A Bipartite Nuclear Localization Signal Is Required for p53 Nuclear Import Regulated by a Carboxyl-terminal Domain*

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Shun-Hsin Liang and Michael F. Clarke‡
From the Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, Michigan 48109-0936

Abnormal p53 cellular localization has been considered to be one of the mechanisms that could inactivate p53 function. To understand the regulation of p53 cellular trafficking, we have previously identified two p53 domains involved in its localization. A basic domain, Lys305–Arg306, is required for p53 nuclear import, and a carboxyl-terminal domain, namely the cytoplasmic sequestration domain (CSD), can block the nuclear import of Lys305 or Arg306 mutated p53. To characterize further the function of these two domains, we demonstrate in this report that the previously described major nuclear localization signal works together with Lys305–Arg306 to form a bipartite and functional nuclear localization sequence (NLS) for p53 nuclear import. The CSD could block the binding of p53 to the NLS receptor, importin α, and reduce the efficiency of p53 nuclear import in MCF-7, H1299, and Saos-2 cells. The blocking effect of the CSD is not due to the enhancement of nuclear export or oligomerization of the p53. These results indicate that the CSD can regulate p53 nuclear import by controlling access of the NLS to importin α binding.

The p53 tumor suppressor is a nuclear phosphoprotein whose activities have been linked to cell cycle control, apoptotic cell death, DNA repair, stress responses, and genomic stability. p53 is thought to regulate cell growth and viability through transcriptional dependent and independent pathways. Since a defect in p53 nuclear translocation would impair p53 function. To understand the regulation of p53 cellular trafficking, we have previously identified two p53 domains involved in its localization. A basic domain, Lys305–Arg306, is required for p53 nuclear import, and a carboxyl-terminal domain, namely the cytoplasmic sequestration domain (CSD), can block the nuclear import of Lys305 or Arg306 mutated p53. To characterize further the function of these two domains, we demonstrate in this report that the previously described major nuclear localization signal works together with Lys305–Arg306 to form a bipartite and functional nuclear localization sequence (NLS) for p53 nuclear import. The CSD could block the binding of p53 to the NLS receptor, importin α, and reduce the efficiency of p53 nuclear import in MCF-7, H1299, and Saos-2 cells. The blocking effect of the CSD is not due to the enhancement of nuclear export or oligomerization of the p53. These results indicate that the CSD can regulate p53 nuclear import by controlling access of the NLS to importin α binding.

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‡ To whom correspondence should be addressed: Dept. of Internal Medicine, University of Michigan Medical Center, 4210 CCGC, 1500 East Medical Center Dr., Ann Arbor, MI 48109-0936 Tel.: 734-764-8195; Fax: 734-763-4226; E-mail: mclarke@umich.edu.

1 The abbreviations used are: NLS, nuclear localization sequence; NLSI, nuclear localization signal; NES, nuclear export sequence; CSD, cytoplasmic sequestration domain; PCR, polymerase chain reaction; PK, pyruvate kinase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; LRSC, lissamine rhodamine B sulfonyl chloride; LMB, leptomycin B; GST, glutathione S-transferase; WT, wild type.

EXPERIMENTAL PROCEDURES

Cell Culture—The MCF-7 breast cancer cell line, which expresses wild-type p53, and two p53-null cell lines, H1299 lung adenocarcinoma and Saos-2 osteosarcoma, were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% (v/v) fetal bovine serum at 37 °C in a humidified 5% CO2 atmosphere.

Plasmid Construction and Mutagenesis—To analyze the function of p53 nuclear localization signals (NLS), the DNA fragments corresponding to p53 amino acid residues 316–322, 305–322, and K305N-322 were amplified by PCR and subcloned into the KpnI and NotI sites in the 3’ end of a Myc-tagged chicken muscle pyruvate kinase (PK) cDNA expression plasmid (25). To replace the NLSI (residues 316–322; PQQKKKP) of p53 with the SV40 large T antigen nuclear localization signal (PKKKRKV), a two-step PCR mutagenesis protocol was performed as described previ-
ously (23) using pC53-SN (26) as the template. The final PCR fragments were subcloned into the BamHI and EcoRI sites of the pcK7-GFP plasmid (27). To analyze the relationship between p53 oligomerization and nuclear import, the FLAG-tagged DNA fragment corresponding to p53 residues 305–322 was amplified by PCR with a 5′ primer containing FLAG sequences, and the PCR product was ligated to the BamHI and EcoRI sites of the pcDNA3 plasmid. All other mutated p53 constructs were made by two-step PCR and inserted into the plasmid pcK7-GFP.

Cell Transfection and Immunofluorescence Microscopy—Cells were grown on glass coverslips in well-plates and transfected using LipofectAMINE (Life Technologies, Inc.) as described previously (23). After transfection, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. After three washes with PBS, the fixed cell were then permeabilized with 0.2% Triton X-100 in PBS for 2 min and incubated in PBS containing 0.5% bovine serum albumin for 20 min. Where indicated, cells were incubated for 1 h with an anti-FLAG M2 monoclonal antibody (Sigma) or an anti-c-Myc monoclonal antibody (9E10, Santa Cruz Biotechnology) followed by 50 min incubation with fluorescein isothiocyanate or lissamine rhodamine B sulfonyl chloride (LRSC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). After a final wash with PBS, coverslips were mounted with antifade solution (100 mM NaHCO3, pH 9.0, 4 mg/ml p-phenylendiamine, 50% glycerol) and examined by fluorescence microscopy.

Analysis of Leptomycin B-treated Cells—MCF-7 cells were transiently transfected with plasmid p53K305N-GFP. Two days after transfection, cells were treated with 100 mM leptomycin B (LMB) for 4 h. The localization of cyclin B1, a control for LMB-inhibited nuclear export (28), was then determined by indirect immunostaining using a combination of monoclonal antibody to human cyclin B1 (GNS1, Santa Cruz Biotechnology) and LRSC-conjugated goat anti-mouse IgG. The cellular localizations of exogenous p53K305N-GFP and endogenous cyclin B1 in a sample were visualized and differentiated by fluorescence microscopy.

In Vitro Binding Assay of p53 and Importin α—The wild-type or mutated p53 DNA fragments were ligated into the BamHI and EcoRI sites of vector pGEX-2T (Amersham Pharmacia Biotech) and transformed into the Escherichia coli strain DH5α. The cell culture and batch purification of glutathione S-transferase (GST) fusion proteins were performed according to manufacturer’s instructions. The E. coli strain BLR containing GST fusions of a functional SV40 large T antigen nuclear localization signal (Tag NLS) or an inverse version of Tag NLS (Tag NLSinv) were cultured, and the proteins were purified as described (29). The plasmid pRSET-hSRP1 containing a cDNA of human importin α (30) was used to produce a [35S]methionine-labeled protein using the Promega TNT T7 Quick Coupled Translation System. Fifteen micrograms of GST-p53 or p53 mutants and 7 μg of GST-Tag NLS or GST-TagNLSinv were incubated with 40 μl of glutathione-agarose beads (Santa Cruz Biotechnology) in 0.5 ml of binding buffer (20 mM Hepes, pH 6.8, 150 mM KCl, 2 mM Mg(OAc)₂, 2 mM dithiothreitol, 0.1% Tween 20) for 2 h at 4 °C. The beads were collected and washed three times with binding buffer. After washing, one-twentieth of the beads were removed to analyze the amount of immobilized GST fusion proteins by immunoblotting with the anti-p53 pAb122 hybridoma supernatant (ATCC TIB116) or the anti-GST mouse IgG1 monoclonal antibody (Santa Cruz Biotechnology). The rest of the beads were incubated with 90 μl of in vitro translated importin α reaction mixture for 4 h at 4 °C. The beads were then washed six times with binding buffer, boiled in 30 μl of sample buffer, and the immobilized proteins were resolved on a SDS-10% polyacrylamide gel. The [35S]-labeled importin α bound to the GST fusion proteins was detected by fluorography using Amplify Reagent (Amersham Pharmacia Biotech). The relative intensity of importin α was measured by NIH Image software.

RESULTS

Nuclear Export Is Not Associated with Cytoplasmic Sequestration of Lys305-mutated p53—Due to the fact that p53 cellular localization is a dynamic equilibrium between rapid nuclear import and ongoing export, it is possible that the cytoplasmic sequestration effect of the Lys305 or Arg306 mutations is a result of enhancement of p53 nuclear export, probably by increasing the interaction with CRM1. To address this possibility, MCF-7 cells transiently transfected with the p53K305N-GFP plasmid were treated with leptomycin B (LMB). The transfected cells were immunostained for cyclin B1 as the control of LMB inhibition of CRM1-mediated nuclear export (28). The results showed that although cyclin B1 shifted from the the cytoplasm to the nucleus after LMB treatment, Lys305-mutated p53 (p53K305) still accumulated in the cytoplasm (Fig. 1). This indicates that cytoplasmic sequestration of p53K305 is not a result of an increase in nuclear export but a result of a defect in nuclear import.

Lys305-Arg306 and NLSI Form a Bipartite-like NLS—Previously we showed that a spacer between the Lys305-Arg306 motif and NLSI is necessary for entrance of p53 into the cell nucleus (24). Thus, it is possible that the Lys305-Arg306 motif and NLSI work together as a bipartite NLS. To test this possibility, the NLSI alone or the complete sequence from Lys305 to NLSI were fused with a cytoplasmic protein, Myc-tagged pyruvate kinase, to analyze their nuclear targeting efficiency. The NLSIs were fused to a Myc-tagged pyruvate kinase (Myc-PK, Ref. 25) at the carboxyl terminus, and all constructs were transfected into MCF-7 cells. The results showed that although the sequence composed of residues 305–322 was sufficient to direct a complete nuclear import of Myc-PK, neither NLSI alone (residues 316–322) nor Lys305-mutated sequences were able to do so (Fig. 2). To determine further the effect of NLSI in its native context within the p53 protein, we replaced NLSI in p53K305 with the SV40 large T antigen NLS (Tag NLS, PKKRRKV), a well-defined monopartite NLS. If NLSI functions the same way as that of Tag NLS, this replacement should not change the cytoplasmic sequestration of the protein. The result, however, showed that p53K305 restored nuclear import when NLSI was substituted by Tag NLS (Fig. 3), suggesting that NLSI is a weak monopartite NLS compared with the Tag NLS. These data confirmed that two basic domains, Lys305-Arg306 and NLSI, are both required for nuclear import of p53. Taken together, these data indicate that a bipartite-like NLS is required for p53 nuclear import.

Masking of p53 Nuclear Import Signals by a Cytoplasmic Sequestration Domain—It has been shown that deletion of any region from residues 326 to 355, the so-called cytoplasmic sequestration domain (CSD), results in entry of Lys305 or Arg306 mutated p53 into the nucleus (23, 24). It is possible that the CSD could inhibit the nuclear import of p53 by masking the NLS and block the binding of importin α. To test this hypothesis, we first performed a binding assay using in vitro translated importin α and recombinant GST fusion proteins of either wild-type (WT) or p53 mutations of Lys305, the CSD, or both. The GST fusions with SV40 Tag NLS (GST-NLS) and an inverse version of Tag NLS (GST-NLSinv) served as positive and negative controls, respectively, for the importin α binding. As
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The different fusion constructs were transiently transfected into MCF-7 cells, and the cellular localization of fusion proteins was determined by immunostaining with a monoclonal anti-c-Myc antibody and a fluorescein isothiocyanate-conjugated goat anti-mouse IgG.

The subcellular localizations of p53 nuclear localization signals and PK fusion derivatives. The p53 residues 316–322 (NLSI), 305–322, or K305N-322 were linked to the KpnI sites in the carboxyl terminus of a Myc-tagged PK cDNA. The different fusion constructs were transiently transfected into MCF-7 cells, and the cellular localization of fusion proteins was determined by immunostaining with a monoclonal anti-c-Myc antibody and a fluorescein isothiocyanate-conjugated goat anti-mouse IgG.

The GFP fusion proteins of wild-type (WT) p53 and CSD-deleted mutants were expressed in MCF-7 cells, and the subcellular localization of each fusion protein was determined by GFP fluorescence, were scored and divided into three groups as nuclear (N), cytoplasmic (C), and both nuclear and cytoplasmic (N + C) accumulation. The percentage of each localization was determined from a total of ~500 fluorescent cells observed in several fields of a slide. The results showed that although WT p53 was distributed predominantly in both the nucleus and the cytoplasm of an individual cell, the CSD-deleted mutants primarily exhibited the nuclear localization, although the percentage of distribution varied in different cell lines (Table II). The cellular localization of a p53 mutant with the deletion outside of the CSD region (residues 356–365) did not show a significant difference from that of WT p53 (Table II). This result supports the observation that the CSD could block the binding of importin α to the NLS and hence reduce the efficiency of p53 nuclear import.

p53 Can Enter the Nucleus as an Oligomerized Complex— Due to the fact that the CSD contains the p53 oligomerization domain (31, 32), it is possible that the blocking effect of the CSD on nuclear import is a result of oligomerization of p53 protein. To test this possibility, MCF-7 cells were co-transfected with the GFP fusion of WT p53 or p53K305 and a FLAG-tagged construct (residues 325–369) consisting of the oligomerization domain. The localization of WT p53 or p53K305 and the oligomerization domain peptide were differentiated by GFP fluorescence and immunostaining of FLAG tags. The results showed that the oligomerization domain itself was distributed to both the nucleus and the cytoplasm (Fig. 5). When co-transfected with WT p53-GFP, the oligomerization domain exclusively accumulated in the nucleus as did WT p53-GFP (Fig. 5). Because this oligomerization domain contains no NLS, it must rely on the p53 NLS to enter the nucleus, suggesting that oligomerization of p53 does not block the p53 nuclear import. Our data also showed that the oligomerization domain accumulated in the cytoplasm together with p53K305 (Fig. 5). Taken together, these data indicate that the blocking effect of the CSD on p53 nuclear import is not associated with p53 oligomerization.

**FIG. 2.** Subcellular localizations of p53 nuclear localization signals and PK fusion derivatives. The p53 residues 316–322 (NLSI), 305–322, or K305N-322 were linked to the KpnI sites in the carboxyl terminus of a Myc-tagged PK cDNA. The different fusion constructs were transiently transfected into MCF-7 cells, and the cellular localization of fusion proteins was determined by immunostaining with a monoclonal anti-c-Myc antibody and a fluorescein isothiocyanate-conjugated goat anti-mouse IgG.

**FIG. 3.** Effect of NLSI replacement by SV40 large T antigen (Tag NLS) on the subcellular localization of Lys305-mutated p53. The GFP fusion proteins of wild-type (a) and mutated p53 proteins including K319A/K320A/K321A (b), K325N (c), and replacement of NLSI by Tag NLS (d) were expressed in MCF-7 cells, and the subcellular localization of each fusion protein was determined by GFP fluorescence. Two basic domains required for p53 nuclear import are underlined. The italic letters indicate the mutated amino acid residues.

Three novel findings about p53 subcellular localization are demonstrated in this study. First, the nuclear localization signal of p53 is bipartite. Second, a CSD from residues 326 to 355 can reduce the efficiency of p53 nuclear import by blocking the binding of NLS to importin α. Third, p53 can enter the nucleus as an oligomerized complex.

**FIG. 4.** Binding of importin α to wild-type and mutated p53 proteins. Immobilized GST-p53 (15 μg per 40 μl agarose beads) or GST-NLS and GST-NLSinv (7 μg per 40 μl agarose beads) were incubated with 90 μl of in vitro translated importin α labeled with [35S]methionine for 4 h at 4 °C. The proteins were separated by SDS-10% PAGE gel, and bound importin α was analyzed by fluorography. The bottom panel is the immunoblot showing the relative amount of immobilized GST fusion proteins subjected to the binding assay. The difference of importin α binding between wild-type (WT) and the mutant p53 proteins was quantitated by measuring the intensity of each band with NIH Image software. The intensity is relative to the binding of WT p53 to importin α. The results are representative of two independent experiments.

**DISCUSSION**

Three novel findings about p53 subcellular localization are demonstrated in this study. First, the nuclear localization signal of p53 is bipartite. Second, a CSD from residues 326 to 355 can reduce the efficiency of p53 nuclear import by blocking the binding of NLS to importin α. Third, p53 can enter the nucleus as an oligomerized complex.
The NLS motif may function as an NLS (19), but due to the CSD in the p53 protein, it is an extremely weak NLS resulting from a weak binding to importin α. Therefore, the activity of the p53 NLS is dependent on the protein context within which it is located. The replacement of NLSI with the Tag NLS could restore the nuclear import of PK when it was linked at the amino terminus of PK and transfected into COS-7 cells (19). The NLSI motif function as an NLS (19), but due to the CSD in the p53 protein, it is an extremely weak NLS resulting from a weak binding to importin α. Therefore, the activity of the p53 NLS is dependent on the protein context within which it is located. The replacement of NLSI with the Tag NLS could restore the nuclear import of PK through this domain.

The NLSI was originally considered as a functional NLS based upon the observation that NLSI is sufficient to direct the nuclear import of PK when it was linked at the amino terminus of PK and transfected into COS-7 cells (19). The NLSI motif may function as an NLS (19), but due to the CSD in the p53 protein, it is an extremely weak NLS resulting from a weak binding to importin α. Therefore, the activity of the p53 NLS is dependent on the protein context within which it is located. The replacement of NLSI with the Tag NLS could restore the nuclear import of PK through this domain.

Deletion of the CSD enhances the binding of p53 to importin α and increases the efficiency of p53 nuclear import, indicating that the CSD can regulate p53 cellular trafficking by controlling its entrance into the nucleus. The CSD also contains an oligomerization domain (31, 32) and a functional nuclear export signal (NES, see Ref. 20). Several lines of evidence suggest that oligomerization and nuclear export of p53 are not involved in the control of p53 nuclear import by the CSD. First, inhibition of protein nuclear export mediated by CRM1 could not rescue the nuclear import of Lys305-mutated p53 which, however, happened when the CSD was deleted (Fig. 1 and Ref. 23). Second, a deletion in the CSD region (residues 326–335), but not within the NES (residues 340–351), increased the efficiency of p53 nuclear import (Table I), indicating that the blocking of p53 nuclear import by the CSD is not solely the counter effect of the NES residing in the CSD. Third, association of a peptide (residues 325–369) consisting of the p53 oligomerization domain with the p53 protein has no effect on p53 nuclear import (Fig. 5), indicating that the blocking of p53 nuclear import by the CSD does not result from oligomerization of p53 through this domain.

The in vitro binding assay indicated that the CSD can structurally mask the access of importin α to the NLS of p53. It is likely that this masking is regulated in the cell via a change in protein conformation, thus the CSD may serve as a guarding domain.

These observations extend our previous studies that identified a basic domain, Lys305–Arg306, required for p53 nuclear import. We show here that this domain works together with NLSI (PQPKKKP) to bind to importin α and direct p53 to the nucleus. The NLSI was originally considered as a functional NLS based upon the observation that NLSI is sufficient to direct the nuclear import of PK when it was linked at the amino terminus of PK and transfected into COS-7 cells (19). The NLSI motif may function as an NLS (19), but due to the CSD in the p53 protein, it is an extremely weak NLS resulting from a weak binding to importin α. Therefore, the activity of the p53 NLS is dependent on the protein context within which it is located. The replacement of NLSI with the Tag NLS could restore the nuclear import of PK through this domain.

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### TABLE I

| Protein | Sequence<sup>a</sup> | Ref. |
|---------|---------------------|-----|
| Monopartite NLSs | SY40 T antigen: PKKKRKKV | 36 |
| | Yeast histone H2B: GKKSKV | 37 |
| | Human c-MYC: PAARKVXL| 38 |
| | Human p53: PQPKKKP| 18, 19 |

<sup>a</sup> Bold letters indicate the basic amino acid residues required for the nuclear import.

### TABLE II

Subcellular localization of mutated p53 in MCF-7, H1299, and Saos-2 cells

| Cells | Mutations of p53 | Localization<sup>b</sup> |
|-------|-----------------|--------------------------|
| | | N | N + C | C |
| MCF-7 | Wild-type | 12 | 84 | 4 | 6 |
| | Δ326–335 | 91 | 9 | 3 |
| | Δ336–345 | 97 | 3 |
| | Δ346–350 | 90 | 10 |
| | Δ356–365 | 12 | 82 | 6 |
| H1299 | Wild-type | 14 | 86 |
| | Δ326–335 | 70 | 30 |
| | Δ336–345 | 69 | 31 |
| | Δ346–350 | 76 | 24 |
| | Δ356–365 | 21 | 79 |
| Saos-2 | Wild-type | 34 | 66 |
| | Δ326–335 | 55 | 45 |
| | Δ336–345 | 59 | 41 |
| | Δ346–350 | 62 | 38 |
| | Δ356–365 | 31 | 69 |

<sup>b</sup> Cells were analyzed 1 day after transfection with GFP fusion constructs using LipofectAMINE. Subcellular localizations are divided into nuclear (N), both nuclear and cytoplasmic (N + C), and cytoplasmic (C) accumulation. The percentage of each accumulation is the average of three independent experiments.

<sup>a</sup> Not detected.
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