Phospholipid Scramblase 1 Contains a Nonclassical Nuclear Localization Signal with Unique Binding Site in Importin α*

Received for publication, November 22, 2004, and in revised form, December 17, 2004
Published, JBC Papers in Press, December 17, 2004, DOI 10.1074/jbc.M413194200

Min-Hsuan Chen‡, Iris Ben-Efraim§, Gregory Mitrousis‡, Nancy Walker-Kopp‡, Peter J. Sims§, and Gino Cingolani**

From the ‡Department of Biochemistry and Molecular Biology, State University of New York Upstate Medical University, Syracuse, New York 13210 and the §Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

Nuclear import of proteins containing a classical nuclear localization signal (NLS) is an energy-dependent process that requires the heterodimer importin α/β. Three to six basic contiguous arginine/lysine residues characterize a classical NLS and are thought to form a basic patch on the surface of the import cargo. In this study, we have characterized the NLS of phospholipid scramblase 1 (PLSCR1), a lipid-binding protein that enters the nucleus via the nonclassical NLS (5/7/KISKHI-WTGI266). This import sequence lacks a contiguous stretch of positively charged residues, and it is enriched in hydrophobic residues. We have determined the 2.2 Å crystal structure of a complex between the PLSCR1 NLS and the armadillo repeat core of vertebrate importin α. Our crystallographic analysis reveals that PLSCR1 NLS binds to armadillo repeats 1–4 of importin α, but its interaction partially overlaps the classical NLS binding site. Two PLSCR1 lysines occupy the canonical positions indicated as P2 and P5. Moreover, we present in vivo evidence that the critical lysine at position P2, which is essential in other known NLS sequences, is dispensable in PLSCR1 NLS. Taken together, these data provide insight into a novel nuclear localization signal that presents a distinct motif for binding to importin α.

Nuclear transport is an active signal-mediated process that requires, in most cases, soluble transport factors and specific import signals. Two families of transport receptors have been identified in the importin β superfamily, which is involved in both nuclear import and export, and the TAP superfamily that mediates nuclear export. Transport receptors recognize specific nuclear localization signals (NLSs) and nuclear export signals exposed on the molecular surface of cargoes. In the classical nuclear import pathway proteins bearing a classical SV40-like NLS (PKKKRKV) are recognized by the importin α/β heterodimer (also known as karyopherin α/β (1–3). Importin α (4) acts as an adaptor that recognizes NLS sequences after association with the receptor importin β (5). The importin α/β-NLS cargo complex is then translocated through the nuclear pore complex in a process that requires multiple rounds of interaction of the receptor importin β with nucleoporins, likely via their exposed hydrophobic FG-rich motifs (6, 7). Release of the import complex from nuclear pore complex binding sites as well as the final release of the import cargo into the nucleoplasm is mediated by the small GTPase Ran, which binds to importin β in its nuclear GTP-bound form (8).

Structural and biochemical investigations of importins have provided great help in dissecting the molecular determinants for recognition of NLS sequences (9). Importin β presents a modular structure built of 19 tandem HEAT repeats (10). Each HEAT repeat is a simple secondary structural motif formed by two helices (A and B) connected by a short loop (11, 12). In the tertiary structure of importin β, 19 HEAT repeats are arranged to form a superhelix of helices, which exposes two structurally and functionally distinct surfaces to the solvent (10). The outer surface of the proteins contains at least two major nucleoporin binding sites (7, 13), whereas the internal face presents an extended cargo binding surface that also binds Ran (8). Three different cargoes have been visualized in complex with importin β using crystallographic techniques: the importin β binding (IBB) domain of importin α which binds to the C-terminal HEAT repeats 7–19 (10); the parathyroid hormone-related protein NLS, which interacts with the N-terminal HEAT repeats 2–11 (14); and the sterol regulatory element-binding protein 2, which binds as a dimer to HEAT repeats 7–17 (15).

The adaptor importin α also consists of two functionally and structurally distinct domains. The highly basic IBB domain is located N-terminally of a helical core built by ten armadillo (Arm) repeats (16). The C-terminal Arm repeat of the protein also contains an “acidic tail” that is necessary for binding to the export factor CAS/Cse1 (17). The Arm repeat structurally resembles a HEAT repeat and differs from it by being composed of three helices (A, B, and C) connected by two loops (12). The helical Arm core of importin α provides a structurally rigid scaffold that exposes a concave and a convex surface. Two NLS binding sites have been identified in the concave surface of the protein. A major binding site is located between Arm repeats 2 and 4 and a minor binding site, which presents a smaller area of binding, between Arm repeats 7 and 8 (16). Crystallographic analysis of yeast and vertebrate importin α in complex with NLS peptides has revealed that two monopartite NLS peptides bind simultaneously to both major and minor NLS binding sites (18–20). It is unclear, however, if the binding to the minor NLS binding site is physiologically relevant or reflects a crys-

* The costs of this publication were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1Y2A) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† Supported by the NHLBI, National Institutes of Health.

‡ Supported by Grants HL36846 and HL63819 from the NHLBI, National Institutes of Health.

** To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, SUNY Upstate Medical University, 750, E. Adams St., Syracuse, NY 13208. Tel.: 315-464-8744; Fax: 315-464-8750; E-mail: cingolag@upstate.edu.

The abbreviations used are: NLS, nuclear localization signal; Arm, armadillo; GFP, green fluorescent protein; His6, hexahistidine; IBB domain, importin β binding domain; PLSCR1, phospholipid scramblase 1.
tallization artifact resulting from the large excess of NLS peptide used for co-crystalization with importin α. In contrast, a single nucleosplasmid bipartite NLS interacts with both NLS binding sites via two basic boxes, which are spaced apart by ~10 nonconserved residues (KRPAATKKAGQAKKKK) (20, 21).

In addition to providing a high affinity for importin β, the IBB domain of importin α also contains an autoinhibitory NLS-like sequence that regulates binding of cargoes to the adaptor (22, 23). When importin α is not bound to importin β, the autoinhibitory sequence within the IBB domain occupies the NLS binding pocket, resulting in a low affinity (~μM) for NLS cargo. Binding of the IBB domain to importin β relieves the autoinhibition with the result that the affinity for NLS cargo increases into the nm range (24–26).

In this paper, we have studied the molecular basis for the recognition of phospholipid scramblase 1 (PLSCR1) NLS by the adaptor importin α. PLSCR1 is a lipid-binding protein that was proposed to accelerate transbilayer movement of phospholipids at high calcium concentrations (27–29). In the cytoplasm PLSCR1 is localized mostly at the endofacial plasma membrane via multiple palmitoylations, where it binds calcium via a direct EF hand-like calcium binding domain (29). It was reported recently that PLSCR1 has the potential to enter the nucleus (30). This nuclear activity was observed either in the presence of inhibitors of palmitoyl-CoA, which block PLSCR1 anchoring to the plasma membrane, or by treatment with interferon α (31), which is known to activate the PLSCR1 gene expression transcriptionally (32, 33). In the nucleus PLSCR1 might function in the overall cellular response of those cytosines and growth factors that can induce expression of the PLSCR1 gene. The PLSCR1 NLS was mapped to the C terminus region 257–266, and it was reported to be sufficient for nuclear import when conjugated to bovine serum albumin via association with the importin α/β heterodimer (30).

We have used x-ray crystallography to define the interaction between the import adaptor importin α and the nonclassical PLSCR1 NLS. Remarkably, our data indicate that the PLSCR1 NLS binds the N-terminal region of importin α between Arms 1 and 4. This region spans the classical NLS binding domain (Arm 3–4) and also includes a novel binding interaction with Arm 1–2.

**MATERIALS AND METHODS**

**Expression and Purification of Recombinant Proteins**—The gene encoding truncated importin α-2 (residues 70–529) lacking the IBB domain (ΔIBB-importin α) was cloned in a pET-30 vector (Novagen) and expressed in *Escherichia coli* BL21 (DE3) strain. Purification was carried out as described previously (19). The SV40 NLS was cloned in-frame to the green fluorescent protein (GFP) in a pET-28a vector. The fusion protein His<sub>6</sub>-SPKKKKKKVAE-NLS-GFP was expressed and purified as described previously (34). The PLSCR1 NLS fused to GFP (His<sub>6</sub>-SGKISHKWTGIEAS-GFP) was generated by ligating two oligonucleotides encoding the PLSCR1 NLS sequence into a unique Not site of the pET plasmid pET-28a-GFP. His<sub>6</sub>-SGKISHKWTGIEAS-GFP was expressed in *E. coli* BL21 (DE3) strain for 3 h at 30 °C after induction with 1 mM isopropyl-β-D-thiogalactopyranoside. Recombinant His<sub>6</sub>-SGKISHKWTGIEAS-GFP was purified by metal chelate affinity chromatography using nickel-nitritotriacetic acid-agarose beads (Qiagen).

**Fluorescence Anisotropy Assay**—Fluorescence anisotropy measurements were carried out using a Spex FluoroMax-3 fluorometer (Horiba Group). Binding of SV40 NLS-GFP and PLSCR1 NLS-GFP to importin α were measured in phosphate-buffered saline (135 mM NaCl, 2.7 mM KCl, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 44 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) as described previously (34). Changes in GFP anisotropy as a function of the importin α concentration were used to calculate the fraction of the GFP fluorophore bound, yielding a binding isotherm for the reaction. The binding isotherm was then fit through nonlinear regression and K<sub>d</sub> values calculated using the program Prism.

### Table 1

| Crystallographic statistics for ΔIBB-importin α-PLSCR1 NLS complex
| Outer shell statistics are in parentheses (2.28–2.20 Å).
| Data collection statistics
| Space group | P<sub>2</sub>1<sub>1</sub>2<sub>1</sub>2<sub>1</sub>
| Unit cell dimensions (Å) | a = 78.24, b = 91.10, c = 97.25
| Resolution range (Å) | 500–2.29
| Mosaicity (degrees) | 0.49
| Total observations | 391,309
| Unique observations | 36,323
| Completeness (%) | 92.0 (60.4)
| R<sub>w</sub> (%) | 9.2 (25.0)
| I/σ<sub>i</sub> (Refinement statistics)
| No. of reflections (working, test) | 28,953/3,255 (2,171/775)
| R<sub>work</sub> | 22.9/25.8 (26.9/30.2)
| Total number of non-H atoms | 3441
| No. of water molecules | 134
| r.m.s. deviation from ideal bond length (Å) | 0.007
| r.m.s. deviation from ideal bond angles (degrees) | 1.48
| Ramachandran plot (%) |
| Core region | 91.9
| Allowed region | 7.1
| Generously allowed region | 1.0
| Disallowed region | 0.0

* Root mean square.

**Crystallographic Analysis—X-ray data were collected on beam-line 9–2 at the SSRL on a Quantum-3 × 3 CCD detector. Data were processed with the HKL suite (35) and analyzed further using CCP4 programs (36). Crystals of ΔIBB-importin α-PLSCR1 NLS complex belong to space group P<sub>2</sub>1<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, with one complex/asymmetric unit. The structure was determined by molecular replacement using crystallography NMR software (CNS; 37) and importin α-2 (70–529) as search model (PDB code 1EJL).**

Initial rigid body refinement of the model using program CNS (37) yielded working and free R factors of 30.49% and 29.93% (calculated using 10% of the observed reflections). The PLSCR1 NLS was built in Fe<sub>o</sub>–Fe<sub>e</sub> and 3Fe<sub>o</sub>–2Fe<sub>e</sub> electron density difference maps with several rounds of manual building in O (38) followed by simulated annealing and grouped B factor with CNS (37). The final model, which includes all residues for importin α-2 (70–529), PLSCR1 NLS (257–266), and 134 water molecules, has a R<sub>work</sub> of 22.9% and R<sub>free</sub> of 25.8%. The average B factors for importin α-2 (70–529) and PLSCR1 NLS (257–266) are 28.77 and 48.043 Å<sup>2</sup>·Å<sup>3</sup>, respectively. Stereochemistry was checked with PROCHECK (36) and revealed no residues in disallowed regions. Data collection and refinement statistics are summarized in Table 1. Structural figures were made using Bobscript (39) and Raster3d (40). Coordinates for the ΔIBB-importin α-PLSCR1 NLS complex were deposited in the Protein Data Bank with accession code 1Y2A.

**Plasmid Construction, Cell Culture, Transient Transfection, and Fluorescence Microscopy—Human PLSCR1 cDNA or mutant PLSCR1 (K<sub>184AAPA</sub>) or the PLSCR1 (K<sub>184AAPA</sub>, W<sub>263AAPA</sub>) in pcDNA3 vector were constructed as described previously (30, 31). Mutant PLSCR1 (K<sub>184AAPA</sub>, W<sub>263AAPA</sub>, K258A), PLSCR1 (K<sub>184AAPA</sub>, W<sub>263AAPA</sub>, K261A), or PLSCR1 (K<sub>184AAPA</sub>, W<sub>263AAPA</sub>, W263A) was constructed by oligonucleotide-directed mutagenesis with appropriate oligonucleotide primers, and the PCR-derived DNA was cloned into pcDNA3 vector as EcoRI and BamHI fragment. The sequences of the constructs were verified by DNA sequencing. Mouse fibroblast cell line SVT2 was cultured in Dulbecco’s modified Eagle’s medium (Mediotech) containing 10% fetal bo-
PLSCR1 NLS Binds Importin α with High Affinity—To characterize the recognition of the PLSCR1 NLS by importin α, we first determined the binding affinity of the minimum PLSCR1 NLS (257–266) for the import adaptor importin α. The binding study was carried out using the fluorescence depolarization anisotropy assay developed by Hodel et al. (34) to study the binding of classical NLSs to importin α. Briefly, the PLSCR1 NLS region (257–266) was fused to the GFP, and the fusion protein PLSCR1 NLS-GFP was assayed for binding to a truncated version of importin α-2 (residues 70–529) which lacks the IBB domain. The increase of GFP anisotropy that results from the binding of PLSCR1 NLS to ΔIBB-importin α was measured as a function of ΔIBB-importin α concentration, yielding a $K_d \approx 45.8$ nM (Fig. 1B). This value is comparable with that observed for the binding of full-length PLSCR1 to importin α (30), thus confirming that region 257–266 genuinely represents the minimum NLS of PLSCR1. As a positive control, we used the SV40 NLS-GFP, which, under identical experimental conditions was found to bind ΔIBB-importin α with $K_d \approx 10$ nM (Fig. 1B). This value agrees well with the dissociation constants measured using Biacore or microtiter binding assay (43), suggesting that the apparent affinities derived by our fluorescence anisotropy-based binding assay are consistent with those estimated using other methods.

Crystallographic Analysis of Importin α Bound to the PLSCR1 NLS—We have used x-ray crystallography to determine the molecular basis of the interaction between PLSCR1 NLS and importin α. As the IBB domain is not required for this interaction, we have crystallized a truncated construct of importin α (residues 70–529) in complex with a chemically synthesized peptide comprising the PLSCR1 NLS sequence (257–266). Crystals of the complex were grown in the presence of a 2-fold molar excess of PLSCR1 NLS, using 0.6 M sodium citrate at pH 6.0. Using synchrotron radiations, we found that ΔIBB-importin α-PLSCR1 NLS complex crystals diffract x-ray beyond 2 Å resolution (Table I). The structure was determined by molecular replacement, using mouse ΔIBB-importin α-2 (70–529) (PDB code 1EJL) as a search model. The PLSCR1 NLS was built in unbiased electron density difference maps calculated using phase angles from the refined importin α model and structure factor amplitudes derived from the observed x-ray data. After several rounds of manual building and refinement all PLSCR1 NLS residues were clearly modeled in the electron density. The final model, which includes residues (70–529) of importin α, (257–266) of PLSCR1 NLS, and 134 water molecules, was refined to a working and free $R$ factors of 22.9 and 25.8%, respectively, using all diffraction data between 40 and 2.2 Å. A ribbon diagram of the ΔIBB-importin α-PLSCR1 NLS complex structure is shown in Fig. 2A. The Arm core of importin α consists of nine Arm repeats that pack into a right-handed superhelix of helices. As observed previously for yeast and vertebrate importin α (18, 20, 22), Arm 1–2 and 9–8 are slightly curved in opposite directions with respect to a central helical core formed by Arm repeats 3–7. The nonclassical PLSCR1 NLS is bound to the N terminus of importin α where it interacts with Arm repeats 1–4. In contrast to previous complexes of importin α with monoparticle NLSs (18–20), a single copy of the PLSCR1 NLS is bound to importin α between Arms 1 and 4, in a region...
that includes the major NLS binding sites located between Arms 2 and 4. No significant density is seen at the minor binding site (Arm 2–4). The final PLSCR1 NLS model (Fig. 2B) has an average B factor of 48 Å$^2$, slightly higher than importin α (28.8 Å$^2$). The former value is in line with the B factor observed for the classical SV40 NLS, which was determined to a comparable resolution of 2.2 Å in complex with yeast importin α (20). The conformation of importin α adopted upon binding to the PLSCR1 NLS is virtually identical to those in complex with both classical monopartite NLS (18–21) and bipartite NLS (19, 20). This suggests that the binding of the PLSCR1 NLS is not accompanied by significant conformational changes in the protein.

The binding interface between importin α and the PLSCR1 NLS is remarkably extended. The NLS buries ~654.4 Å$^2$ of surface between Arm repeats 1 and 4. The PLSCR1 peptide is bound in a fully extended conformation, which resembles the binding of the nonclassical parathyroid hormone-related protein NLS to the import receptor importin α (14). We identify two distinct areas of contact between importin α-2 and the PLSCR1 NLS (Fig. 2C). First, the N-terminal moiety $^{257}$GKISR$^{262}$ occupies the conventional NLS binding site between Arm 2 and 4 which is transversed to the importin α superhelical axis. In analogy with the nomenclature used to describe classical SV40 NLS residues, we will refer to these residues as P1–P5. Second, the C-terminal sequence PLSCR1 $^{262}$HWTGI$^{266}$ spans Arm repeats 1–2 where it binds importin α laterally, adopting a conformation that is almost parallel to the superhelical axis. These residues will be indicated as S1–S5 (Fig. 3A).

**Interaction of Importin α with the N-terminal PLSCR1 Sequence** $^{257}$GKISR$^{262}$—The N-terminal PLSCR1 NLS moiety $^{257}$GKISR$^{262}$ spans the major NLS binding site between Arm repeats 2 and 4 (19, 22). As seen in the recognition of the SV40 NLS (18, 19), the NLS backbone is held in the binding groove by intimate contacts with three conserved asparagines Asn$^{235}$, Asn$^{188}$, and Asn$^{145}$. These residues make hydrogen bonding interactions with the main chain amide groups of PLSCR1.
Interestingly, CaPTURE predicts that both residues at position P1, P3, and P5, respectively (Fig. 2C). The PLSCR1 residues occupying positions P1–P5 are dramatically different from the SV40 NLS polybasic stretch127KKKR131. At position P1 an invariant residue Gly257 is probably important to provide high conformational flexibility to the following Lys261, which, together with Lys258, occupies the conventional positions annotated as P2 and P5, respectively (Fig. 2C). Both PLSCR1 Lys258 and Lys261 contact importin α intimately by inserting their N-terminal basic group inside acidic pockets of the protein. Whereas the basic N-terminal group of Lys258 directly contacts the C-terminal group of Asp192, Lys261 at position P5 makes a salt bridge with εGlu181 (Fig. 3A). Notably, three tryptophans surround the PLSCR1 Lys261, two from importin α, Trp194 and Trp142, and one, Trp263, from the PLSCR1 (Fig. 3B). This tryptophan “cage” locks the ε-N-terminal group of Lys261 in a conformation ideal to interact with εGlu181. However, there is a significant difference in the chemical interactions engaged by the three tryptophans. Whereas the indole rings of Trp194 and αTrp142 point directly toward the ε-N-terminal groups of PLSCR1 Lys261, PLSCR1 Trp263 is oriented more toward the aliphatic side chain of Lys261. The former interaction is consistent with a cation–π interaction, whereas the latter is presumably more a hydrophobic contact. To investigate further this prediction, we used the program CaPTURE (Cation–π Trends Using Realistic Electrostatics) (44), which predicts and quantifies cation–π interactions based on ab initio calculations from three-dimensional structures. Interestingly, CaPTURE predicts that both αTrp194 and αTrp142 engage in energetically significant intermolecular cation–π interactions with Lys261. The energetic contributions were estimated to be approximately −7.40 and −2.41 kcal/mol for αTrp194 and αTrp142, respectively. As predicted by visual examination of the three-dimensional structure, the potential energetic contribution resulting from cation–π interaction for the pair Trp263-Lys261 was not significant (≤2 kcal/mol), as the ring of the tryptophan points more toward the aliphatic portion of the side chain of the Lys261 than to the cationic N-terminal group.

Finally, PLSCR1 residues Ile259 and Ser260 at position P3 and P4 show a pattern of molecular interactions completely different from classical SV40-like sequences. Instead of basic Arg/Lys, P3 presents the aliphatic side chain of residue Ile259, which makes strong hydrophobic contacts with the indole rings of αTrp194 and αTrp142. In turn, the hydroxy group of Ser260 at position P4 makes hydrogen bond to importin αSer149 (Fig. 2C).

Interaction of Importin α with the C-terminal PLSCR1 Sequence 262HWTGI266—The NLS region upstream the P5 site includes the sequence 262HWTGI266. Here three of five residues (Trp263, Gly265, and Ile266) are both highly conserved and nonbasic, strengthening the idea that this region represents a strong nonbasic determinant for import (Fig. 1A). Similar to the C-terminal moiety, the molecular recognition of this heptapeptide is based on both main chain and side chain contacts with importin α. The 262HWTGI266 main chain is held in place by four contacts with importin α residues, Gin263, Arg101, Lys102, and Ser105 (Fig. 2C). These residues orient the PLSCR1 backbone along the binding site, in a way analogous to the asparagine arrays observed in the major NLS binding site. Intriguingly, analysis of the importin α primary sequence conservation indicates that all four residues, Gin263, Arg101, Lys102, and Ser105, are highly conserved within vertebrates. This suggests that the extended binding interaction observed for the PLSCR1 NLS may not be a unique feature of this cargo and that Arm 1–2 potentially represent a general recognition site for nonclassical NLSs.

In addition to backbone contacts, importin α makes at least three specific side chain-side chain contacts with PLSCR1 NLS residues (Fig. 2C). Notably, PLSCR1 His260 and Thr264 at positions S1 and S3 make electrostatic interactions with εGlu107 and aArg101, respectively. In turn, the conserved Trp263 at S2 engages in an intramolecular interaction with the aliphatic portion of Lys261 (Fig. 3B), which, as described previously, sits at position P5.

In Vivo Analysis of the PLSCR1 NLS—Ben-Efraim et al. (30) have shown that the classical SV40 NLS competes with PLSCR1 NLS in a nuclear import assay in digitonin-permeabilized cells, suggesting that PLSCR1 NLS might bind to importin α in the same fashion as does the SV40 NLS (30). This hypothesis is fully supported by our three-dimensional structure. The structural alignment in Fig. 4 shows that the conformation adopted by PLSCR1 NLS main chain between residues

![Fig. 3. Analysis of the ΔIBB-importin α-PLSCR1 NLS binding interface. A, electrostatic surface potential of importin α. Acidic and basic surfaces of the protein are in red and blue, respectively. The PLSCR1 NLS, in blue sticks, is docked to the importin α surface. B, recognition of PLSCR1 Lys261. The conserved lysine at position P5 is sandwiched by two importin αTrp194/αTrp142 (shown in yellow) and by the PLSCR1-Trp263 (in orange).](image-url)
257 and 261 (257GKISK261) is virtually identical to that observed in classical NLSs (Fig. 4). In addition, Lys258 and Lys261 align well with the canonical P2 and P5 sites of other NLS peptides solved in complex with importin α (18–21) (Table II). However, as discussed previously, PLSCR1 NLS residues at position P1, P3, and P4 are nonbasic, as well as the N-terminal sequence 262HWTGI266 neither matches nor aligns to any NLS sequence discovered to date.

To characterize further the PLSCR1 NLS in vivo, we have studied the effect of single point mutations in the PLSCR1 NLS in a transient transfection assay. SVT2 fibroblasts were transfected with cDNA for wild-type PLSCR1 or the PLSCR1 mutant, which bears mutations of the palmitylated amino acid residues, Gln98, Arg101, Lys102, and Ser105, between Arm 1–2. As reported previously, wild type PLSCR1 showed a distinctive immunofluorescent staining at the endo-}

| NLS       | Minor binding site | Major binding site |
|-----------|--------------------|--------------------|
| SV40      | A K K A A          | P K K K R K V      |
| H28       | G K K R S K A      |                   |
| v-jun     | K S R K R K L      |                   |
| c-Myc     | K R V K L          |                   |
| NfKb p50  | Q R K R Q K        |                   |
| Prothymosin α | K R P R P |                   |
| Adenovirus E1a | G K I S K |                   |

Red indicates PLSCR1 residues S1–S5.

We have studied the molecular basis for the recognition of poorly basic PLSCR1 NLS by importin α. This poorly basic NLS deviates significantly from the polybasic SV40-like NLS consensus, but binds importin α with high affinity (Kd < 45 nM). Our structural analysis indicates that despite the paucity of basic amino acids, PLSCR1 NLS recognition of importin α is intimate and extensive. A complex network of side chain and main chain interactions is seen in the crystal structure, which spans Arm repeats 1–4 of the adaptor. Multiple structural determinants contribute to the recognition of the nonclassical PLSCR1 NLS. First, the entire N-terminal portion of the importin α Arm core domain provides an extended binding surface that extends and orients the PLSCR1 backbone along the classical NLS binding site (Arm 2–4) as well as on the lateral surface of Arm 1–2. The PLSCR1 backbone is contacted by a complex set of hydrogen bonding with importin α side chains. These interactions are largely mediated by three conserved asparagines within Arm 2–4 and four conserved importin α residues, Gln98, Arg101, Lys102, and Ser105, between Arm 1–2. Second, two basic residues at position P2 and P5 provide the essential electrostatic binding complementarily with importin α. Notably, both Lys258 and Lys261 are flanked by two small amino acid at positions P1 (Gly257) and P4 (Ser260). This may reduce the steric hindrance introduced by a bulky side chain and contribute to provide higher conformational flexibility to basic side chains of Lys258 and Lys261, hence enhancing their binding specificity for importin α. Although the recognition of Lys258 at positions P2 closely resembles that described previously in the recognition of the SV40 NLS (13–15), our in vivo assay demonstrates that this residue is important but not essential for nuclear import. Third, hydrophobic residues in the PLSCR1 NLS engage in intramolecular contacts within the NLS as well as intramolecular contacts with importin α. For instance, the hydrophobic side chain of PLSCR1 Ile259 is held in place by the two aTrp184 and aTrp231. Similarly, PLSCR1 Trp261 packs in close proximity (−4 Å) to the aliphatic portion of nuclear import sequences. Moreover, a conserved lysine at position P2 (Lys258 in the classical SV40 NLS) is crucial for nuclear import. Mutation of this residue completely abolishes nuclear import (45, 46) and reduces the binding affinity for importin α by more than 100-fold (34). Mutation of one of the adjacent basic residues at P1, P3, P4, or P5 reduces but does not completely abolish nuclear import (46).

DISCUSSION

The prototypical NLS of the SV40 large T antigen consists of a single cluster of five contiguous positively charged residues (126PKKKRKV132) which is recognized by transport adaptor importin α. Although other nuclear localization signals such as the human c-myc NLS (329PAAKRKVLKD338) are somewhat less basic, a clustering of consecutive three to five positive charged residues appears to be the main diagnostic feature of most
of Lys261, sealing the lysine side chain in a tryptophan cage.

The structural prediction that multiple binding determi-
nants provide high affinity binding specificity to importin
/H9251 in the absence of a cluster of positively charged residues is also
corroborated by our in vivo transfection assay. As mentioned
previously, a well accepted paradigm in the nucleocytoplasmic
transport field lies in the absolute necessity of a Lys at position
P2 (45, 46). A large body of work has shown that this residue is
responsible for most of the binding energy (34). Deletion or
substitution of this residue dramatically reduces the binding
affinity. In contrast, our in vivo mutational data reveal that a
single point mutation of P2 or P5 or at the conserved Trp263 can
reduce nuclear import of PLSCR1 but not abolish it. At least
three mutations are necessary to render the PLSCR1 distribu-
tion fully cytoplasmic. The somewhat dispensable role of Lys258
at position P2 is also indirectly confirmed by the observation
that human PLSCR4 lacks a basic residue at this position (Fig.
1A). The only two basic side chains for this phospholipid scram-
blase homolog are found at position P5 and S1. However, it
should be pointed out that the PLSCR4 NLS remains putative,
as the nuclear import of PLSCR4 has not been demonstrated.

The ability of the importin α binding groove to orient the
PLSCR1 NLS backbone and efficiently contact its side chains
suggests that the clustering of positively charged residues
within classical NLS sequence is not a mandatory feature for
all nuclear import signals. For instance, both PLSCR1 residues
at positions P3 and P4 are nonbasic, yet still efficiently bound
to importin α via a combination of hydrophobic and polar con-
tacts. This emphasizes the idea that the recognition of distinct
NLS sequences is highly plastic. The Arm scaffold represents a
versatile binding surface that can efficiently accommodate di-
verse sequences. By fixing and orienting the NLS backbone,
importin α can recognize different NLS sequences and maxi-
mize the number of side chain contacts. As seen in the recog-
nition of the PLSCR1 NLS, the moderately basic sequence
257GKISK262 is bound within the major NLS binding site,
whereas the nonbasic hydrophobic region 262HWGIC266 is ac-
commodated on the lateral surface of Arm 1–2. Such backbone-
controlled binding maximizes the number of side chain contacts
between the protein and the NLS, thus achieving high
binding specificity.

Acknowledgments—We thank Bostjan Kobe for the clone of mouse
Δ1BB-importin α-2 and Anita Corbett for the SV40 NLS-GFP construct.
We also thank the staff of Stanford Synchrotron Radiation Laboratory
(SSRL), a national user facility operated by Stanford University on
behalf of the United States Department of Energy, Office of Basic

---

**Fig. 5.** Nuclear import of PLSCR1 is blocked by triple mutation of the res-
ides K258A, K261A/H263A, but not by single point mutations. Confocal
fluorescence images of murine SVT2 fibroblasts transiently transfected with
(top to bottom): pcDNA3 containing cDNA for wild-type (WT) human PLSCR1 or the
mutants PLSCR1 (184AAAPAA189) or
PLSCR1 (184AAAPAA189, K258A), or (184,
AAAPAA189, K261A) or PLSCR1(184AA-
APAA189, W263A) or (184AAAPAA189, W263,
GAISAATGTG1266). First column PL-
SCR1 label, second column nuclear
label, third column merge. Expressed
human PLSCR1 antigen was detected
with monoclonal antibody 4D2 and fluo-
rescein isothiocyanate-conjugated goat
anti-mouse IgG. Following immuno-
staining and DNA labeling with pro-
pidium iodide (PI), cells were visualized
by confocal fluorescence microscopy.
Data are from a single experiment, rep-
resentative of four so performed.
Energy Sciences, for assistance with synchrotron data collection. The SSRL Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research, and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program, and the National Institute of General Medical Sciences. The technical assistance of Lilin Li, Jo-Lawrence Bigcas, and Daniela Junquiera is gratefully acknowledged.

REFERENCES
1. Gorlich, D., and Kutay, U. (1999) *Annu. Rev. Cell Dev. Biol.* 15, 607–660
2. Macara, I. G. (2001) *Microbiol. Mol. Biol. Rev.* 65, 570–594
3. Wei, K. (2003) *Cell* 112, 441–451
4. Gorlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994) *Cell* 79, 767–778
5. Chi, N. C., Adam, E. J., and Adam, S. A. (1995) *J. Cell Biol.* 130, 265–274
6. Stewart, M., Baker, R. P., Baxi, A., Clayton, L., Grant, R. P., Littlewood, T., and Matsuzuka, Y. (2001) *FEBS Lett.* 498, 145–149
7. Bedneno, J., Cingolani, G., and Gerace, L. (2003) *Traffic* 4, 127–135
8. Vetter, I. R., Arndt, A., Kutay, U., Gorlich, D., and Wittinghofer, A. (1999) *Cell* 97, 635–646
9. Conti, E., and Izaurralde, E. (2001) *Curr. Opin. Cell Biol.* 13, 310–319
10. Cingolani, G., Petosa, C., Weis, K., and Muller, C. W. (1999) *Nature* 399, 221–229
11. Kobe, B., Gleichmann, T., Horne, J., Jennings, I. G., Scotney, P. D., and Teh, T. (1999) *Struct. Fold Des.* 7, R91–R97
12. Andrade, M. A., Petosa, C., O’Donoghue, S. I., Muller, C. W., and Bork, P. (2001) *J. Mol. Biol.* 309, 1–18
13. Bayliss, R., Littlewood, T., and Stewart, M. (2000) *Cell* 102, 99–108
14. Cingolani, G., Bedneno, J., Gillespie, M. T., and Gerace, L. (2002) *Mol. Cell* 10, 1345–1353
15. Lee, S. J., Sekimoto, T., Yamashita, E., Nagoshi, E., Nakagawa, A., Imamoto, N., Yoshimura, M., Sakai, H., Cheng, K. T., Tsukihara, T., and Yoneda, Y. (2003) *Science* 302, 1571–1575
16. Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., and Adam, S. A. (2004) *Trends Cell Biol.* 14, 505–514
17. Parashar, E., Izaurralde, E., Bischoff, F. R., Huber, J., Kutay, U., Hartmann, E., Luhmann, R., and Gorlich, D. (1999) *J. Cell Biol.* 145, 255–264
18. Conti, E., Uy, M., Leighton, L., Blebel, G., and Kuriyan, J. (1998) *Cell* 94, 193–204
19. Fontes, M. R., Teh, T., and Kobe, B. (2000) *J. Mol. Biol.* 297, 1183–1194
20. Conti, E., and Kuriyan, J. (2000) *Struct. Fold Des.* 8, 329–338
21. Fontes, M. R., Teh, T., Jans, D., Brinkworth, R. I., and Kobe, B. (2003) *J. Biol. Chem.* 278, 27081–27087
22. Kobe, B. (1999) *Nat. Struct. Biol.* 6, 388–397
23. Fanara, P., Hodel, M. R., Corbett, A. H., and Hodel, A. E. (2000) *J. Biol. Chem.* 275, 21218–21223
24. Caturel, B., Teh, T., Fontes, M. R., Jennings, I. G., Jans, D. A., Howlett, G. J., Nice, E. C., and Kobe, B. (2001) *J. Biol. Chem.* 276, 34189–34198
25. Harreman, M. T., Cohen, P. E., Hodel, M. R., Truscott, G. J., Corbett, A. H., and Hodel, A. E. (2003) *J. Biol. Chem.* 278, 21361–21369
26. Harreman, M. T., Hodel, M. R., Fanara, P., Hodel, A. E., and Corbett, A. H. (2003) *J. Biol. Chem.* 278, 5854–5863
27. Basse, F., Stout, J. G., Sims, P. J., and Wiedmer, T. (1996) *J. Biol. Chem.* 271, 17205–17210
28. Zhou, Q., Zhao, J., Stout, J. G., Luhm, R. A., Wiedmer, T., and Sims, P. J. (1997) *J. Biol. Chem.* 272, 18240–18244
29. Sims, P. J., and Wiedmer, T. (2001) *Thromb. Haemostasis* 86, 266–275
30. Ben-Efraim, I., Zhou, Q., Wiedmer, T., Gerace, L., and Sims, P. J. (2004) *Biochemistry* 43, 3518–3526
31. Wiedmer, T., Zhao, J., Nanjundan, M., and Sims, P. J. (2003) *Biochemistry* 42, 1227–1233
32. Zhou, Q., Zhao, J., Al-Zaghaibi, F., Zhou, A., Wiedmer, T., Silverman, R. H., and Sims, P. J. (2000) *Blood* 95, 2593–2599
33. Zhou, Q., Zhao, J., Wiedmer, T., and Sims, P. J. (2002) *Blood* 99, 4030–4038
34. Hodel, M. R., Corbett, A. H., and Hodel, A. E. (2001) *J. Biol. Chem.* 276, 1317–1325
35. Otwinowski, Z., and Minor, W. (1997) *Methods Enzymol.* 276, 307–326
36. Collaborative Computational Project 4 (1994) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 50, 760–763