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Cloning of a cDNA Encoding a Novel Importin-α Homologue, Qip1: Discrimination of Qip1 and Rch1 from hSrp1 by Their Ability to Interact with DNA Helicase Q1/RecQL

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We isolated two cDNA clones encoding human proteins which interact with DNA helicase Q1/RecQL, a human homologue of *Eschelichia coli* RecQ protein, by two-hybrid screening. One of these proteins, named Qip1, was a novel protein homologous to the nuclear localization signal (NLS) receptor importin-α, and the other was the known protein Rch1, which is also a homologue of importin-α. DNA helicase Q1 in human cell lysates was coprecipitated with bacterially expressed Qip1 and Rch1 fused with glutathione-*S*-transferase with glutathione Sepharose beads, confirming the interaction between these proteins and DNA helicase Q1. Two-hybrid experiments revealed that Qip1 interacted with the NLS of SV40 T antigen similar to Rch1 and hSrp1. In addition, interaction of the putative NLS in DNA helicase Q1 with Qip1 and Rch1 but not with hSrp1 was confirmed by the two-hybrid system.

The nuclear import of karyophilic proteins is directed by short amino acid sequences termed nuclear localization signals (NLSs) (1-3). A cytoplasmic protein such as *Xenopus* importin-α was found to recognize NLSs and to dock NLS-containing proteins to the nuclear pore complex (4-6). *Xenopus* importin-α is homologous to *Saccharomyces cerevisiae* Srp1 whose gene was first identified as a suppressor of RNA polymerase I mutations (7).

In human and mouse cells, two Srp1 homologues, namely, importin-α homologues have been identified. Human Srp1 (hSrp1), also called karyopherin α1 and nucleoprotein interacter 1 (Npi1) and mouse Srp1 (mSrp1), which constitute one group of importin-α homologues, bind specifically to proteins containing NLS and together with other components reconstitute nuclear protein import (8-11). Rch1, also called karyopherin α2 and hSrp1α and mouse importin-α, which constitute another group of importin-α homologues, have similar properties as those of hSrp1 and mSrp1 (11-14). These two groups of importin-α homologues are 45% identical at the amino acid level. Physiological meaning of the existence of two subtypes of importin-α homologues is not clear at present. Recently, mRNAs of mSrp1 and mouse importin-α have been found differentially expressed in various tissues (15).

DNA helicase Q1/RecQL is a human homologue of *Escherichia coli* RecQ protein (16-18). *E. coli* RecQ is known as a member of the RecF recombination pathway (19, 20), but its function has not been clarified precisely.

In this study, we carried out two-hybrid screening (21) to identify proteins which interact with DNA helicase Q1. We isolated two human cDNA clones encoding proteins named Qip1 and Qip2. Qip2 is identical to Rch1. Qip1 is a novel homologue of *Xenopus* importin-α and the amino acid sequence of Qip1 is about 50% identical to those of hSrp1 and Rch1 indicating that Qip1 is the third homologue of importin-α. Thus we examined whether Qip1 interacts with the typical NLS of SV40 T antigen and the putative NLS in DNA helicase Q1. In addition, we also examined whether the putative NLS of DNA helicase Q1 discriminates among three importin-α homologues.

MATERIALS AND METHODS

Yeast strains. The yeast strain used for the library screening was HF7c (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-*

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The abbreviations used are: GST, glutathione-S-transferase; NLS nuclear localization signal; PCR, polymerase chain reaction; Qip1, DNA helicase Q1 interacting protein 1.

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3, 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mers)$_3$-CYC1-lacZ) and that used for β-galactosidase assays was SFY526 (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can^r, gal4-542, gal80-538, URA3::GAL1-lacZ*). Both strains were obtained from Clontech Laboratories, Inc.

Two-hybrid plasmid constructions. Plasmid vectors for the two-hybrid system (MATCHMAKER Two-Hybrid System) were purchased from Clontech Laboratories, Inc. The GAL4 DNA-binding domain vector was pGBT9 and the GAL4 activation domain was pGAD424. The DNA helicase Q1 hybrid with GAL4 DNA-binding domain, pGBT9-Q1 was constructed by ligating the *Bam*H I-*Eco*R V fragment from pBluescript II KS (+)-Q1 into *Bam*H I-*Sma* I digested pGBT9 (17). pGAD424-hSRP1 was constructed by ligating the *EcoR* I-*Xho* I fragment from pJG4-5-hSRP1 into *Eco*R I-*Sal* I digested pGAD424 (8).

Two-hybrid screening. Yeast strain HF7c was sequentially transformed with pGBT9-Q1 and the GAL4 activation domain pGAD GH HeLa cDNA library (Clontech Laboratories, Inc.). Transformants were plated on yeast drop-out media lacking leucine, tryptophan and histidine. After 3-5 days of growth, potentially positive clones were patched onto filter papers (Whatman, #1) layered over the fresh plates. The patches were allowed to grow on the filters for one day and assayed for β-galactosidase activity.

β-Galactosidase assay. β-Galactosidase activity was measured by the filter assay and the liquid assay using *o*-nitrophenyl-galactopyranoside according to the protocol provided by Clontech Laboratories, Inc.

Production of glutathione-S-transferase (GST) -Qip1 and GST-Rch1 fusion proteins. The *Sma* I-*Xho* I fragment of QIP1 cDNA (full-length) or RCH1 cDNA (amino acids 36-529) was blunted with Klenow fragment and ligated into the *Sma* I site of the expression vector pGEX-2T (Pharmacia) to generate pGEX-QIP1 or pGEX-RCH1. *E. coli* (HB101) cells transformed with pGEX-QIP1 or pGEX-RCH1 were grown to an $OD_{600} = 1.0$, and isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 3 h of additional growth, cells were washed with PBS (50 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4). The cells were resuspended in 50 ml of sonication buffer (PBS, 1% Triton X-100) and lysed by sonication for 5 min with 1 min intervals in every minute with a sonifier equipped with a micro tip (BRANSON Model 185). The lysates were centrifuged at 10,000 $\times$ g for 20 min at 4°C and the supernatants used as *E.coli* lysates. *E.coli* lysates containing GST alone were also prepared by the same method. Glutathione Sepharose 4B (Pharmacia) beads were equilibrated in PBS and 30 μl of a 50% slurry was added to 750 μl of *E. coli* lysate. The mixture was incubated for 20 min at 4°C on a shaker, and beads were collected by centrifugation for 5 sec, then washed three times with TET buffer (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM PMSF).

Assay for binding between GST-fusion proteins and DNA helicase Q1. HeLa cells (5×10^7) were washed with PBS and resuspended in 2.8 ml of TET buffer. The cell suspension was left on ice for 20 min, and centrifuged at 15,000 rpm for 20 min at 4°C. Fifteen-microliter aliquots of washed beads containing GST-Qip1 or GST-Rch1 were added to 900 μl of the supernatant and incubated for 1 h at 4°C on a shaker. Beads were collected by centrifugation, and washed three times with TET buffer. Washed beads were resuspended in 25 μl of sample buffer, boiled for 90 sec, and pelleted by centrifugation. A portion of the supernatant (20 μl) was developed by SDS-PAGE on a 7.5% gel. After electrophoresis, proteins were transferred onto a nitrocellulose membranes at 2 mA/cm^2. The membranes were soaked in TBS (20 mM Tris-HCl pH8.0, 100 mM NaCl) containing 1% skim milk for 1 h, then incubated for 1 h with 100-fold diluted affinity-purified rabbit anti-DNA helicase Q1 antibody in TBS containing 0.5% skim milk. The membranes were washed with TBS containing 0.5% skim milk and 0.1% Triton X-100, then incubated with 0.3 μCi/ml of ^{125}I-protein A. Following repeated washing with TBS containing 0.5% skim milk and 0.1% Triton X-100, radioactivity was visualized with an image analyzer (BAS 2000 ; Fuji Photo Film, Tokyo, Japan).

Construction of mutant DNA helicase Q1 gene lacking the putative nuclear localization signal (NLS) and the NLS region of SV40 large T antigen. DNAs encoding the carboxyl-terminal region of DNA helicase Q1 lacking the putative NLS (amino acids 642-649) and the NLS region of SV40 large T antigen were amplified by polymerase chain reaction . Oligonucleotides used for the PCR to prepare the mutant DNA helicase Q1 gene were 5′-ACTGCTGTAAAGACAGTG-CAT-3′ and 5′-AACTGCAGTTAAGCTCCTGTATTCTTAGAACC-3′. The fragment amplified by PCR was ligated into *Pst* I digested pGBT9-Q1 to yield pGBT9-Q1 NLS (−). Oligonucleotides used in the PCR for preparing the DNA encoding the NLS region of SV40 large T antigen were 5′-CGGAATTCTATGGAACTGATGAATGGGAG-3′ and 5′-AACTGCAGGAGTTCTATTACTAAACACAGC-3′. The fragment amplified by PCR was ligated into *EcoR* I-*Pst* I digested pGBT9 yielding pGBT9-SV40 T-ag NLS. DNA sequences in the regions amplified by PCR in both plasmids were checked by sequence analysis.

Assay for binding between GST-fusion proteins and the NLS peptide of T antigen and the putative NLS peptide of DNA helicase Q1. The NLS peptide of T antigen and the putative NLS peptide of DNA helicase Q1 were conjugated to bovine serum albumin (BSA) to make BSA-T and BSA-Q1. The peptides used for preparing BSA-T and BSA-Q1 were CYGGPKKKRKVEDP and CYFQKKAANMLQQSG-SKNTGAKKRKIDDA, respectively. Fifty microliter of 60 μg/ml of BSA-T or 120 μg/ml of BSA-Q1 was fixed to the wells of a microtiter plate by an incubation at 4°C overnight, and PBS containing 5% BSA was poured into the wells. After an incubation at 37°C for 1 h, 50 μl of 0.1 μM GST-fusion proteins were poured into the wells and incubated for 1 h. After the plate was washed with PBS and PBS containing 0.05% Tween 20 three times each, 50 μl of 1/300 diluted anti-GST antibody conjugated horseradish peroxidase (SantaCruz #SC459HRP) was poured into the wells and incubated at room temperature for 1 h. After washing as above, 100 μl of 0.1 M acetate/citrate buffer (pH6.0) containing 0.1 mg/ml of 3, 3′, 5, 5′-tetramethylbenzidine and 0.02% of H_2O_2 was poured into the wells and incubated at room temperature until color was developed sufficiently. The reaction was stopped by adding 50 μl of 0.5 N H_2SO_4. The absorbance at 455 nm was measured using a multiscan photometer.

RESULTS AND DISCUSSION

Isolation of cDNAs Encoding Proteins Interacting with DNA Helicase Q1

To identify proteins that specifically interact with DNA helicase Q1, we used the yeast two-hybrid system. The full-length Q1 gene was subcloned into pGBT9 to generate pGBT-Q1. The yeast reporter strain HF7c carrying pGBT-Q1 was transformed with a HeLa cell cDNA library for screening. Before screening, we ascertained that DNA helicase Q1 was expressed in yeast reporter strains carrying pGBT9-Q1 by immunoblotting (data not shown). The transformants (8×10^6) were screened and potentially positive clones were patched onto filter papers and assayed for β-galactosidase activity. Thirty-one positive clones were isolated, and plasmids were recovered from these clones. *E. coli* HB101 was transformed with the plasmids and transformants were selected using plates without leucine because of the *leuB* mutation in HB101. The cloned plasmids were reintroduced into yeast reporter strain

HF7c or SFY526 with pGBT9-Q1 or pGBT9 and the β-galactosidase activities induced by all of the cloned plasmids were confirmed to be dependent on the presence of the DNA helicase Q1 gene. Sequencing, restriction analysis and PCR analysis of the plasmids revealed that all of the 31 clones encoded one of two genes; QIP1 or QIP2.

QIP1 Encodes a Novel Human Homologue of Saccharomyces cerevisiae Srp1 and QIP2 Is Identical to RCH1 Gene

Sequence analysis of 2.2-kbp QIP1 cDNA revealed that it encoded a 521-amino acid protein with a calculated molecular weight of 57,885 and a pI = 4.66. Comparison of the cDNA sequences or the deduced amino acid sequences in GenBank and EMBL data bases using BLAST program demonstrated that Qip1 is a human homologue of *S. cerevisiae* Srp1. SRP1 was cloned as an allele-specific suppressor of temperature-sensitive mutations in the zinc-binding domain of the A190 subunit of RNA polymerase I (7). The amino acid sequence is conserved between these two proteins with 46% identity at the amino acid level. Qip1, like Srp1, contains a series of seven consecutive motifs, which are degenerated 42-amino acid protein subsequences originally identified in the *Drosophila* armadillo protein. The motif, named the ARM motif, was found in various proteins such as APC (adenomatous polyposis coli), β-catenin, smgGDS, and is thought to be involved in specific protein-protein interactions (22, 23). The amino acid sequence of Qip1 is shown in Fig. 1. The QIP1 cDNA clone obtained by two-hybrid screening contains a putative initiation codon (ATG) at the position corresponding to that of other SRP1 homologues. This putative initiation codon is 10 nucleotides from the 5′ terminus and it has a favorable context for effi-

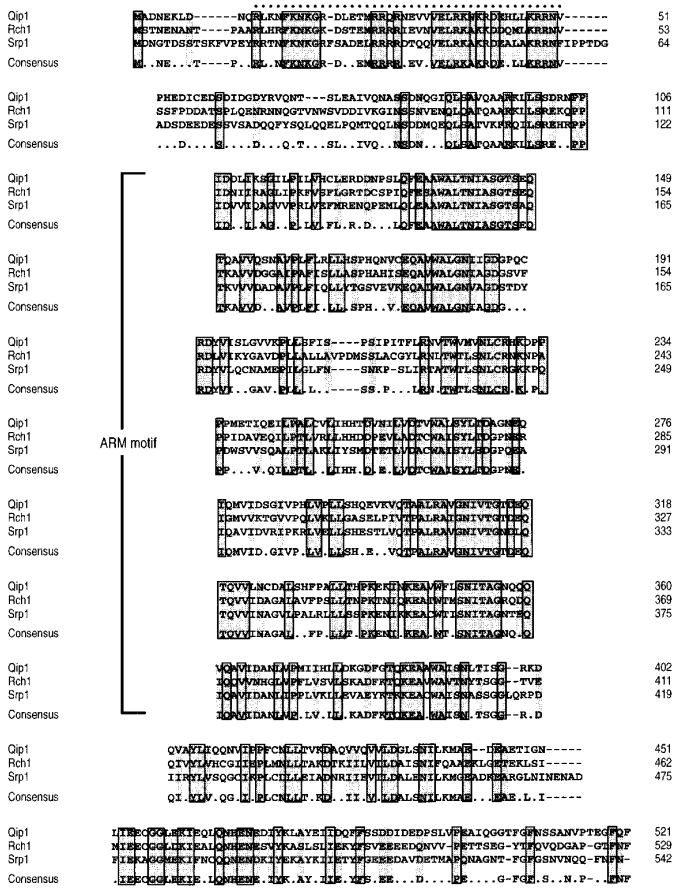

FIG. 1. Amino acid sequence of Qip1 and its homologues. Deduced amino acid sequence alignment of Qip1, Rch1, and Srp1. Boxing and shading indicate conserved amino acid residues as defined by GeneWorks (IntelliGenetics, Inc.). Qip1 shows 50% amino acid identity with Rch1, 46% with *S. cerevisiae* Srp1. The 42-amino acid repeat structure, the ARM motif, is shown. The dotted region indicates the conserved importin-β binding site in importin-α.

TABLE I

Amino Acid Identity Matrix of Srp 1 Homologues

		Qip1	Rch1/ karyopherin α2	m-importin	mSrp1	hSrp1/Npi1/ karyopherin α1
Qip1	human	—				
Rch1/ karyopherin α2	human	50	—			
m-importin	mouse	50	95	—		
mSrp1	mouse	47	44	45	—	
hSrp1/Npi1/ karyopherin α1	human	47	44	45	97	—
importin 1	frog (*Xenopus*)	49	63	62	44	43
Srp1	yeast (*S. cerevisiae*)	46	45	45	50	50

(% of Amino Acid Identity)

Xenopus importin 1 is one of two cloned importin α proteins. Classification of Srp 1 homologues into three groups was based on amino acid identity. Shading indicates identity more than 50%.

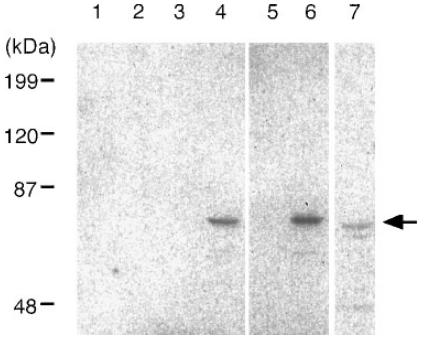

FIG. 2. Coprecipitation of DNA helicase Q1 with GST-Qip1 or GST-Rch1. GST (lane1, 2), GST-Qip1 (lane 5, 6) and GST-Rch1 (lane 3, 4) were expressed in *E. coli* and bound to glutathione Sepharose 4B. The beads were incubated with (lane 2, 4, 6) or without (lane 1, 2, 5) HeLa cell lysates. Precipitated proteins were resolved by SDS–PAGE and detected by immunoblot analysis using anti-DNA helicase Q1 polyclonal antibody. HeLa cell lysate (15 μl) was directly applied to electrophoresis and immunoblotted (lane 7).

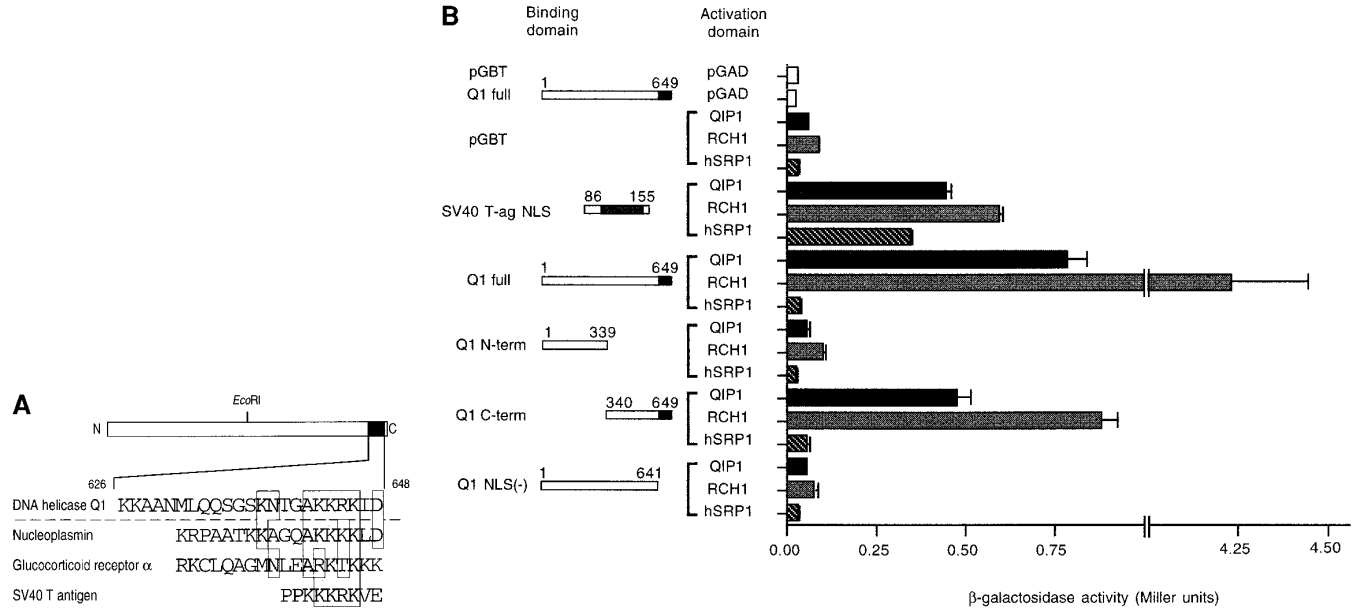

FIG. 3. Binding of importin-α homologues to NLSs. (A) Amino acid sequence of putative NLS of DNA helicase Q1 in carboxy-terminus compared with NLSs of *Xenopus* nucleoplasmin, human glucocorticoid receptor α and SV40 T antigen. Shading indicates identical amino acids. (B) The yeast strain SFY526 was cotransformed with various vectors shown in the figure, and assayed for β-galactosidase activity. β-Galactosidase activity was determined by liquid assay performed in triplicate.

cient translation initiation since there are C residues at the -1, -2 position, and a G residue at the $+4$ position (24, 25). In addition, QIP1 cDNA has a potential polyadenylation signal (AATAAA) followed by a poly (A) sequence in 3′ untranslated region of 599 bp.

Sequence analysis and homology search of 2.1-kbp QIP2 cDNA indicated that QIP2 is identical to the human RCH1 gene. QIP2 cDNA contained partial RCH1 gene lacking its amino-terminal region (amino acids 1-35). This region (dotted region in Fig. 1) has been recently shown to be necessary for the interaction with importin-β but not for that with proteins containing NLSs (26, 27). RCH1 was cloned as a gene encoding a protein interacting with Rag1, one of the most important factors for V(D)J recombination (12). Rch1 is also a homologue of Srp1, and has the 42-amino acid repeat structure. Qip1, Rch1 and Srp1 sequences are aligned in Fig. 1. The identity between Qip1 and Rch1 is 50% at the amino acid level. *Xenopus* importin-α which is required for the import of nuclear proteins into the nucleus has also been shown to be homologous to Srp1 (5). Identities among known Srp1 homologues are indicated in Table I. As demonstrated in this matrix, Srp1 homologues in higher eukaryotes can be classified into 3 groups; one including Rch1/karyopherin α2/hSrp1α, mouse importin-α, one including hSrp1/karyopherin α1/Npi1 and mSrp1, with Qip1 belonging to another group. Besides these, *Drosophila* and mouse pendulin (28, 29) are known as Srp1 homologues.

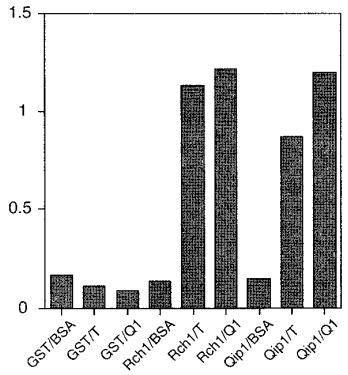

FIG. 4. Interaction of Qip1 and Rch1 with NLS peptides. The interaction of GST-Qip1 or GST-Rch1 with the NLS peptide of T antigen (CYGGPKKKRKVEDP) and the putative NLS peptide of DNA helicase Q1 (CYFQKKAANMLQQSGSKNTGAKKRKIDDA) was examined as described in MATERIALS AND METHODS. The amount of interacting GST-fusion proteins was measured by enzyme-linked immunosorbent assay using anti-GST antibody. T, the NLS peptide of T antigen conjugated to BSA; Q1, the putative NLS peptide of DNA helicase Q1 conjugated to BSA; Rch1, GST-Rch1; Qip1, GST-Qip1.

Qip1 and Rch1 Bind to DNA Helicase Q1 in Vitro

To demonstrate that Qip1 and Rch1 bound to DNA helicase Q1, QIP1 and RCH1 were subcloned into the bacterial expression vector pGEX-2T to yield GST fusion genes. The expressed fusion proteins in bacterial lysates were bound to glutathione Sepharose 4B beads. HeLa cell lysate was incubated with the beads to examine whether DNA helicase Q1 bound to Qip1 and Rch1.

DNA helicase Q1 was coprecipitated with GST-Qip1- or GST-Rch1-bound glutathione beads, but not with those bound with GST alone (Fig. 2). These results indicate that Qip1 and Rch1 interact with DNA helicase Q1 *in vitro*. As Qip1 and Rch1 were isolated by two-hybrid screening, these proteins bind directly to DNA helicase Q1.

Qip1 Interacts with NLSs

S. cerevisiae SRP1 was originally isolated as a suppressor of a temperature-sensitive mutations of RNA polymerase I (7). However, its higher eukaryotic homologues have been recently confirmed to function as NLS receptors (6). Thus we examined whether Qip1 interacted with the NLS of SV40 T antigen. As shown in Fig. 3B, Qip1 interacted with the NLS as did hSrp1 and Rch1.

DNA helicase Q1 has a putative NLS similar to that of nucleoplasmin and of SV40 T antigen in its carboxy terminus (Fig.3A). We divided the DNA helicase Q1 gene into two fragments at the *EcoR* I site near the center of the gene, and subcloned both fragments into pGBT9 to yield pGBT9-Q1 N-term and pGBT9-Q1 C-term, which contained the amino- and carboxy-terminal fragments of DNA helicase Q1, respectively. The carboxy-terminal fragment of DNA helicase Q1 interacted with Qip1 and Rch1 but the amino-terminal fragment did not (Fig. 3B). The interaction between the carboxy-terminal fragment and Qip1 or Rch1, however, was weaker than that of intact DNA helicase Q1, indicating that the amino terminal fragment of DNA helicase Q1 somehow affected the interaction.

We next examined whether the interaction of Qip1 and Rch1 with DNA helicase Q1 was dependent on the existence of the putative NLS of DNA helicase Q1. A mutant DNA helicase Q1 gene lacking NLS was constructed and ligated into pGBT9 to yield pGBT9-Q1 NLS (−). The yeast reporter strain SFY526 was cotransfected with pGBT9-Q1 NLS (−) and GAL4 activation domain vector carrying Qip1 or Rch1. β-Galactosidase activity was not detected in either case, although it was detected with the intact DNA helicase Q1 gene (Fig. 3B), indicating that the interaction requires the putative NLS. It must be noted that hSrp1, which is now known as a human homologue for importin-α, did not interact even with intact DNA helicase Q1 although hSrp1 interacted with the NLS of SV40 T antigen.

To confirm direct interaction of Qip1 with the NLS, we examined whether GST-Qip1 interacted with the NLS peptide of SV40 T antigen and the putative NLS peptide of DNA helicase Q1. As shown in Fig. 4, GST-Qip1 as well as GST-Rch1 interacted with the NLS peptide of T antigen. In addition, these importin-α homologues also interacted with the putative NLS peptide of DNA helicase Q1. These results indicate that Qip1, like other importin-α homologues, might function in nuclear transport of proteins.

In the present study, we isolated a cDNA encoding a novel importin-α homologue, Qip1, which interacts with the NLS motifs of SV40 T antigen and of DNA helicase Q1. Qip1 contains the conserved amino acid sequence required for the interaction with importin-β. Thus, it seems very likely that Qip1 functions as an NLS receptor like *Xenopus* importin -α in the process of nuclear protein transport into the nucleus. Although we have not confirmed that the putative NLS of DNA helicase Q1 functions as NLS in the cell, it seems likely that a certain type of NLS discriminates importin-α homologues.

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