Differential Modes of Nuclear Localization Signal (NLS)
Recognition by Three Distinct Classes of NLS Receptors*

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The targeting of karyophilic proteins to nuclear pores is mediated via the formation of a nuclear pore-targeting complex, through the interaction of nuclear localization signal (NLS) with its NLS receptor. Recently, a novel human protein, Qip1, was identified from a yeast two-hybrid system with DNA helicase Q1. This study demonstrates that Qip1 is a novel third class of NLS receptor that efficiently recognizes the NLS of the helicase Q1. Moreover, the data obtained in this study show that the specific interaction between Qip1 and the NLS of the helicase Q1 requires its upstream sequence of the minimal essential NLS. By using purified recombinant proteins alone in the digitonin-permeabilized cell-free transport system, it was demonstrated that the two known human NLS receptors, Rch1 and NPI-1, are able to transport all the tested NLS substrates into the nucleus, while Qip1 most efficiently transports the helicase Q1-NLS substrates, which contain its upstream sequence in so far as we have examined the system. Furthermore, in HeLa cell crude cytosol, it was found that endogenous Rch1 binds to all the tested NLS substrates, while the binding of endogenous NPI-1 is restricted to only some NLSs, despite the fact that NPI-1 itself shows binding activity to a variety of NLSs. These results indicate that at least three structurally and functionally distinct NLS receptors exist in the human single cell population, and suggest that the nuclear import of karyophilic proteins may be controlled in a complex manner at the NLS recognition step by the existence of a variety of NLS receptors with various specificities to each NLS.

In eukaryotic cells, the selective transport of karyophilic proteins to the nuclei is mediated by short amino acid sequences, which are commonly referred to as nuclear localization signals (NLSs)1 and which are characteristically rich in basic amino acids (1–3). NLSs can be classified into two major groups. The first is a single type containing 3–5 basic amino acids with the weak consensus Lys-Arg/Lys-X-Arg/Lys, which is similar to the simian virus 40 large T antigen (SV40 T) NLS. The other is a bipartite type NLS containing two clusters of basic regions of 3–4 residues, each separated by approximately 10 amino acids, similar to nucleoplasmin NLS. The NLS functions at various positions within the protein and is capable of directing a non-karyophilic protein into nuclei when conjugated genetically or chemically (4).

It is generally thought that the NLS-mediated nuclear transport of karyophilic proteins occurs in at least two steps (5–7). The first step is the NLS-dependent, but energy- and temperature-independent, binding to the cytoplasmic face of the nuclear pore complex. The second step is an energy and temperature-dependent translocation through the nuclear pore complex.

In earlier studies, we found that a karyophilic protein forms a stable complex, the nuclear pore-targeting complex (PTAC) in the cytoplasm to target nuclear pores (4, 8). The complex consists of a karyophilic protein and two essential factors, PTAC58 and PTAC97, which were originally isolated from mouse Ehrlich ascites tumor cells. PTAC58, also named mouse pendulin, directly recognizes the NLS (9). A number of proteins related to PTAC58 have been identified from other species using various biological screening techniques. These include proteins such as SRP1p/Kap60 (10, 11) from yeast, importin-α (12) from Xenopus, Rch1/hSRP1α (13, 14) and NPI-1/karyopherin-α/hSRP1 (15–17) from human, OHO-31/pendulin (18, 19) from Drosophila, and aLMpα (20) from Arabidopsis. Rch1 and NPI-1 were found to have about 50% amino acid identity. These NLS receptors contain Armadillo repeating motifs in their primary structure (21, 22). The motifs consist of about 40 residues, which are rich in hydrophobic amino acids. On the other hand, PTAC97 (23), which is also called p97 (24, 25), importin-β (26), and karyopherin-β (27), does not recognize the NLS, but mediate the first step in the transport by binding directly to the PTAC58, which is bound to the karyophilic, and nuclear pore complex. Moreover, it has been found that Ran/TC4 (28, 29), which is a Ras superfamily of small GTPases, supports the translocation step of the transport in conjunction with its interacting protein, p10/NTF2 (30, 31). In the Ran-mediated translocation step, although importin-α enters the nucleus together with the cargo, importin-β remains on the nuclear envelope, suggesting that the two subunits of importin dissociate during the import reaction (32, 33). It has been clearly demonstrated that guanosine triphosphate (GTP) hydrolysis by Ran is required for the translocation step, but the exact mechanism of translocation remains unclear (34–36).

Although, for example, two major groups, Rch1 and NPI-1, are found in the human single cell population, the number of groups that exist in the same species and the variety of NLS
receptors that are actually required for the nuclear transport remains unknown. Further, if the variety is essential for cellular functions, then it is essential to know whether each isoform recognizes different classes of NLSs and how each one distinguishes the NLSs in the cytoplasm. Recently, it was shown by Northern blot analysis that mouse pendlumin/PTAC58 is very highly expressed in thymus, spleen, and heart (37). This result suggests that the expression of the isoform is regulated in a tissue-specific manner, although the physiological significance for the tissue-specific expression is presently unknown.

More recently, consistent with these results, it was found that Rch1/hSRP1 and NPI-1/hSRP1 are differentially expressed in various human leukocyte cell lines and can be induced in normal human peripheral lymphocytes (38), indicating that this expression is regulated in a cell type-specific manner. Very recently, a novel human protein, named DNA helicase Q1-interacting protein 1 (Qip1; GenBank accession number AB002533) was identified by using a yeast two-hybrid system with DNA helicase Q1 (39). DNA helicase Q1 is a human homologue of Escherichia coli RecQ, which is known to be a member of the RecF recombination pathway and has an intrinsic DNA-dependent ATPase activity (40–43). Since Qip1 has Armadillo repeating motifs in its primary structure and shows about 50% amino acid identity to both Rch1 and NPI-1, it was suspected to be a third class of human NLS receptor.

In this study, we demonstrate that Qip1 is, in fact, a novel third class of human NLS receptor, which efficiently recognizes the NLS of DNA helicase Q1. Further, we found that the recognition of helicase Q1-NLS by Qip1 requires the upstream amino acid sequences of helicase Q1-NLS as well as a single basic amino acid cluster of the NLS itself. Moreover, we found that PTAC58 (~Rch1) interacts with all the NLSs tested, but NPI-1 can interact with only limited types of NLSs, as evidenced by solution binding assays of the crude cytosol. In contrast, NPI-1 showed binding ability for all the tested NLSs when the solution binding assays were performed using recombinant proteins alone. These findings provide evidence that at least three distinct classes of NLS receptors exist in human cells, each of which shows NLS recognition in a different manner. The results suggest that a regulatory mechanism may exist for nuclear transport of karyophilic proteins at the NLS recognition step, and that the binding of some NLS receptors to exist for nuclear transport of karyophilic proteins at the NLS receptors alone. These findings provide evidence that at least three distinct classes of NLS receptors exist in human cells, each of which shows NLS recognition in a different manner.

**NLS Recognition by Three Distinct Classes of NLS Receptors**

**Preparation of Peptide Conjugates**—Bovine serum albumin (BSA) (Sigma), biotinylated BSA (bBSA), or allophycocyanin (APC) (Calbiochem) was chemically conjugated to synthetic peptides as described previously (8, 46). All the conjugates contained 5–9 peptides/carryer molecule, as judged from SDS-polyacrylamide gel electrophoresis.

**Expression and Purification of Recombinant Proteins**—Qip1 was constructed into pGEX2T (Pharmacia Biotech Inc.), was expressed as glutathione S-transferase (GST)-fused Qip1 in E. coli BL21 (DE3). The E. coli cells were grown in LB medium containing 50 μg/ml ampicillin at 37 °C to a density of A660 = 1.0. Expression was induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, followed by incubation at 20 °C. Lysis of bacteria and purification of the fusion protein with glutathione-Sepharose were performed as described previously (8). The GST portion of chimeras was cleaved by incubation for 2 h at 25 °C with 1 NIH unit of thrombin/100 μg of chimeras. GST and thrombin were separated from recombinant proteins by ion-exchange chromatography (MonoQ) column at a flow rate of 0.5 ml/min, with a linear gradient from 0.5 to 1.0 M NaCl in 20 m M Hepes-NaOH, pH 7.3, 2 mM diithothreitol (DTT), 1 μg/ml each of aprotinin, leupeptin, and pepstatin A, and checked on 10% SDS-polyacrylamide gel electrophoresis. The purified proteins were dialyzed against 20 mM Hepes, pH 7.3, 100 mM CH3COOK, 2 mM DTT, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin A. Human NPI-1 gene was amplified from a HeLa cell cDNA library using the polymerase reaction with appropriate oligonucleotides. Polymerase chain reaction products were confirmed by DNA sequencing. Recombinant PTAC58 and NPI-1 were purified as described previously (9). Recombinant Ran (47, 48) and p10 (49) were prepared as described previously.

**Preparation of Cytochalasin Cells**—HeLa S3 cells were grown in culture medium (RPMI 1640/Life Technologies, Inc.), 5% fetal bovine serum, 10 m M Hepes- NaOH, pH 7.3) with an isoviscous factor obtained from the Peptide Institute (Japan). ShortT and ShortQ1, and ShortQ1 peptides were purified by reversed-phase high-performance liquid chromatography (MonoQ) column at a flow rate of 0.5 ml/min, with a linear gradient from 0.5 to 1.0 M NaCl in 20 m M Hepes-NaOH, pH 7.3, 2 mM diithothreitol (DTT). They were then homogenized with lysin buffer (5 mM Hepes-NaOH, pH 7.3, 10 mM CH3COOK, 2 mM DTT, 2 μg/ml cytochalasin B, 1 μ M ethidium bromide, 1 μg/ml each of aprotinin, leupeptin, and pepstatin A). The extract was dialyzed against transport buffer (20 mM Hepes, pH 7.3, 110 mM CH3COOK, 2 mM (CH3COO)2Mg, 5 mM CH3COONa, 0.5 mM EDTA, 2 mM DTT, 1 μg/ml each of aprotinin, leupeptin, and pepstatin A).

**Antibodies**—The purified recombinant Qip1 was used to immunize two rabbits. Immunizations were performed as described previously (9) except that 0.75 mg of recombinant protein was injected into each rabbit on each occasion. Anti-Qip1 antibodies were purified independently from antisera of each rabbit, by passage over GST-Qip1 immobilized to glutathione-Sepharose with glutaraldehyde. Antibodies bound to Qip1-Sepharose were eluted with 100 mM glucose-HIC, pH 2.5, and neutralized. After dialysis against phosphate-buffered saline (PBS), containing 300 mM NaCl, the purified antibodies were concentrated in a Micro Centrin 30. Anti-PTAC58 and anti-NPI-1 antibodies were purified as described previously (9, 50).

**Microinjection**—MDBK cells were plated on coverslips, and microinjection experiments of DNA helicase Q1-related peptide-bBSA (1 mg/ml) were performed as described previously (46). After injection into the cytoplasm and incubation for 30 min at 37 °C, cells were fixed with 3.7% HCHO in phosphate-buffered saline. The injected biotinylated peptide conjugates were detected by fluorescein isothiocyanate-avidin (Pierce).

**Solution Binding Assay for Recombinant or Endogenous NLS Receptor**—Ten μl of each peptide-conjugated bBSA (1 mg/ml) was immobilized on 15 μl of avidin-agarose gel, and mixed with affinity-purified recombinant GST-PTAC97 (100 pmol) in addition to GST-Qip1, GST-PTAC58, or GST-NPI-1 (100 pmol), respectively, and the total reaction volume was adjusted to 100 μl with TB. After incubation for 1 h at 4 °C, materials bound to the immobilized peptide conjugates were washed with TB, and eluted with elution buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2% SDS) for 30 min at 37 °C. Each NLS receptor immobilized peptide conjugate was analyzed by Western blotting with anti-PTAC58, anti-NPI-1, or anti-Qip1 antibodies. Immunoblotting was performed as described previously.

One ml of HeLa cell cytosol (8 mg/ml) containing a final concentration of 2 μg/ml cytochalasin B was added to 10 μl of peptide-bBSA conjugates (1 mg/ml), and the mixture was incubated with 15 μl of avidin-agarose gel for 1 h at 4 °C. After the agarose was washed with...
TB, the bound proteins were eluted with incubation with elution buffer for 30 min at 37 °C. NLS receptors bound to immobilized NLS substrates were detected by anti-NLS receptor antibodies.

Cell-free Transport Assay—Digitonin-permeabilized MDBK cells were prepared as described previously (8, 44). The 10-μl sample solution contained 1 μl of peptide-APC conjugate (1 mg/ml) and appropriate transport factors were diluted with TB. For the nuclear-binding (first step) assay, the incubation was performed on ice for 20 min in the presence of each NLS receptor (7 pmol) and PTAC97 (6 pmol), the concentration of which was adjusted with TB containing 2% BSA. For the nuclear import (second step) assay, the incubation was performed at 30 °C for 20 min in the presence of each NLS receptor (7 pmol), PTAC97 (6 pmol), GDP-Ran (42 pmol), p10/NTP2 (14 pmol), 1 mM ATP, ATP regeneration system (20 units/ml creatine phosphokinase, 5 mM creatine phosphate), and 1 mM GTP, the concentration of which was adjusted with TB containing 2% BSA. After incubation, cells were fixed with 3.7% HCHO in TB. Peptide-APC conjugates were detected by Axiopt fluorescence microscopy (Carl Zeiss, Inc.).

RESULTS

Determination of the NLS of DNA Helicase Q1—To determine whether Qip1 actually functions as an NLS receptor, we first attempted to determine the NLS of DNA helicase Q1. Based on the observation that Qip1 failed to bind DNA helicase Q1, which lacks the C-terminal basic amino acid cluster in a yeast two hybrid system (39), we postulated that DNA helicase Q1-NLS involves the 4 C-terminal basic amino acids (KKK626). Furthermore, since another basic amino acid cluster (KK626) was located 16 amino acids upstream from this C-terminal basic cluster, we also considered the possibility that the helicase Q1-NLS is a bipartite type. Therefore, considering these two possibilities, we prepared four types of synthetic peptides containing only the C-terminal basic amino acid cluster (ShortQ1, termed in Table I), the C-terminal basic cluster and its 19-amino acid upstream sequence (LongQ1), a peptide identical to LongQ1 except for the amino acid substitution K625,626A (LongQ1(KK-AA)), and the 17 amino acids upstream from the C-terminal basic amino acid cluster containing KK626 (UpstreamQ1), and conjugated these to bBSA. We then examined whether these conjugates migrate into the nucleus, when injected into the cytoplasm of cultured mammalian cells. As shown in Fig. 1, we found that ShortQ1, LongQ1, and LongQ1(KK-AA) efficiently directed bBSA into the nucleus to the same extent, but UpstreamQ1 did not. From these findings, we conclude that the C-terminal basic amino acid cluster of DNA helicase Q1 is necessary and sufficient for NLS activity. As a result, the NLS of helicase Q1 can be classified into a conventional single basic type.

Qip1 Is a Novel NLS Receptor That Requires Upstream Sequences from the NLS of DNA Helicase Q1 for Its NLS Recognition—We next examined whether Qip1 directly binds to the helicase Q1-NLS in a solution binding assay. Purified recombinant Qip1 was added to the peptide-bBSA conjugates immobilized to avidin-agarose, and the interaction was examined by Western blotting of bound materials with affinity-purified anti-Qip1 antibodies. Unexpectedly, as shown in Fig.

TABLE I

| Name       | Sequence |
|------------|----------|
| ShortQ1    | CYGSKNTGAKKKPK1DDA |
| LongQ1     | CYFQKAANLQGSKGNTGAKKKPK1DDA |
| LongQ1(KK-AA) | CYFQKAANNLQGSKGNTGAKKKPK1DDA |
| UpstreamQ1 | CYFQKAANNLQGSKGNTGAKKKPK1DDA |
| ShortT     | CYGGPKKKRKVVEDP |
| LongT      | GCYMPSSDDEATDSQHSTPPKKKRKVEDP |
| ReverseT   | CYGGPDEVKRRKKP |
| CBP80      | ACCYMRRRHSNDENDGQPHKRRKRTSDANED |
| Q1T        | CYFQKAANNLQGSKGNTGAKKKPK1DDA |

FIG. 1. Identification of the NLS of DNA helicase Q1. MDBK cells were grown on coverslips in Dulbeco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. bBSA only (A) and ShortQ1- (B), LongQ1- (C), LongQ1(KK-AA)- (D), or UpstreamQ1- (E) bBSA conjugates (1 mg/ml) were microinjected into the cytoplasm of MDBK cells, followed by incubation for 30 min at 37 °C. After the cells were fixed with 3.7% HCHO, they were permeabilized with 0.5% Triton X-100. Intracellular distribution of the conjugates was detected by fluorescein isothiocyanate-avidin.

2A, Qip1 bound to LongQ1- and LongQ1(KK-AA)-bBSA efficiently, but only weakly to ShortQ1- and not at all to UpstreamQ1-bBSA. Differential binding activity of Qip1 with ShortQ1- and LongQ1-bBSA indicates that the upstream sequences contained in LongQ1 peptide play an important role in the recognition of helicase Q1-NLS by Qip1.

To confirm whether these upstream sequences actually contribute to the NLS recognition by Qip1, we prepared three additional peptides containing minimal essential sequences of SV40 T-antigen NLS (ShortT), ShortT sequences plus its own upstream sequences of the same length as those of LongQ1 (LongT), and SV40 T-antigen NLS combined to the 17-amino acid upstream sequence of DNA helicase Q1-NLS (Q1T). Furthermore, we prepared a synthetic peptide, CBP80, which contains a typical bipartite type NLS for a nuclear CAP-binding protein 80. As shown in Fig. 2B, Qip1 was found to bind efficiently to Q1T-bBSA, but only slightly to ShortT-, LongT-, and CBP80-bBSA, indicating that the NLS binding of Qip1 requires not only the C-terminal basic amino acid cluster of DNA helicase Q1, but also its upstream sequences, and that the basic amino acid sequence in the NLS is essential but not sufficient for the binding of Qip1. On the other hand, recombinant PTAC58 and NPI-1 efficiently bound to all the tested functional NLS substrates (ShortQ1-, LongQ1-, LongQ1(KK-AA)-, ShortT-, LongT-, CBP80-, and Q1T-bBSA), suggesting that the upstream sequences are not required for the NLS recognition by PTAC58 and NPI-1.

To further investigate whether Qip1 actually functions as an NLS receptor, we examined the transport activity of recombinant Qip1 using an in vitro digitonin-permeabilized cell-free transport assay. As expected, as shown in Fig. 3, Qip1 transported LongQ1- and Q1T-APC conjugates into the nucleus efficiently, but ShortQ1-APC was transported only weakly, and UpstreamQ1-APC conjugate not at all (data not shown), which is consistent with the solution binding assay described above. These results indicate that Qip1 acts as a novel class of NLS.
receptor, requiring the upstream sequences of the helicase Q1-NLS for its NLS recognition.

Different Classes of NLS Receptors Are Functionally Distinct from One Another—From this and previous studies, it is evident that at least three different classes of NLS receptors, Rch1, NPI-1, and Qip1, are present in HeLa cells. However, the issue of whether distinct classes of NLS receptors show different NLS recognition specificity to a variety of NLSs remains poorly understood. To address this question, we compared the ability of three classes of recombinant NLS receptors to transport various NLS substrates into the nucleus using the digitonin-permeabilized cell-free transport system. Since mouse PTAC58 is closely related to human Rch1, showing an amino acid identity of 94%, this recombinant mouse PTAC58 was used instead of human Rch1. The data on ShortT- and LongQ1(KK-AA)-APC transport are not shown, because their fluorescence images were the same as those of LongT- and LongQ1-APC, respectively.

As mentioned above, the recombinant Qip1 targeted LongQ1- and Q1/T-APC conjugates to the nuclear pores in the presence of recombinant PTAC97 under the first step assay condition, but transported ShortQ1-, LongT-, and CBP80-APC hardly at all (Fig. 3). Moreover, under the second step assay condition, the recombinant Qip1 efficiently transported LongQ1- and Q1/T-APC into the nucleus.

On the other hand, the recombinant PTAC58 efficiently transported all the tested NLS substrates into the nucleus, except for ReverseT-APC, both in the first and second step of transport. Recombinant NPI-1, like PTAC58, targeted all the NLS substrates examined to nuclear pores in the first step of the transport except ReverseT-APC. Further, under the second step assay conditions, all the transport substrates were efficiently transported by the recombinant NPI-1 into the nucleus.
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Although some of the transport substrates (ShortQ1-, LongQ1-, and Q1/T-APC) showed an unusual dotlike pattern of fluorescence image within the nucleus. The dot pattern image was not observed when the assay was carried out in the presence of a lectin, wheat germ agglutinin, indicating that the dot pattern observed in the in vitro transport assay may be due to aggregation, which, for some unknown reason, occurred in the nucleus after translocation (discussed below).

These findings indicate that the NLS receptors can be divided into two major groups, judged from the viewpoint of their own specificities for NLS recognition, i.e. Rch1 and NPI-1 have a binding ability for a wide variety of NLSs, while Qip1 has high specificity for binding to only certain NLSs. These results also give rise to the problem of whether Rch1 and NPI-1 may act as the same NLS receptor in cells.

**NPI-1 Is Restricted to Binding to a Particular NLS in the Crude Cytosol**—We next examined whether endogenous NLS receptors bind to the NLS substrates in the crude cytosol as efficiently as do the purified recombinant proteins alone. For this, immobilized biotin-labeled NLS conjugates were incubated with crude cytosolic extracts prepared from HeLa cells, and the bound materials were blotted with affinity-purified anti-PTAC58, anti-NPI-1, and anti-Qip1 antibodies, respectively. These affinity-purified antibodies have high specificities for each molecule, and anti-PTAC58 antibodies cross-react with human Rch1 but not NPI-1 and Qip1 in HeLa cells.

As shown in Fig. 4, it was found that endogenous Rch1 detected by affinity-purified anti-PTAC58 antibodies efficiently bound to all the tested functional NLS-bBSA substrates (ShortT-, LongT-, ShortQ1-, LongQ1-, LongQ1(KK-AA)-, and CBP80-bBSA). These results show that Rch1 is capable of binding to many different classes of NLSs, even in the presence of other endogenous cytosolic proteins, which is consistent with the results, based on the in vitro transport assay.

On the other hand, it was found that endogenous NPI-1 bound only slightly to SV40 T-NLS-bBSA (ShortT- and LongT-bBSA) (Fig. 4), although purified recombinant NPI-1 alone, like recombinant Rch1 alone, efficiently transported SV40 T-NLS-APC (Fig. 3) into the nucleus to the same extent as in the case of the other tested NLS substrates in the in vitro transport assay.

These results indicate that these three classes of NLS receptors behave in quite different manners in recognizing a variety of NLSs in cells. This finding suggests that NLS receptors can be structurally and functionally divided into three distinct classes. Moreover, since the specificity for the NLS recognition as was observed for NPI-1 in the presence or absence of other cytosolic proteins changed drastically, it is plausible to conclude that the NLS recognition specificity of the NLS receptors may be regulated by other cytosolic proteins (see "Discussion").

**DISCUSSION**

The present study describes the determination of the NLS of DNA helicase Q1. Although the primary sequences of helicase Q1 suggest that the NLS may be a bipartite type, as shown in Fig. 1, its C-terminal 15 amino acids containing only a single basic amino acid cluster (KKKR(KK)-ShortQ1) and its C-terminal 27 amino acids, which included amino acid substitutions (K625,626AA) (LongQ1(KK-AA)) were found to act as an NLS in living cells. However, the upstream 17 amino acids alone (UpstreamQ1) do not support the transport, which indicates that the NLS of helicase Q1 can be classified into a conventional single basic type, similar to the SV40 T-antigen NLS. These in vitro results were confirmed by using the in vitro semi-intact cell-free transport system. The C-terminal 15-amino acid containing peptide-APC (ShortQ1-APC) was efficiently transported into the nucleus in the presence of the known transport factors (Fig. 3) or crude HeLa cell cytosol (data not shown).

Since Qip1 has an Armadillo repeating motif, characteristic of the known NLS receptors, in its primary structure, and shows about 50% amino acid identity to both of the known human NLS receptors, Rch1 and NPI-1, it would be expected that Qip1 would interact with the NLS of helicase Q1, ShortQ1 sequence. As shown in Fig. 2A, however, the data clearly show that Qip1 binds to LongQ1- and LongQ1(KK-AA)-bBSA efficiently, but only weakly to ShortQ1-bBSA in the solution binding assay. Accordingly, we examined whether Qip1 transports these substrates into the nucleus, in the presence of the known transport factors alone in the in vitro transport assay. As shown in Fig. 3, Qip1 efficiently carries LongQ1-APC, but not ShortQ1-APC, into the nucleus, which is consistent with the solution binding assay data, shown in Fig. 2A. These results indicate that Qip1 acts as an NLS receptor for helicase Q1 but that its efficient NLS recognition requires sequences that are upstream from the functional NLS.

Since it was shown that Qip1 is a functional NLS receptor for helicase Q1, we examined whether Qip1 functions as an NLS receptor for other NLS substrates. For this, we prepared synthetic peptides containing SV40 T-NLS as a representative for single basic type NLSs and CBP80-NLS as that for bipartite basic type NLSs. As shown in Figs. 2B and 3, Qip1 bound only weakly to ShortT-bBSA, LongT-bBSA, and CBP80-bBSA, and transported negligible amounts of LongT- and CBP80-APC into the nucleus in the in vitro assay. These results suggest the possibility that Qip1 acts as a novel NLS receptor, which has high recognition specificity for helicase Q1-NLS, although the possibility remains that Qip1 is capable of transporting other untested karyophilic proteins into the nucleus as efficiently as helicase Q1.

To exclude the possibility that the efficient and specific NLS recognition by Qip1 requires the NLS to only extrude from the molecule, we prepared synthetic peptides containing SV40 T-NLS plus its upstream sequences (LongT) whose length is same as that of LongQ1, and a peptide in which the basic amino acid cluster of the LongQ1 was substituted by the minimal essential
sequence of SV40 T-NLS (Q1/T). As shown in Figs. 2B and 3, it was found that neither LongT-bBSA nor ShortT-bBSA bound efficiently to Qip1, and LongT-APC was barely transported by Qip1 in the in vitro assay. On the other hand, Q1/T-bBSA and -APC were efficiently recognized and transported into the nucleus by Qip1. The number of peptides conjugated to bBSA was nearly the same for both Q1/T- and LongT-bBSA, as judged from electrophoretic mobility shifts. Therefore, it is most likely that the upstream sequence of helicase Q1 required for NLS recognition by Qip1 does not function simply to extrude the NLS from the molecule, but plays a role in the efficient recognition of NLS by Qip1. However, since UpstreamQ1 peptides, which contain only the upstream 17 amino acids, were not recognized by Qip1, the upstream region is necessary but not sufficient for efficient NLS recognition by Qip1.

Evidence that Qip1 efficiently recognizes both LongQ1 and Q1/T conjugates indicates that the upstream region, rather than the basic cluster of the NLS, plays a critical role in the efficient recognition of helicase Q1-NLS by Qip1, although both are required for Qip1 to act as an NLS receptor for the helicase Q1. The issue of which portion of the 17-amino acid upstream sequence is required for the efficient NLS recognition by Qip1 is now under investigation.

From this and earlier studies, it appears likely that there are at least three classes of NLS receptors, Rch1, NPI-1, and Qip1 in HeLa cells. From the results involving their recombinant proteins, it is also clear that Rch1 (−PTAC58) and NPI-1 can recognize and transport all the functional NLS-containing substrates into the nucleus as evidenced by studies to date. Therefore, the NLS receptors can be divided into two major functional classes: (i) a class of low stringency NLS receptors, such as Rch1 and NPI-1; and (ii) a class of high stringency NLS receptors, such as Qip1. The high stringency of NLS recognition by Qip1 was found to require the upstream sequences of the NLS, rather than the NLS itself. However, how Qip1 specifically recognizes this upstream region near the helicase Q1-NLS and whether it is capable of recognizing other untested NLSs or not remain unknown. Furthermore, whether the region of Qip1 required for the recognition of the upstream sequences is different from that for the binding of the basic amino acid cluster of the NLS remains to be elucidated.

As described above, Rch1 and NPI-1 were functionally indistinguishable when their recombinant proteins were used in the in vitro assay, which raises the possibility that both Rch1 and NPI-1 actually function as equivalent NLS receptors in living cells. To answer this question, we examined whether endogenous Rch1 and NPI-1 behave in a manner similar to that of recombinant NLS receptors. As shown in Fig. 4, endogenous Rch1 efficiently bound to all the tested NLS substrates, while the binding affinity of endogenous NPI-1 to SV40 T-NLS, but not other tested NLSs, was significantly reduced in the HeLa cell crude cytosol. There are three possibilities to explain these phenomena. First, endogenous Rch1 may have a much higher affinity for SV40 T-NLS than NPI-1 and, thus, compete with endogenous NPI-1. However, it seems likely that both receptors would be occupied by SV40 T-NLS substrates, since excess amounts of the NLS substrates were added to the crude cytosol. Second, endogenous NPI-1 may bind tightly to another NLS-containing karyophile in the crude cytosol, which might have a much higher affinity for NPI-1 than the SV40 T-NLS substrates. In this case, the binding of other artificial substrates, such as ShortQ1- and CBP80-bBSA to endogenous NPI-1, should also be inhibited, provided the NLS-hinding site of NPI-1 is the same. Third, other cytosolic factor(s) may exist that modulate the binding affinity of NPI-1 to a variety of NLSs. Very recently, Nadler et al. (38) showed that the two NLS receptors, Rch1 and NPI-1, bind with varied specificities in a sequence-specific manner to different NLSs and that the sequence specificity is altered by other cytosolic proteins in leukocyte cell lines. Although the NLSs used in this study are different from those of Nadler et al. and, as a result, we cannot compare our results with theirs directly, our results are not inconsistent with their findings.

Moreover, the substrates related to the helicase Q1 such as LongQ1-APC showed an unusual dotlike pattern within the nucleus only when recombinant NPI-1 was used. Unlike the in vitro results using purified recombinant proteins alone, when the substrates were injected into the cytoplasm of living cells, this dotlike pattern was not observed. As described under “Results,” these dots appeared to occur within the nucleus after translocation, which may point to other properties of NLS receptors with respect to intranuclear events, such as the dissociation of the substrates from NPI-1.

The present study suggests that there are at least three distinct classes of NLS receptors, which have different NLS specificities. The question of why such a divergence of NLS receptors exists in cells is an interesting one. Our results, and those of others, show that the NLS specificity of each receptor is modulated in a cell type-specific manner, i.e. there is a possibility that the same NLS receptor transports different karyophiles into the nucleus when it acts in different types of cells. Furthermore, although Qip1 is a unique example that binds efficiently to limited number of NLSs, it is possible that other NLS receptors that recognize unique and specific NLSs may be further identified in the future. On the other hand, it has been shown that the expression of various NLS receptors is regulated in both tissue-specific and cell type-specific manners (37, 38). The findings herein suggest that nuclear protein transport is differentially regulated at the NLS recognition step through the expression level and/or the controls of NLS binding specificities of NLS receptors in various cells, which, in turn, may lead to the regulation of development or cell differentiation. Therefore, it is important to understand precisely how many isoforms of NLS receptors are expressed in the same species, how their expression and NLS specificities are controlled at the molecular level, and which karyophiles they transport into the nucleus in each cell or tissue.

REFERENCES
1. Dingwall, C., and Laskey, R. A. (1991) Trends Biochem. Sci. 16, 478–481.
2. Garcia-Bustos, J., Heitman, J., and Hall, M. N. (1991) Biochim. Biophys. Acta 1071, 83–101.
3. Makker, J. P. S., Dingwall, C., and Laskey, R. A. (1996) Curr. Biol. 6, 1025–1027.
4. Yoneda, Y. (1996) Arch. Histol. Cytol. 59, 97–107.
5. Newmeyer, D. D., and Forbes, D. J. (1988) Cell 52, 641–653.
6. Richardson, W. D., Mills, A. D., Dibworth, S. M., Laskey, R. A., and Dingwall, C. (1988) Cell 52, 655–664.
7. Akery, C. W., and Goldfarb, D. S. (1989) J. Cell Biol. 109, 971–982.
8. Imamoto, N., Tachibana, T., Matsubae, M., and Yoneda, Y. (1995) J. Biol. Chem. 270, 8559–8565.
9. Imamoto, N., Shimamoto, T., Takao, T., Tachibana, T., Kose, S., Matsubae, M., Sekimoto, T., Shimoshini, Y., and Yoneda, Y. (1995) EMBO J. 14, 3617–3620.
10. Yano, R., Oakes, M., Yamagishi, M., Dodd, J. A., and Nomura, M. (1992) Mol. Cell. Biol. 12, 5640–5651.
11. Enenkel, C., Blobel, G., and Rexach, M. (1995) J. Biol. Chem. 270, 16499–16502.
12. Görlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994) Cell 79, 767–778.
13. Newmeyer, D. D., and Forbes, D. J. (1988) Arch. Histol. Cytol. 51, 1025–1027.
14. Wels, K., Mattaj, I. W., and Lamond, A. I. (1995) Science 268, 1049–1053.
15. Cortes, P., Ye, Z.-S., and Baltimore, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7633–7637.
16. O’Neill, R. E., and Palese, P. (1995) Virology 206, 116–125.
17. Morishima, J., Blobel, G., and Radu, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2008–2011.
18. Torok, I., Strand, D., Schmitt, R., Tick, G., Torok, T., Kiss, I., and Mechler, Y. Kamei, S. Yuba, and Y. Yoneda, manuscript in preparation.
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B. M. (1995) J. Cell Biol. 129, 1473–1489
19. Kuszel, P., and Frasch, M. (1995) J. Cell Biol. 129, 1491–1507
20. Hicks, G. R., Smith, H. M., Lobreaux, S., and Raikhel, N. V. (1996) Plant Cell 8, 1337–1352
21. Peifer, M., Berg, S., and Reynolds, A. B. (1994) Cell 76, 799–791
22. Yano, R., Oakes, M. L., Takao, T., Tachibana, T., Matsubae, M., Sekimoto, T., Shimonishi, Y., and Yoneda, Y. (1995) FEBS Lett. 368, 415–419
23. Adam, E. J., and Adam, S. A. (1994) J. Cell Biol. 125, 547–555
24. Chi, N. C., Adam, E. J. H., and Adam, S. A. (1995) J. Cell Biol. 130, 265–274
25. Gorlich, D., Kostka, S., Kraft, R., Dingwall, C., Laskey, R. A., Hartmann, E., and Prehn, S. (1995) Curr. Biol. 5, 383–392
26. Radu, A., Blobel, G., and Moore, M. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1769–1773
27. Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993) J. Cell Biol. 123, 1649–1659
28. Moore, M. S., and Blobel, G. (1993) Nature 365, 661–663
29. Moore, M. S., and Blobel, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10212–10216
30. Paschal, B. M., and Gerace, L. (1995) J. Cell Biol. 129, 925–937
31. Morenau, J., Hijkata, M., Blobel, G., and Radu, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6532–6536
32. Gorlich, D., Vogel, F., Mills, A. D., Hartmann, E., and Laskey, R. A. (1995) Nature 377, 246–248
33. Melchior, F., and Gerace, L. (1995) Curr. Opin. Cell Biol. 7, 310–318
34. Gorlich, D., and Mattaj, I. W. (1996) Science 271, 1513–1518
35. Koepp, D. M., and Silver, P. (1996) Cell 87, 1–4
36. Prieve, M. G., Guttridge, K. L., Mungua, J. E., and Waterman, M. L. (1996) J. Biol. Chem. 271, 7654–7658
37. Nadler, S. G., Tritschler, D., Hassar, O. K., Blake, J., Bruce, A. G., and Cleaveland, J. S. (1997) J. Biol. Chem. 272, 4310–4315
38. Seki, T., Tada, S., Katada, T., and Enomoto, T. (1997) Biochem. Biophys. Res. Commun. 234, 48–53
39. Seki, S., Miyazawa, H., Tada, S., Yanagisawa, J., Yamaoka, T., Hoshino, S., Ozawa, K., Eki, T., Nogami, M., Okumura, K., Taguchi, H., Hanashka, F., and Enomoto, T. (1994) Nucleic Acids Res. 22, 4566–4573
40. Seki, M., Miyazawa, H., Tada, S., Yanagisawa, J., Yamaoka, T., Hoshino, S., Ozawa, K., Eki, T., Nogami, M., Okumura, K., Taguchi, H., Hanashka, F., and Enomoto, T. (1994) J. Biol. Chem. 271, 657–668
41. Yoneda, Y., Arioka, T., Imamoto-Sonobe, N., Sugawa, H., Shimonishi, Y., and Uchida, T. (1987) Exp. Cell Res. 170, 439–452
42. Melchior, F., Sweet, D. J., and Gerace, L. (1995) Methods Enzymol. 257, 279–291
43. Sekimoto, T., Nakajima, K., Tachibana, T., Hirano, T., and Yoneda, Y. (1995) J. Biol. Chem. 271, 31017–31021
44. Sekimoto, T., Nakajima, K., Tachibana, T., Hirano, T., and Yoneda, Y. (1996) J. Biol. Chem. 271, 31017–31021
45. Tachibana, T., Hieda, M., Sekimoto, T., Yoneda, Y. (1996) FEBS Lett. 397, 177–182
46. Sekimoto, T., Nakajima, K., Tachibana, T., Hirano, T., and Yoneda, Y. (1996) J. Biol. Chem. 271, 31017–31021
47. Sekimoto, T., Nakajima, K., Tachibana, T., Hirano, T., and Yoneda, Y. (1996) FEBS Lett. 397, 177–182
48. Imamoto, N., Matsuo, Y., Kurihara, T., Kohno, K., Miyagi, M., Sakiyama, F., Okada, Y., Tsunawasawa, S., and Yoneda, Y. (1992) J. Cell Biol. 119, 1047–1061