A Role for Hsc70 in Regulating Nucleocytoplasmic Transport of a Temperature-sensitive p53 (p53Val-135)*

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Shin Akakura‡, Minoru Yoshida§§§, Yoshihiro Yoneda¶, and Sueharu Horinouchi‡

From the ‡Department of Biotechnology, Graduate School of Agriculture and Life Sciences, the University of Tokyo, Bunkyo-ku, Tokyo 113-8657, the §Department of Anatomy and Cell Biology, Osaka University Medical School, Suita, Osaka 565, and ¶CREST Research Project, Japan Science and Technology Corporation, Saitama 332-0012, Japan

Mouse temperature-sensitive p53Val-135 accumulates in the nucleus and acts as a “wild-type” at 32 °C while it is sequestered in the cytoplasm at 37 °C. The cytoplasmic p53Val-135 relocated into the nucleus upon inhibition of the nuclear export at 37 °C, whereas a mutation in a major bipartite nuclear localization signal (NLS) caused constitutive cytoplasmic localization, indicating that it shuttled between the cytoplasm and the nucleus by its own nuclear export signal and NLS rather than tethered to cytoplasmic structures. Although the full-length p53Val-135 did not bind the import receptor at 37 °C, a C-terminally truncated p53Val-135 lacking residues 326–390 did bind it. Molecular chaperones such as Hsc70 were associated with p53Val-135 at 37 °C but not at 32 °C. When the nuclear export was blocked by leptomycin B, only a fraction lacking Hsc70 was specifically accumulated in the nucleus. Immunodepletion of Hsc70 from the reticulocyte lysate caused p53Val-135 to bind the import receptor. This binding was blocked by supplying the cell extract containing Hsc70 but not by the addition of recombinant Hsc70 alone. We suggest that the association with the Hsc70-containing complex prevents the NLS from the access of the import receptor through the C-terminal region of p53Val-135 at 37 °C, whereas its dissociation at 32 °C allows rapid nuclear import.

Cellular DNA damage caused the increased stability and accumulation of the tumor suppressor protein p53 in the nucleus, which leads to cell cycle arrest or apoptosis by transcriptional activation of specific growth-inhibitory target genes (1–3). Cells lacking this pathway are more resistant to chemotherapeutic agents and exhibit increased genomic instability, allowing them to gain a selective advantage during tumor progression. It is therefore proposed that the physiological function of p53 is a “guardian of the genome” (4). In ~50% of human tumors, the p53 gene is inactivated by a point mutation that gives rise to a missense protein. In cells expressing both the wild-type and the mutant alleles, mutant p53 may lead to a dominant negative inactivation of the remaining wild-type protein through oligomerization (5–7). In other cases, wild-type p53 is functionally inactivated by sequestration in the cytoplasm or by associating with other proteins such as MDM2 and viral oncoproteins (8–10). Thus, appropriate subcellular localization is crucial for regulating the p53 function.

Recently, p53 was shown to shuttle between the nucleus and cytoplasm (11), and a functional leucine-rich nuclear export signal (NES)1 was identified in the p53 tetramerization domain (12). This finding led to an intriguing model in which wild-type p53 subcellular localization is established through tetramerization-regulated exposure of the NES to the export machinery CRM1; p53 shuttles between the nucleus and the cytoplasm through its intrinsic nuclear localization signal (NLS) and NES, but tetramerization inhibits nuclear export by masking the NES, resulting in the p53 nuclear retention. Several tumor types such as neuroblastoma have wild-type p53 which is inactivated due to its cytoplasmic sequestration (13). At least in neuroblastoma cells, the enhanced nuclear export, rather than a tether retaining it in cytoplasmic structures, is ascribed to the cytoplasmic localization (12). In some cases, however, overproduction of MDM2, a p53-target gene product, may play an important role in the p53 cytoplasmic sequestration (14, 15).

Like wild-type p53, the subcellular localization of mutant p53 proteins is subject to variation, ranging from exclusively cytoplasmic to exclusively nuclear in tumors and transformed cells (16–19). It seems likely that the subcellular localization of mutant p53 is not determined by the mutation itself but may be dependent on the intracellular environment (20, 21). Mutant p53 proteins, independent of the mutation sites, exhibit the same epitopes recognized by the mutant-specific antibodies and associate with heat-shock proteins to prolong the half-lives, probably due to a conformation similar to that among the mutant proteins but not to the wild-type one. It is therefore unclear whether the mechanism by which mutant p53 is sequestered in the cytoplasm is the same as that for the wild-type protein. It was previously proposed that p53 with an aberrant conformation is sequestered from the nucleus by Hsp70/Hsc70 binding (22–24). In some cases, however, Hsc70 binding may not effectively sequester p53 to the cytoplasm, since similar proportions of Hsc70-p53 complexes have been found in both the cytoplasm and the nucleus (20).

The mouse temperature-sensitive mutant p53Val-135 serves as a useful system to analyze the mechanism underlying the subcellular localization of both mutant and wild-type p53 (25).

1 The abbreviations used are: NES, nuclear export signal; CRM1, chromosome region maintenance 1; DAPI, 4,6-diamidino-2-phenylinidole; GFP, green fluorescent protein; Hsc70, heat-shock protein 70 cognate protein; LMB, leptomycin B; NLS, nuclear localization signal; PBS, phosphate-buffered saline; pAb, polyclonal antibody; PCR, polymerase chain reaction; CHX, cycloheximide.
p53Val-135 is present in the nucleus with the "wild-type" conformation at the permissive temperature (32 °C), whereas it redistributes in the cytoplasm at the nonpermissive temperature (37 °C) in the cells transformed with p53Val-135 plus ras. Since cycloheximide induced rapid nuclear import of the mutant form of p53Val-135 at 37 °C, it has been postulated that p53Val-135 is anchored to the cytoplasmic structures, which is mediated by short lived protein(s) (26). This model is apparently different from that for wild-type p53, explained by the hyperactive nuclear export. In this study, using leptomycin B, a specific inhibitor of nuclear export (27–29), we show that p53Val-135 at 37 °C still shuttles between the nucleus and the cytoplasm as does wild-type p53 but that the rate of nuclear import is markedly reduced. Our data suggest that the formation of a multi-protein complex containing Hsc70 causes NLS masking, resulting in the cytoplasmic retention. This model is in contrast to the oligomerization-induced NES masking of wild-type p53 for nuclear retention.

**EXPERIMENTAL PROCEDURES**

### Cells, Antibodies, and Reagents—Clone 6, a rat fibroblast cell line transformed with p53Val-135 and activated ras (25), was kindly provided by S. Khochbin. 2KO, a mouse embryonic fibroblast cell established from a p53−/−/Mdm2−/− mouse (30), was kindly provided by G. Lozano. These cells were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (HyClone Laboratories). Cells were grown at 37 °C in a 5% CO2 atmosphere, and the temperature was shifted to 32 °C when necessary. An anti-p53 monoclonal antibody (pAb421) and an anti-p53 polyclonal antibody (anti-p23 monoclonal antibody JJ3) was a gift from D. Toft (Mayo Clinic). Recombinant Hsc70 was purchased from HyClone Laboratories. Leptomycin B (LMB) was prepared as described previously (31). All other chemical reagents were purchased from Sigma Laboratories. Anti-mouse IgG, Amersham Pharmacia Biotech). The coverslips were washed with PBS, rinsed with water, and mounted with 1 μg/ml DAPI in Vectashield for microscopic observation.

### Immunoprecipitation, Immunoblotting, and Immunodepletion—Cells collected were sonicated for 5 s twice in ice-cold immunoprecipitation (IP) buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM MgCl2, 0.2% (v/v) Tween 20, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 mM sodium molybdate, which stabilizes the unstable chaperone complex (32). The lysates were centrifuged at 15,000 × g for 20 min at 4 °C, and the protein concentrations of the supernatants were determined by the Bradford method (Bio-Rad). Immunoprecipitation was performed with 1 μg of total protein. After the supernatants had been preclarified with protein A/G-agarose beads (Sigma, St. Louis, MO, USA), immunodepletion was performed with primary antibodies for 1 h with gentle agitation and with the protein A/G-agarose beads for 1 h. The agarose pellets were then washed three times with lysis buffer, and the bound proteins were extracted with 5× Laemmli sample loading buffer by heating to 95 °C for 5 min. Proteins eluted were separated by sodium dodecyl sulfate, 10% polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Millipore Co, Bedford, MA) by electroblotting. After the membranes had been incubated from http://www.jbc.org/ by guest on July 24, 2018http://www.jbc.org/Downloaded from
Cytoplasmic Sequestration of Mutant p53 by NLS Masking

RESULTS

LMB Causes Nuclear Accumulation of Temperature-sensitive p53—To assess the proposed model in which a tether of mutant p53 to cytoplasmic structures via short lived protein(s) prevents its release and subsequent movement into the nucleus, we analyzed the effect of leptomycin B (LMB), an inhibitor of the nuclear export signal (NES)-dependent protein nuclear export, on the subcellular localization of p53 in rat embryonic fibroblast clone 6 cells (25) expressing an activated Ras and temperature-sensitive p53Val-135 at a high level (Fig. 1). If p53 in these cells is tethered in the cytoplasm at 37 °C, it should remain there even when the nuclear export is blocked by LMB. The subcellular localization of p53Val-135 in clone 6 cells, as determined by immunofluorescent staining, is divided into three groups as follows: predominantly nuclear (N), cytoplasmic (C), and both nuclear and cytoplasmic (N = C), localization. More than 80% of cells showed p53 cytoplasmic localization (C and N = C) at 37 °C. In contrast, when the clone 6 cells were transfected with 10 nM LMB for 12 h, and p53Val-135 was detected by indirect immunofluorescent staining with an anti-p53 antibody pAb421. Effect of transient expression of CRM1 or CRM1-K1 on the LMB-induced nuclear localization was examined by transfection of clone 6 cells with 10 μg per dish of the CRM1 or CRM1-K1 mutant construct before the treatment with LMB. The fixed cells were stained with 1 μg/ml DAPI, and their localization of p53Val-135 was analyzed by immunofluorescent microscopy. At least 300 cells were counted, and the results of three independent experiments are shown as percentages with the standard errors.

N
N=C
C

|        | 37°C | 32°C |
|--------|------|------|
| 37°C   |      |      |
| 32°C   |      |      |

As the cells extracts. One mg of total protein was added to the mixture 3 h before p53 immunoprecipitation.

Fig. 1. Nuclear accumulation of p53Val-135 by LMB treatment.

Clone 6 cells cultured at 37 °C were treated with 10 nM LMB for 12 h. Since LMB covalently bound a cysteine residue in the central conserved region of CRM1 by a Michael-type addition and that CRM1-mediated nuclear export may be involved in the cytoplasmic localization of p53Val-135 at 37 °C. To rule out the possibility that LMB induced a general stress response thereby leading to the nuclear translocation of p53 independently of CRM1, we tested whether the expression of the LMB-resistant CRM1 mutant causes the recovery of the cytoplasmic localization of p53Val-135 in the presence of LMB. We previously showed that Lp83 covalently bound a cysteine residue in the central conserved region of CRM1 by a Michael-type addition and that the mutation at this residue caused the protein to be insensitive to LMB (35). When the clone 6 cells were transfected with the human CRM1-K1 mutant having Ser instead of Cys-528, the cells containing nuclear p53 in the presence of LMB decreased to about 40% (Fig. 1, LMB+CRM1-K1). Since the transfection efficiency was about 50% in this particular experiment, the cells showing p53 cytoplasmic localization were reasonably expressing CRM1-K1. In agreement with this, only a marginal increase in the cells showing p53 cytoplasmic localization was observed for the wild-type CRM1 construct (Fig. 1, LMB+CRM1). We assume that the CRM1-dependent nuclear export of p53Val-135 is responsible for the cytoplasmic localization of p53Val-135 at 37 °C. Transcriptional activity of p53 was not recovered even when p53Val-135 was accumulated in the nucleus by LMB treatment (data not shown).

Temperature-sensitive p53 Shuttles between the Nucleus and the Cytoplasm by Its Own NLS and NES—The clone 6 cells express MDM2 that can bind and functionally inactivate p53 (data not shown). MDM2 has been shown to promote the nuclear export of p53 in a CRM1-dependent manner by its NES (36, 37) or stimulating the p53 NES activity (38, 39). We therefore tested whether the cytoplasmic localization of p53Val-135 was mediated by MDM2. To this end, we expressed the GFP-p53 fusion in p53Val-135/mdm2−/− mouse embryonic fibroblast (2KO) cells (Fig. 2 A). As in the clone 6 cells, p53Val-135 showed the temperature-sensitive nuclear localization in 2KO cells; it localized in the cytoplasm at 37 °C but became present in the nucleus when cultured at 32 °C. Cycloheximide treatment rapidly induced nuclear translocation of p53Val-135. These patterns of subcellular localization were identical to those in the clone 6 cells (26). LMB treatment induced its nuclear accumulation

Fig. 2. Nucleocytoplasmic shuttling of p53Val-135. A, effect of LMB and cycloheximide (CHX) on the localization of GFP-p53Val-135 which is transiently expressed in 2KO cells (mdm2−/− p53−/−). Effect of co-expression of CRM1 or CRM1-K1 on the LMB-induced nuclear localization was examined by transfection of 2KO cells with the CRM1 or CRM1-K1 mutant construct before the treatment with LMB for 12 h. The fixed cells were mounted with 1 μg/ml DAPI, and their localization was analyzed by immunofluorescent microscopy. B, the C-terminal sequences containing the NLS and NES in mouse and human p53. Possible bipartite-type NLS sequences consisting of two clusters of basic amino acids are underlined. The leucine-rich NES sequences (12) are shown by boxes. C, subcellular localization of GFP-p53Val-135 containing mutations in the NES and NLS. An NES mutant (L345A/L347A) and an NLS mutant (K302N) were transiently expressed in 2KO cells, and the effects of CHX and LMB were analyzed by immunofluorescent microscopy.

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also in this cell line, and LMB-induced nuclear transport of p53Val-135 was almost completely suppressed by co-transfection of the CRM1-K1 construct. These results clearly indicate that p53Val-135 is exported in an MDM2-independent, CRM1-mediated manner, although they do not rule out the possibility that other NES-bearing protein partners participate in the nuclear export of p53Val-135.

An NES, which is sufficient to mediate the export of wild-type human p53, has recently been identified in the tetramerization domain of p53 encompassed by the proposed cytoplasmic sequestration domain (12). We therefore constructed a mutant GFP-p53Val-135 fusion consisting of leucine to alanine conversions at residues 345 and 347, which corresponded to the leucine residues at 348 and 350 essential for the NES of wild-type human p53 (Fig. 2B), and we analyzed the subcellular localization of the putative NES mutant. The 2KO cells expressing the GFP-p53Val-135 L345A/L347A had exclusively nuclear p53 at not only 32 °C but also 37 °C (Fig. 2C). This finding demonstrates that p53Val-135 uses its own NES for the nuclear export.

Three potential NLSs have been proposed to reside in the C terminus of p53 (40). A mutation in the major one, NLS1 (1113 to KKK in mouse p53, caused a defect in the nuclear localization. In addition to NLS1, however, Lys-305 in human p53 has recently been shown to be essential for the nuclear import of p53 (41, 42), suggesting that the two basic amino acid clusters, 305–306 (KR) and 319–321 (KKK), constitute a bipartite-type NLS (Fig. 2B). To see if the NLS is necessary for the nuclear localization of p53Val-135, we constructed a GFP-p53Val-135 fusion containing a K302N mutation corresponding to the human K305N, and we expressed it in 2KO cells. As shown in Fig. 2C, the K302N mutant localized in the cytoplasm even at 32 °C. Cycloheximide or LMB treatment could not induce the nuclear accumulation of the K302N mutant. The constitutive cytoplasmic localization of this mutant indicates that the NLS directly mediates the nuclear import of p53Val-135.

The Rate of Nuclear Import of Temperature-sensitive p53 Is Reduced at 37 °C—The subcellular localization of shuttling proteins reflects the equilibrium of the nuclear import and export rates. Since LMB does not affect the nuclear import of proteins (27, 29), specific inhibition of nuclear export by LMB allows us to determine the kinetics of the nuclear import of shuttling proteins. The percentage of cells with p53 nuclear localization (nuclear > cytoplasmic), as calculated from total 400–500 fluorescent cells, was ~15% in the control clone 6 culture at 37 °C, and it was almost unchanged during the further cultivation at 37 °C for 12 h (Fig. 3A). Cells having nuclear p53 rapidly increased upon the temperature shift to 32 °C and the cycloheximide treatment. About only a 1- or 2-h treatment was sufficient for the half-maximum nuclear import. When the clone 6 cells were treated with LMB at 37 °C, the cells showing p53 nuclear localization gradually increased to reach a plateau 12 h after the LMB addition. The time for the half-maximum induction was 7 h, indicating that the import rate is greatly reduced at 37 °C. In contrast, the import rate of p53 during the temperature shift and cycloheximide treatment was almost unaffected by LMB. These results suggest that the increase in the import rate is responsible for the rapid nuclear accumulation of p53Val-135 upon the temperature shift and cycloheximide treatment.

Association of p53Val-135 with the Import Receptor Complex Is Reduced at 37 °C—NLS-dependent nuclear translocation of proteins is mediated by importins α and β (also known as karyopherins α and β or PTACs), which together constitute the NLS receptor, in a manner dependent on the GTPase Ran/TC4 and p10/NTF2 (nuclear transport factor 2) (43, 44). At least three major forms of importin α (NPI-1, Rch-1, and Qip1) have been shown to bind conventional NLSs rich in the basic amino acids and interact specifically with importin β1. We next tested whether the reduced nuclear import of p53Val-135 at 37 °C is due to the reduced association with the NLS receptor. Since it is unknown which form of importin α carries the p53 NLS, we monitored the p53 binding to importin β. p53 was immunoprecipitated from the lysates of clone 6 cells cultured at 32 or 37 °C, and importin β in the precipitates was detected by immunoblotting with an anti-importin β antibody (Fig. 3B). The amount of importin β associated with p53Val-135 was analyzed by immunoprecipitation using the anti-p53 antibody followed by autoradiography.
may be seen only in the living cells.

Addition of the p53 NLS to the N Terminus Suppresses the Temperature-sensitive Nuclear Import—If the NLS is masked by interacting with other proteins or p53 itself, then the addition of the NLS motif to the unmasked region would rescue the temperature-sensitive nuclear import. To test this possibility, we conjugated the 26-amino acid sequence (residues 297–322) containing both Lys-302 and NLS1 (Fig. 2B) to the N terminus of p53Val-135, and we examined the subcellular localization of the fusion proteins (Fig. 4). The p53 NLS was able to direct the nuclear import of GFP alone or GFP-p53Val-135 at both 32 and 37 °C. We also tested whether the constitutive cytoplasmic sequestration of the K302N mutant can be rescued by the NLS supply at its N terminus, to rule out the possible activation of endogenous NLS by a conformational change upon the fusion. The GFP-p53 NLS-K302N also showed nuclear localization even at 37 °C. These results clearly indicate that the NLS does not work irrespective of temperatures in culture, when localized at the N terminus of p53Val-135. The NLS is probably masked at 37 °C only when it is localized in the C-terminal region.

Part of the C-terminal Domain Is Involved in the NLS Masking—The C-terminal region of p53 containing the NES has been described previously to be responsible for the cytoplasmic sequestration of wild-type p53 (41, 46). To determine whether the C-terminal region of p53Val-135 plays a role in the NLS masking, we synthesized three C-terminally truncated mutants of p53Val-135 with the rabbit reticulocyte lysate, and we examined their ability to bind importin β in vitro at 37 °C (Fig. 5). Like the full-length protein, p53Val-135 and p53Val-135-K302N showed nuclear localization in the cytoplasm (22, 23, 47–49). The multiprotein complex dissociates from p53Val-135 upon its nuclear translocation (25, 26, 48). To obtain a clue to understanding the mechanism by which the NLS is masked at 37 °C, we compared the association of p53Val-135 with these chaperones between cytoplasmic and nuclear p53Val-135. We first determined whether Hsp90 could be co-precipitated with p53Val-135 in the presence of sodium molybdate that stabilized the chaperone complex (32). As described by others (48, 49), Hsp90 and its co-chaperone p23 became almost undetectable in the precipitates containing the wild-type conformation of p53Val-135 when cultured at 32 °C (Fig. 6A). On the other hand, when the nuclear export was blocked by LMB or the nuclear import was stimulated by cycloheximide, p53Val-135 accumulated in the nucleus was still associated with both Hsp90 and p23. We next asked if the Hsc70 binding to p53Val-135 was affected by drug-induced nuclear translocation (Fig. 6B). p53Val-135 with the wild-type conformation at 32 °C did not interact with Hsc70. In contrast to the case of Hsp90, the amount of p53Val-135 co-precipitated with Hsc70 was greatly reduced in the cells treated with LMB for 12 h, at which time in more than 80% of cells p53Val-135 were accumulated in the nucleus (Fig. 3A). As shown in Fig. 6C, the time course of the dissociation of p53Val-135 from the Hsc70 complex during LMB treatment coincided well with the increase in the p53 nuclear accumulation (see Fig. 3A). In the cycloheximide-treated cells, however, the p53Val-135 was slightly decreased but still present in the precipitate (Fig. 6B). These results demonstrate that the composition of molecular chaperones in the nuclear p53 complex is different between the two drug treatments. Namely, the inhibition of the nuclear export by LMB causes selective nuclear import of the complexes containing Hsp90 but lacking Hsc70, whereas cycloheximide treatment can induce nuclear translocation of the complexes containing Hsp90, Hsc70, or both. To test the possibility that LMB directly promotes dissociation of the p53Val-135-Hsc70 complex, we examined the effect of LMB on in vitro interaction between p53 and Hsc70 in the rabbit reticulocyte lysate. As shown in Fig. 6D, the association of the in vitro translated proteins was unaffected by LMB.

Depletion of Hsc70 from the Reticulocyte Lysate Restores the p53 Association with the Import Receptor—To investigate further the role of Hsc70 in the regulation of p53Val-135 subcellular localization, we examined the effect of Hsc70 removal from the reticulocyte lysate by immunoadsorption on the in vitro binding of p53Val-135 to importin β. Since in vitro translation in the
lysate was strongly inhibited by the depletion of Hsc70, we synthesized labeled p53 and importin β in the presence of Hsc70, and then Hsc70 was removed by an anti-Hsc70 antibody at 4 °C, at which temperature p53 was completely dissociated from the Hsc70 complex (data not shown). The successful depletion was monitored by immunoblot analysis using the antibodies to Hsc70 and Hsp90 (Fig. 7 A). In the presence of Hsc70, in vitro binding of p53 Val-135 to importin β was detected at 32 °C but not at 37 °C, as shown in Fig. 7 A and Fig. 3 C. When Hsc70 was removed, however, the association of p53 Val-135 with importin β became detectable even at 37 °C. This binding was specific, because the K302N mutant defective in the NLS function did not bind importin β under the same conditions. We next examined if the purified recombinant Hsc70 protein could inhibit the association with importin β, which had been restored by the Hsc70 depletion. As shown in Fig. 7 B, Hsc70 even at a high concentration did not interfere with the protein-protein interaction, suggesting that the Hsc70-containing complex is required but Hsc70 alone is insufficient for masking the NLS at 37 °C. Interestingly, a soluble cell extract prepared from the clone 6 cells could block this interaction, whereas an extract from the cells treated with cycloheximide failed to inhibit it (Fig. 7 C).

**DISCUSSION**

**Temperature-sensitive Nuclear Import of p53**—In this study, we show that cytoplasmic p53 Val-135 at the nonpermissive temperature still shuttles between the nucleus and the cytoplasm by using its own NLS and NES. The specific inhibition of the nuclear export by LMB revealed that the decreased nuclear import is responsible for the cytoplasmic localization of p53 at 37 °C. Binding to the import receptor was not detected at nonpermissive temperature in the rabbit reticulocyte lysate, suggesting that the NLS function in p53 Val-135 is temperature-sensitive. Although three potential monopartite NLS sequences (NLS1–NLS3) have been identified in the p53 C-terminal region, it was recently shown that NLS1, which alone directed the nuclear import of proteins, constituted a strong bipartite-type NLS with an upstream KR sequence, inducing sufficient nuclear transport of p53 (42). The constitutive cytoplasmic localization of the NLS mutant of p53 Val-135 indicates that the bipartite-type NLS containing NLS1 is required for the nuclear import of p53 Val-135 and that NLS2 and NLS3 may have a marginal activity to induce the nuclear import of p53 Val-135 (Fig. 2 C). Since the NLS sequence itself has no mutation in p53 Val-135, it is unlikely that the structure of the NLS itself is impaired at 37 °C. We showed that the defect in the NLS function at 37 °C is completely rescued by the same NLS when attached to the N terminus of p53 Val-135 (Fig. 4). Therefore, the C-terminal region containing the NLS may be locally masked at 37 °C. This mechanism of cytoplasmic retention is in contrast to that for the nuclear retention by the tetramerization-induced NES masking (Fig. 8).

**A Model for the Cytoplasmic Sequestration of p53 Val-135**—We showed that immunodepletion of Hsc70-containing complex from the reticulocyte lysate rescued the p53 Val-135 binding to importin β (Fig. 7). This finding strongly suggests that the formation of the p53 Val-135 multiprotein complex with Hsc70-containing chaperones is important for the NLS masking.
Thus, we present a model for the temperature-sensitive nuclear import in that the NLS is masked by the Hsc70-containing complex (Fig. 8). Dissociation of p53Val-135 from the chaperone complex at 32 °C may result in the rapid nuclear import.

The amounts of Hsc70 in the mixtures were also determined by immunoblotting.
the NLS masking, but the latter is able to shuttle. The subcellular localization of Hsc70 is predominantly cytoplasmic in clone 6 cells, and it was unaffected by LMB treatment. Since reticulocyte lysate does not contain nuclei, the increased dissociation of p53 Val-135 from the Hsc70 complex in the LMB-treated living cells may be due to compartmentalization of p53 Val-135 by the inhibition of the nuclear export after the nuclear import, which prevents re-association with cytoplasmic Hsc70. The dissociation of p53 Val-135 from the Hsc70-containing complex may be the rate-limiting step for the p53 Val-135 nuclear import at 37 °C. It is possible that the NLS-masked, Hsc70-associated complex of p53 is reversibly tethered to a cytoplasmic structure. Thus, the current model is not inconsistent with the observation that the mutant p53 is sometimes tethered to intermediate filaments (50) and mitochondria (51).

It is still unclear how the p53 NLS is masked. It was previously proposed that p53 with an aberrant conformation is sequestered from the nucleus by Hsp70/Hsc70 binding (22–24). Suppression of the high transforming capacity of specific p53 mutants by overexpression of Hsc70 suggests a role of Hsp70/Hsc70 in regulating transformation (52). In some cases, however, Hsc70 binding may not effectively sequester p53 to the cytoplasm, since similar proportions of Hsc70-p53 complexes have been found in both the cytoplasm and the nucleus (20). Our experiments with recombinant Hsc70 ruled out the possibility that Hsc70 itself interacts directly with the NLS (Fig. 7B). It is therefore possible that a protein that masks the p53 NLS depending on the p53 Val-135 C-terminal domain is associated with the Hsc70-containing complex (Fig. 8, protein X). It was recently reported that the NLS in wild-type p53 was masked in some tumor cells probably through its cytoplasmic protein X substituted from reticulocyte lysate (53, 54), the p53 Val-135 containing five proteins (Hsp90, Hsp70, Hop, Hsp40, and p23) reconstituted from reticulocyte lysate (53, 54), the p53 Val-135 polypeptide may be folded by the chaperone complex leading to the NLS masking with the cytoplasmic sequestration domain (Fig. 8).

A Yet Unsolved Question, Cycloheximide-induced Nuclear Import—Since cycloheximide induces nuclear translocation of p53 Val-135, it has long been postulated that a protein(s) that rapidly turns over mediates the anchoring of p53 Val-135 to the cytoplasmic structure (26). However, the present study demonstrated that cycloheximide induced recovery of the NLS binding to the NLS receptor in cells (Fig. 3B) but not in the reticulocyte lysate (Fig. 3C). In agreement with this, the nuclear import rate in the cells treated with cycloheximide was much greater than that in the control cells cultured with LMB at 37 °C (Fig. 3A). It is therefore unlikely that cycloheximide stabilizes p53 Val-135, leading to the tetramerization-mediated inhibition of the nuclear export. These results suggest that de novo protein synthesis is required for the NLS masking. As described in the early study (26), the nuclear p53 Val-135 complex in the cells treated with cycloheximide at 37 °C contained Hsc70 (Fig. 6B), supporting the idea that Hsc70 itself is not the masking protein. Since the cell extract from the untreated cells, but not cycloheximide-treated cells, blocked the NLS binding to p53 Val-135 in the Hsc70-depleted lysate (Fig. 7C), it seems likely that a short lived protein(s), which was lost in the nuclear p53 Val-135 complex in the presence of cycloheximide, is directly responsible for the NLS masking or indirectly associated with specific chaperone activity to form the NLS-masked conformation (Fig. 8). A protein of unknown function, Spot-1, has been shown to interact with NLS1, but the significance of this binding remains to be elucidated (55). Recently, Mot-2, a member of the Hsp70 family (also known as mtHSP70 and Grp75), was shown to interact directly with p53 and induce p53 cytoplasmic sequestration, thereby inactivating the normal p53 function (56). The half-lives of these proteins are unknown. The in vitro importin binding assay in the Hsc70-depleted lysate used in this study provides a useful system to identify the protein involved in the NLS masking.

p53 NLS Masking and Cancer—Besides p53 gene mutations in many human cancers, a substantial fraction of cancers contain p53 that is functionally inactivated by other mechanisms. Aberrant subcellular localization is one such mechanism found in breast carcinoma (9), colorectal adenocarcinoma (57), undifferentiated neuroblastoma (13), hepatocellular carcinoma (58), and retinoblastoma (59). It is also associated with metastasis and poor prognosis (60). p53 cytoplasmic sequestration could result from its anchorage to a cytoplasmic tether or by an imbalance in nucleocytoplasmic shuttling, i.e. increased nuclear export or decreased nuclear import. Discrimination of these mechanisms had been difficult, until LMB became available as a specific nuclear export inhibitor. Although MDM2-dependent nuclear export may be involved in some cases, we showed that the cytoplasmic sequestration of p53 Val-135 was independent of MDM2. It was recently shown that in neuroblastoma cells the energy-dependent shuttling of p53 is altered so that hyperactive nuclear export results in net cytoplasmic accumulation (12). This finding implies that an upstream signaling event which results in nuclear export is constitutively active in these cells or that signals required for nuclear retention are missing. It is currently unclear whether the cytoplasmic sequestration of p53 in other cancers is also a result of hyperactive nuclear export. The present study implies that the NLS masking mechanism should exist in some cases that are believed to be cytoplasmic anchoring. It is apparently important to explore the possibility of the p53 cytoplasmic sequestration by NLS masking for the better understanding of the mechanism by which p53 is inactivated in human cancers.

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A Role for Hsc70 in Regulating Nucleocytoplasmic Transport of a Temperature-sensitive p53 (p53 Val-135)

Shin Akakura, Minoru Yoshida, Yoshihiro Yoneda and Sueharu Horinouchi

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