide chemically coupled to bovine serum albumin (T-BSA) is known to bind directly to importin-α and competitively inhibit the nuclear import of classical NLSs. The N-terminus of importin-α containing IBB domain inhibits importin-β-dependent nuclear import by saturating the importin-α binding site of importin-β. Consistent with

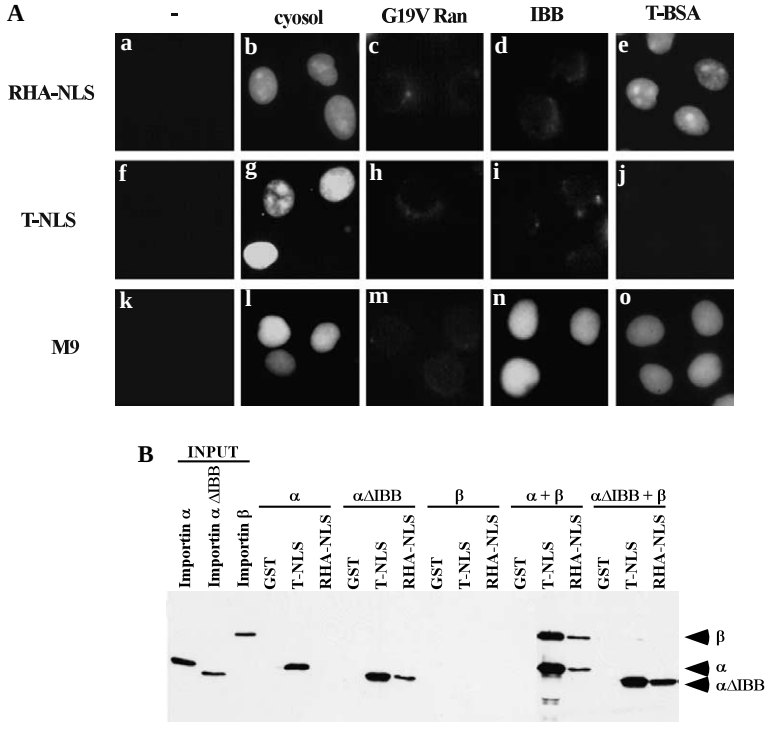

Fig. 3. RHA-NLS is transported into nuclei of digitonin-permeabilized cells. (A) In vitro nuclear import assay. Digitonin-permeabilized HeLa cells were incubated with a reaction mixture containing an ATP regeneration system, each import substrate (GST-T-NLS-GFP, GST-RHA-NLS-GFP, and GSTM9-GFP), and/or indicated effectors, a cytosol (cytosol), cytosol and G19V Ran-GTP (G19V Ran), cytosol and of IBB (IBB), cytosol and of T-BSA (TBSA). (B) Pull-down assay with GST, GST-RHA-NLS or GST-T-NLS-GFP was performed using biotinated importin-α, importin-αΔIBB and importin-β.

previous reports, the nuclear import of T-NLS was markedly inhibited in the presence of excess amount of T-BSA and IBB (panels i and j) while that of M9 was not affected (panels n and o). The nuclear accumulation of RHA-NLS was abrogated by IBB but not by T-BSA (panels e and f). These results suggest that the nuclear import mechanism of RHA-NLS is importin-β-dependent.

To determine whether the RHA-NLS region directly interacts with importin complex, pull down assay was performed. As a probe, GST-NLS expressed in bacterial cells were incubated with biotinated importin-α or -β. As shown as Fig. 3B, RHA-NLS interacted with importin-α/β complex but not with only importin-α alone, whereas T-NLS interacted with importin-α and importin-α/β complex. It is known that the IBB domain has autoinhibitory activity for nuclear import. The IBB domain interacts with its ARM domain which is a binding domain of the NLS of cargo protein. This interaction prevents importin-α to form a complex with cargo proteins and its inhibition is released by interaction of importin-α and -β [36]. To confirm whether the interaction between importin-α and RHA-NLS is inhibited by the autoinhibitory mechanism, importin-α lacking IBB domain (importin-αΔIBB) was used as a probe for pull-down assay. RHA-NLS bound with importin-αΔIBB but not with importin-α. These results suggest that RHA-NLS and importin-α could form a nuclear import complex.

Basic residues in RHA-NLS are important for nuclear import and interaction with importin complex

Although the amino acid sequence of RHA-NLS is unique to RHA homologues, they have basic amino acids as known in the classical NLSs. Previous studies indicated that certain basic amino acids in RHA-NTD are important for the nuclear import of RHA. Mutations were introduced into full-length RHA and the mutants (RHA R1160A, K1163A, and R1166A) were expressed as GFP fusion proteins in HeLa cells (Fig. 4A). The mutant R1160A was localized in the nucleus, whereas K1163A was localized in the cytoplasm. The mutant R1166A was localized in both the nucleus and cytoplasm, although the extent of nuclear accumulation was significantly lower (Fig. 4B). As described in previous studies [16], it is consistent that the two basic residues (K1163 and R1166) are important for the nuclear import activity of RHA-NLS and that K1163 is particularly essential. To test whether the basic amino acids are required for the formation of complex with importin-α and -β, we performed in vitro binding assays using each mutant of RHA-NLS fused to GST. As described previously, the wild type RHA-NLS bound to importin-α and -β. The mutant R1160A interacted with the importin complex similar to the wild type, whereas the mutant R1166A bound weakly and K1163A bound at a level similar to that of GST alone (Fig. 4C). These results emphasize the importance of the basic residues for complex formation of importin-α and β.

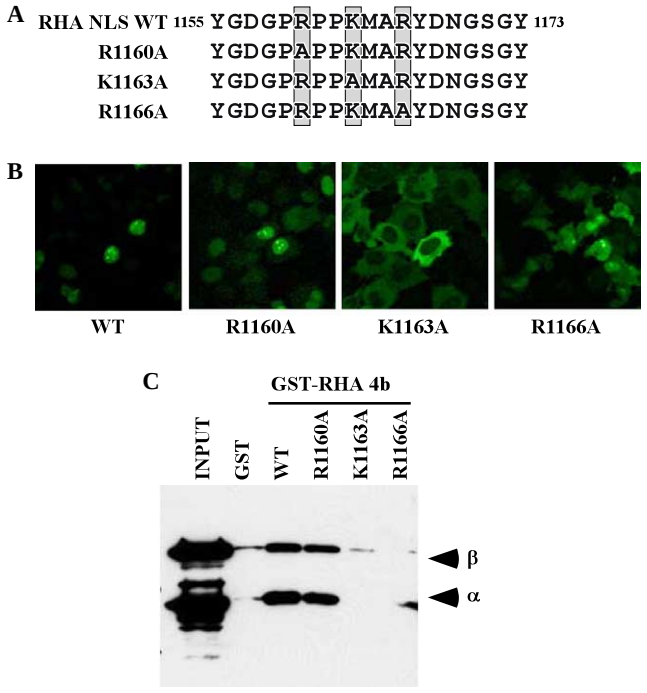

Fig. 4. Alanine substitution of basic residues in RHA-NLS reduces its nuclear migration. (A) Amino acid sequence of RHA-NLS. The mutated three basic residues are indicated by the shaded box. (B) The contribution of basic residues for nuclear import. Full-length RHA and mutant RHAs (R1160A, K1163A, and R1166A) were expressed as GFP-fusion in HeLa cells. (C) Pull-down assay with mutant RHA. RHA 4b with each substitution fused to GST was incubated with biotinated importin-α1 and β.

Importin-α1 and α3 mediate the nuclear import of RHA

Six importin-αs have been identified in human cells, and they are classified into three subfamilies, termed importin-α1, α3, α4, α5, α6, and α7 [27]. We examined the types of importin-α isoforms involved in the nuclear import mechanism of RHA. First, to determine whether RHA-NLS interacts with importin-α1, α3, α4, α5, and α7, pull-down assays were performed using biotinated importin-αs and -β expressed in *E. coli* as probes. Importin-α1, α3, and α4 bound efficiently to RHA-NLS, whereas importin-α5 bound weakly and importin-α7 did not bind (Fig. 5A). The basic residues in RHA-NLS which were important for interaction with importin-α1 had roles for interaction with importin-α3 (data not shown). Next to confirm the specificity of the interaction of RHA-NLS with importin-αs, we carried out in vitro import assays with recombinant transport factors. RHA-NLS and T-NLS substrates were combined with recombinant Ran, importin-β, an ATP-regenerating system, and/or purified importin-αs. As shown in Fig. 5B, incubation without any importin-α failed to induce efficient nuclear import of both substrates. All importin-α isoforms stimulated the nuclear import of T-NLS. For RHA-NLS, importin-α3 showed the best stimulation of the nuclear import and importin-α1 displayed a somewhat weaker effect. Interestingly, importin-α4, α5 or

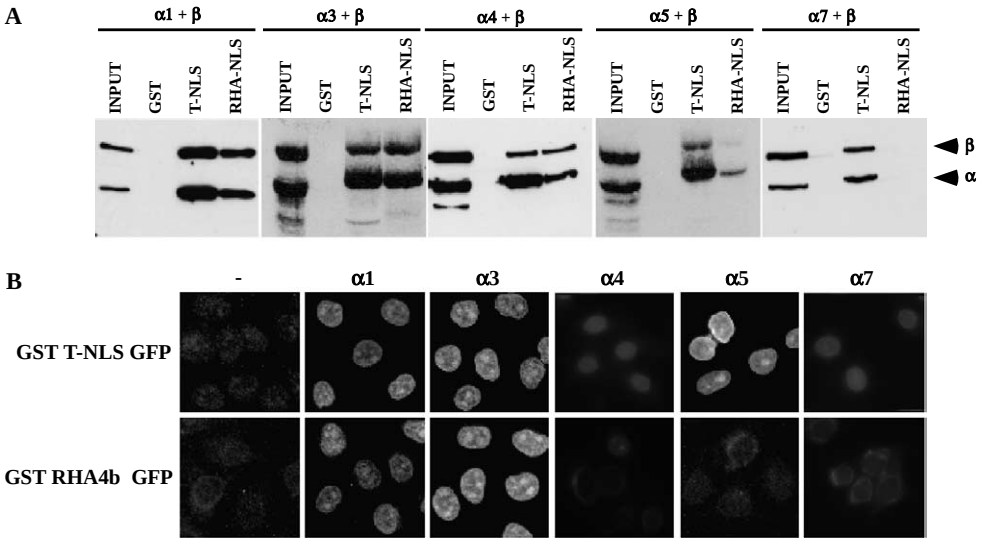

Fig. 5. Qip1 preferentially binds to RHA-NLS. (A) Pull-down assays of recombinant importin isoforms. RHA-NLS fused to GST was incubated with each importin-α, importin-β. (B) In vitro import assay with importin-α isoforms in digitonin-permeabilized HeLa cells.

α7 did not induce nuclear accumulation of RHA-NLS substrate. This was not due to inappropriate preparation of recombinant importin proteins based on the finding that T-NLS was efficiently imported by the addition of these proteins. These results indicate that the nuclear import of RHA is preferentially mediated by importin-α3.

Discussion

In this study, we identified the minimal region of the RHA that is important for its nuclear localization. The NLS of RHA is located at amino acid residue 1155–1173 and consists of 19 amino acids (Fig. 2). RHA formed a nuclear import complex through this region with importin-α and the translocation was mediated by importin-α/β and small GTP-Ran. In this process, importin-α3 was preferentially used for the nuclear import of RHA among six importin-α subfamily members. The 19 amino acid stretch has a few basic residues and the particular residues are important for the interaction with importin complex and the nuclear import of RHA.

It is reported that the NTD comprising 110 amino acids from 1150 to 1250 is essential for the nuclear import and the export of RHA. With regard to nuclear import, it has been indicated that the N terminal half of NTD containing 71 amino acids between 1150 and 1220 is more important, and that the region can transfer into the nucleus [16,28]. The 19 residues identified in the present study are part of the 71 amino acids of NTD and the minimal region for the nuclear import. These results are consistent with previous reports. Furthermore, it is reported that the NTD of RHA is methylated by PRMT1 and the methylation is necessary for the nuclear import mediated by this 110 amino acid stretch [28]. Some proteins, such as heterogeneous nuclear ribonucleoproteins A2 (hnRNP A2) and RNA-binding protein Sam68, are known to have PRMT1-methylated the arginine residues in the RGG domain, and such methylation regulates the translocation [16,37]. However, the relationship between methylation and nuclear import is still not clear. It is thought that the methylation could affect protein interaction. The RHA-NLS region is located next to the RGG domain and does not contain any RGG motifs, although NTD has four RGG motifs. These results suggest that NTD could be divided into two functional regions, the N-terminal for the formation of the import complex and C-terminal for regulation. Methylation in the C-terminal half of NTD may induce conformational change or dissociation from inhibitory factors. These changes may enable the RHA-NLS in the N terminal half of NTD to recruit with importin-α.

The nuclear import of cargo proteins with these classical NLSs utilizes various members of the importin-β families [23]. With regard to RHA-NLS, it has been suggested that the nuclear import is mediated by importin-α/β pathway. Although RHA-NLS directly interacted with importin-α, T-NLS peptide did not compete with RHA-NLS in the nuclear import assay (Fig. 3). While the exact reason for this phenomenon is not clear, several mechanisms could be postulated. One is differences of affinities between NLS and importin-α isoforms. It is reported that importin-α1 and α5 have binding activities for a wide variety of NLS sequences, while importin-α3 exhibits specificity for certain NLSs [38]. Furthermore, it is known that the expression patterns of importin-α subfamilies are tissue-specific and are changed in the differential stages. The binding specificity can be affected by abundance of nuclear import receptors. It is reported that the U1A splicesome protein has two import mechanisms. U1A is translocated through importin-α/β-dependent and cytosol independent pathways. It is suggested that the latter pathway might be negatively regulated under ordinary condition [39,40]. RHA also interacts with various molecules

and has functions in cells. These reports allow us to speculate that nuclear localization mechanisms and the efficiency of the interaction between importin-α and cargo proteins are regulated in the cell type- or signal-dependent manners. These differences in binding specificity may prevent T-NLS from competing with RHA-NLS. The second mechanisms may be related to difference in nuclear import pathway using importin-α/β. NLS interacts with the ARM domains in importin-α. It is known that two T-NLS peptides can bind with each pocket in the ARM domain in vitro binding assay [41]. It is possible that T-NLS and RHA-NLS interact with the different pockets in importin-α. These issues can be addressed by probing the structure of the nuclear import complex.

In the nuclear import process, the two types of classical NLS sequences have been well-analyzed. These sequences are classified into two major groups depending on the numbers of their charged clusters. One is a monopartite sequence with a single consensus motif of basic residues like SV40 T-antigen. The other is a bipartite sequence with two clusters of basic residues with a spacer region like nucleoplasmin. The NLS of RHA which consists of 19 amino acid residues has some basic residues, but it is not consistent with either monopartite or bipartite NLS, basic-basic-X-basic [42]. Comparisons of the amino acid alignment of the NLS sequences show that the RHA-NLS has similarity to that of Ran Binding Protein 3 (RanBP3). Mutational analysis of the NLS of RanBP3 indicated that substitution of the first amino acid in the basic cluster (lysine at amino acid 52) leads to a small nuclear localization and that loss of the double basic residues completely blocked the translocation [43]. In the case of RHA, it has three basic residues and a lysine residue at amino acid 1163 corresponding to lysine at 52 of RanBP3 which is the most important. The next residue of K52 that has only slight effect in RanBP3 is not a basic residue (methionine) in RHA (Fig. 2). RanBP3 NLS also has similarity with c-Myc NLS [43,44]. These results suggest that they could form the same group of NLS.

Our results indicated that the RHA-NLS uses importin-α1 and α3, especially α3 for the nuclear import (Fig. 5). importin-α consists of six isoforms classified into three subgroups. Most cargo proteins, such as SV40 T-antigen, hnRNP K, PCAF [45], and mCRY2 [46], are imported into the nucleus with some efficiency for all importin-α members. On the other hand, some substrate proteins have the specificities for each importin-α isoform. For example, STAT1 [47] and thioredoxin-binding protein 2 (TBP-2) [48] are imported via interaction with importin-α1 or α5. Importin-α3 with which RHA interacts preferentially is also used by other proteins. It was originally isolated as an interactant with DNA helicase Q1/RecQL [49]. STAT3 [50] and RanBP3 [43] have the monopartite of NLS sequences and RCC1 [17,45,51] and mCRY2 [46] have the bipartite NLSs. Their factors are imported into the nucleus using importin-α3. The NLSs of RHA and RanBP3 specifically use importin-α3 for the nuclear import, whereas that of c-Myc does not. The consensus motifs for each importin-α isoforms are unknown. Structural analysis of the cargo proteins could clarify these regulation mechanisms.

Recent studies suggested the involvement of importin-α isoforms in cell-specific functions. Most importins are expressed in several types of tissues except importin-α6 which is limited to the testes [17,25]. The expression levels of importin-α are regulated in cell-specific or signal-dependent manners [52]. For example, importin-α3 is strongly detected in the testes, ovaries and small intestine. Downregulation of importins with small interfering RNAs (siRNAs) revealed that importin-α3 could control cell proliferation and apoptosis [45]. In HL60 cells, the expression levels of each isoform of importin-α are affected during the differentiation stage [52]. Genetic analysis of *Drosophila melanogaster* and *Caenorhabditis elegans* also showed that the germ cells use specific importin isoforms and the individual importins cannot rescue the functions of others [27,53]. For example, *Drosophila* homologue of importin-α3 (Dα3) has a more important role in development, and Dα1 and Dα2 partially rescue the Dα3 [27,54]. However, the relationship between their expression patterns and the selectivity of their cargo proteins for remains to be clarified. RHA is expressed ubiquitously and regulates CREB-dependent transcription [6,8]. The CREB family includes CREB, CREM, and ATF-1 and they are expressed ubiquitously. It is known that CREM has specific functions in the testis. There are tissue-specific splicing isoforms CREM-τ and CREM interacts with testis-specific coactivator [55–57]. These reports suggest that RHA plays important roles as a coactivator of the CREB–CBP complex in the testis and this might be the reason why importin-α3 is used by RHA. We speculate that these may be the underlying mechanisms through which importins regulate the tissue-specific functions of ubiquitously expressed factors. In this study, RHA also bound with importin-α4 as well as importin-α3, which are classified under the same subfamily. Unexpectedly, RHA was not mediated the nuclear localization with importin-α4. It is indicated that some proteins, such as RCC1 and RanBP3 which interact with importin-α3 and α4, are translocated into nuclear by importin-α3 but not by importin-α4 in cells. It is unclear why there are such differences between importin-α3 and α4. They might need the other components for forming the stable import complexes or be competed with factors in cytosol.

In addition to transcription, RHA is involved in the splicing [15] and the export of viral RNA and shuttles between the nuclear and the cytoplasm [17]. It is suggested that RHA does not only export the mRNA but also has functions in the cytoplasm. RHA colocalizes with cytosolic Staufen in the dendrites of differentiated neuroblasts and may regulate the translation [58]. In a preliminary study, we observed that RHA could interact with a ribosomal protein (unpublished data). These findings suggest that the ratio of translocation may regulate the functions

of RHA in cells. RHA uses importin-α1 and α3. The expression level of importin-α3 with which RHA preferentially interacts is stable and are regulated such as housekeeping genes. In contrast, importin-α1 is the most widely expressed in tissues and its levels is regulated by various signals [52]. RHA is predominantly localized in the nucleus and has very important roles in transcription. These might be the reasons for the use of these two importin isoforms (importin-α1 and α3) by RHA-NLS rather than the testis-specific importin-α6. These mechanisms of the nuclear import could allow effective regulation of RHA functions.

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Supplementary data

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