Activation of Wild Type p53 Function by Its Mortalin-binding, Cytoplasmically Localizing Carboxyl Terminus Peptides*

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The Hsp70 family member mortalin (mot-2/mthsp70/GRP75) binds to a carboxyl terminus region of the tumor suppressor protein p53. By in vivo co-immunoprecipitation of mot-2 with p53 and its deletion mutants, we earlier mapped the mot-2-binding site of p53 to its carboxyl terminus 312–352 amino acid residues. In the present study we attempted to disrupt mot-2-p53 interactions by overexpression of short p53 carboxyl-terminal peptides. We report that p53 carboxyl-terminal peptides (amino acid residues 312–390, 312–352, 323–390, and 323–352) localize in the cytoplasm, whereas 312–322, 337–390, 337–352, and 352–390 locate mostly in the nucleus. Most interestingly, the cytoplasminically localizing p53 peptides harboring the residues 323–337 activated the endogenous p53 function by displacing it from p53-mortalin complexes and relocating it to the nucleus. Such activation of p53 function was sufficient to cause growth arrest of human osteosarcoma and breast carcinoma cells.

p53, the "guardian of the genome," is a major player in cell cycle arrest and apoptosis in response to the diverse endogenous and exogenous stress signals (1). Since its first identification three decades ago, it has been studied widely and has been recognized as a most frequently mutated gene in a variety of cancers (2, 3). Loss of p53 function is one of the early events in immortalization of human cells (4, 5). In addition, it confers resistance to radiation and chemotherapy of tumors (6–8). Therefore, studies on the regulation of p53 function and its reactivation are extremely important for cancer therapeutics. p53 is synthesized in the cytoplasm and must translocate to the nucleus to exert its sequence-specific transcription factor function (9). Another function of p53, independent to its nuclear localization, is to regulate mitochondrial membrane potential by interactions with the mitochondrial proteins Bcl2 and mortalin/mthsp70 (10–17).

Nuclear import and export of p53 is regulated by three nuclear localization signals (NLSs), 2 two nuclear export signals, and its binding to nuclear and cytoplasmic binding partners (9, 18, 19). The tetrameric state of p53 in the nucleus masks its nuclear export signal (18) and, thus, favors its nuclear retention. Transcriptional activation of p53 in the nucleus leads to the expression of MDM2 (one of its downstream regulators and antagonists) that results in its degradation by one or both of the following mechanisms: (i) physical export of p53 to the cytoplasm and its subsequent degradation by proteasome pathway; (ii) ubiquitination of p53 in the nucleus, unmasking of the nuclear export signal that favors its export from the nucleus, and subsequent proteasome-mediated degradation (20–22). Other proteins that regulate p53 function include p14ARF, CARF, PML, Parc, mthsp70/mortalin, Bcl2, and cytoskeleton proteins (actin, vimentin, and microtubules) (20, 22–27).

We had previously reported that mortalin causes cytoplasmic sequestration of p53 by binding to its carboxyl terminus amino acid residues 312–352 (31, 34, 35). In the present study, we report an activation of p53 function by overexpression of mortalin binding, cytoplasminically localizing the carboxyl terminus region peptides of p53.

MATERIALS AND METHODS

Plasmid Constructions—Full-length and deletion mutants of mouse p53 were obtained by PCR using p53-specific primers and cloned into the EYFPC1, EGFP/E1 (Clontech), and pTOPO/V5 (Invitrogen) mammalian expression vectors. The integrity of the plasmids encoding various deletion mutant proteins was confirmed by sequencing. Proteins expressed in cells were visualized on a Carl Zeiss microscope and were also examined by Western blotting with GFP, V5, and mouse p53-specific antibodies.

Cell Culture and Transfections—Human osteosarcoma (U2OS) or breast carcinoma (MCF7) cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Transfections were performed using Lipofectamine™ (Invitrogen). Typically, 3 μg of plasmid was used per 6-cm dish. After 24–48 h of transfection, protein expression was visualized by Western blotting or immunostaining as described below.

Cell Fractionation—Transfected cells were harvested and fractionated into nuclear and cytoplasmic fractions using a nuclear/cytosol fractionation kit from BioVision (Mountain View, CA). Protein (20 μg) from each fraction was resolved on a 12% SDS-polyacrylamide gel and Western-blotted with anti-YFP antibody for detection of YFP-tagged p53 proteins.

Immunoprecipitation and Immunodepletion—Cell lysates (600 μg) were incubated with anti-p53 polyclonal antibody for immunoprecipitation of endogenous p53 protein as described (31). p53 immunocomplexes were examined for the presence of mortalin by Western blotting with an anti-mortalin monoclonal antibody. Equal amounts of the lysate taken for immunoprecipitation were ensured by probing with an anti-actin antibody. For mortalin immunodepletion, lysates were immunoprecipitated with polyclonal anti-mortalin antibody for two rounds of precipitation. Supernatants were analyzed for endogenous p53 and YFP-p53 fragments by Western blotting with specific mono-
clonal anti-GFP and anti-p53 antibodies. Endogenous p53 was quantitated by image analysis software.

**Western Blotting**—Cells transfected with various deletion mutants of mouse p53 were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and protease inhibitors (Roche Applied Science Protease Inhibitor Cocktail). The protein concentration in cell lysate was determined by a standard dye binding assay (Bio-Rad). 10 μg of total protein was resolved on a 12.5% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan), and probed with anti-GFP, anti-p53, anti-histone H1, or anti-mortalin antibodies. Immunocomplexes were observed with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (ECL kit, Amersham Biosciences).

**Immunostaining**—Cells were fixed with ice cold methanol/acetone (1:1) for 5 min and stained for endogenous p53 with a human p53-specific antibody (p53-DO1; Santa Cruz Biotechnology) or mortalin with an anti-mortalin antibody (36). Transfected YFP- or GFP-tagged mouse p53 proteins were visualized by their autofluorescence at the same time. The cells were examined on a Carl Zeiss microscope (Axiovert 200 i) in either conventional fluorescence or the ApoTome mode for high optical resolution attached to Photomerics Sensys and AxioCam MRm monochrome charge-coupled device cameras. The extent to which the two proteins overlapped was assessed by combining the two images using either Metamorph or AxioVision software.

**p53-dependent Reporter Assays**—U2OS cells were stably transfected with the p53-responsive luciferase reporter plasmid PG-13luc (kindly provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute)). Cells stably expressing a p53-dependent reporter were then transfected with expression plasmids encoding p53 deletion mutants. As a control, a pRL-TK vector (Promega) was co-transfected in each assay to correct for variations in transfection efficiency. Cells were lysed, and luciferase activity was measured by using the Dual-Luciferase™ reporter assay system (Promega). Results presented are the mean of three transfections. Transfection efficiencies were normalized by co-transfection of a plasmid encoding Renilla luciferase. Luciferase activity was calculated per μg of protein and presented as the percent of activity with untransfected cells taken as 100%.

**Cell Growth Assays**—The growth of U2OS cells was monitored by colony-forming assays. Transfected cells were selected by co-transfection of pClur plasmid and subsequent selection in puromycin-supplemented (2 μg/ml) medium for 48 h. Selected cells (1000) were plated in a 6-cm dish (in triplicate) and left to form colonies for the next 2 weeks with a regular change of medium every third day. Colonies were fixed in methanol, stained with 0.1% crystal violet solution, photographed, and counted.

**RESULTS AND DISCUSSION**

**Subcellular Localizations of GFP- and YFP-tagged Carboxyl-terminal Deletion Mutants of p53**—Expression plasmids encoding GFP- or YFP-tagged full-length and various deletion mutants of murine p53 were constructed by PCR followed by cloning into the pEGFP-C1 or pEYFP-C1 vector (Fig. 1A). Cells transfected with expression plasmids were fixed and visualized under the microscope for the subcellular localization of the exogenous protein. The number of cells with purely nuclear, cytoplasmic, and predominantly nuclear p53 were counted (Fig. 1A). Based on the data, the constructs were divided into three groups as follows: (i) those encoding purely nuclear p53, i.e. YFP-p53 full protein (1–390 aa) and its carboxyl terminus-deleted mutant (1–322 aa); (ii) cytoplasmic p53, i.e. YFP-p53 (312–390, 312–352, 323–390, 323–352, and 323–337 aa); and (iii) predominantly nuclear p53 with some diffuse localization in the cytoplasm, i.e. 312–322, 337–390, 337–352, and 352–390 aa) (Fig. 1A). These data demonstrated the following findings. (i) The carboxyl terminus of p53 (323–390 aa) is responsible for its cytoplasmic localization. (ii) Amino acid residues 323–337 are required for its cytoplasmic localization. (iii) In the absence of 323–337 aa, the carboxyl terminal mutants locate to the nucleus (Fig. 1A). We also confirmed the presence of YFP-tagged p53 mutants in the cytoplasm or the nucleus by cell fractionation and Western blotting. The nuclear and cytoplasmic fractions were probed with antibodies for resident marker proteins (mortalin for the cytoplasmic fraction and histone H1 for nuclear fraction). Similar to the cell visualization results, YFP-p53 full (1–390 aa) was nuclear, two mutants (323–352 and 312–352 aa) were mainly cytoplasmic (Fig. 1B), and one mutant (337–390 aa) was mainly nuclear with a small amount detected in the cytoplasmic fraction (Fig. 1B). Taken together with the above visualization data, p53 amino acid residues 323–337 seemed to be required for its cytoplasmic localization. Consistent with this finding, we had reported earlier (31) that p53 amino acid residues 312–352, but not 352–390, bind to mortalin in immunoprecipitation assays performed on cell lysates (free of subcellular compartments). Although the amino acid residues such as 312–322, 337–390, and 337–352 could also bind to mortalin in immunoprecipitation assays (31), this might not happen in vivo because of their nuclear localization.

**Nuclear Translocation and Stabilization of Wild Type p53**—We demonstrated earlier (31, 34, 35) that mortalin interacts with p53 and sequesters it in the cytoplasm. We next considered examining whether mortalin-binding small p53 peptides could compete with the endogenous p53 and abrogate its cytoplasmic sequestration from the mortalin-p53 complex, resulting in nuclear translocation of p53 (Fig. 1C). Such nuclear translocation would also lead to its stabilization and enhanced transcriptional activation function. As predicted, we found that the cells transfected with p53 fragments (323–390, 312–352, and 312–390) showed stabilization of endogenous p53. Of note, YFP by itself or fused to p53 fragment 1–30, 352–390, 337–390, or 337–352 was neutral (Fig. 2 A, B). Binding of p53 (fragments versus endogenous) to mortalin was examined by immunodepletion assays. Cell lysates immunodepleted with anti-mortalin antibody were examined for the presence of YFP-p53 and endogenous p53. As shown in Fig. 2C, lysates immunodepleted for mortalin showed a decrease in YFP-p53 (312–352) but not in YFP itself or YFP-p53 (352–390). Consistent with this result, the level of endogenous p53 was decreased by 5% in two rounds of mortalin immunoprecipitation (efficiency of immunoprecipitation is ~5% in each round) in cells transfected with YFP or YFP-p53 (352–390). Of note, the level of endogenous p53 remained unchanged in cells expressing amino acid residues 312–352. The data supported the hypothesis that the mortalin-binding p53 fragment competed with the endogenous p53, resulting in its stabilization. We then assayed the p53 activity by p53-dependent reporter assays in cells stably transfected with PG13-Luc (p53-dependent luciferase reporter plasmid) (Fig. 2D). Consistent with our prediction and the data obtained as described above, cells expressing p53 fragments harboring amino acid residues 323–337 caused significant activation of p53 function. The mortalin-nonbinding p53 fragment was neutral.

We also examined the effect of overexpression of mortalin in these assays. The cells expressing each of the two peptides that contained mortalin-binding region (312–352 and 312–390 aa) showed a decreased amount of mortalin in p53 immunocomplexes as compared with an empty vector control or the 352–390 peptide-transfected (devoid of mortalin-binding region) cells (c.f. lanes 2 and 3 with lanes 1 and 4 in Fig. 2, E and F). Most significantly, in mortalin-overexpressing cells the
expression of p53 fragments (312–352 and 312–390 aa) did not cause a decrease in mortalin in p53 immunocomplexes. The data clearly showed that the mortalin-binding p53 peptides disrupt mortalin-p53 interactions by competing out the endogenous p53 from mortalin-p53 complexes as proposed in our model (Fig. 1C) and cause stabilization and activation of p53 function (Fig. 2, A–D).

We next aimed to examine the nuclear translocation of endogenous p53 by direct visualization (Fig. 3A). Because the transfected protein was the murine p53, it was possible to distinguish the exogenous and endogenous (human) proteins by the use of a specific anti-p53 antibody that reacts only to the human protein. Transfected YFP-mouse p53 deletion mutants (Fig. 2A, bottom section) did not cross-react to the anti-human p53 antibody (Fig. 2A, top section).

We next transfected the cells with YFP-tagged p53 deletion mutants and examined the intracellular localization of endogenous p53 by anti-p53 human-specific antibody (Fig. 3A). Time course observations were...
Detection of endogenous and exogenous p53 proteins by anti-p53-specific (top panel) and anti-GFP/YFP-specific (bottom panel) antibodies, respectively. A and B, the membrane was first probed with anti-human p53 antibody (A, top section) and then stripped and probed with anti-YFP and anti-actin antibodies (bottom section). Note that the transfected YFP-tagged mouse p53 was not detected by anti-human p53 antibody and that the carboxyl-terminal deletion mutants (323–390, 312–352, and 312–390 aa) led to increased levels of endogenous p53. C, cells transfected with indicated YFP-p53 fragments were immunoprecipitated with anti-mortalin antibody. Mortalin-immunodepleted
also taken subsequent to the transfection of 312–352 (mortalin-binding) and 352–390 (mortalin-nonbinding) aa fragments (Fig. 3B). We found that ∼90% of the cells transfected with p53 deletion mutants (312–352 and 323–352 aa, located in the cytoplasm) showed intense nuclear staining for endogenous p53 (Fig. 3A). In contrast, cells transfected with vector or the deletion mutants that locate in the nucleus (312–322, 337–390, 337–352 and 352–390 aa) (Fig. 1A) showed intense nuclear staining for p53 in ∼10% of the cells (Fig. 3A and data not shown). Vector transfectants also showed intense p53 staining in a small fraction of cells. Most likely, such variability in p53 levels may be due to the cell cycle stage. Nevertheless, the population of cells showing intense endogenous p53 staining was only 10% when transfected with nuclearly localizing p53 peptides as compared with 90% in the case of cells transfected with peptides localizing in the cytoplasm. Time course observations confirmed that the mortalin-binding p53 fragment could cause the translocation of endogenous p53 to the nucleus, whereas the...
Reactivation of p53 Function

![Graph showing reactivation of p53 function](image)

**FIGURE 4. Effect of cytoplasmically localizing p53 deletion mutants on a colony-forming assay.** A, each of the five mutants was inhibitory as compared with the vector control. Deletion mutant 323–337 caused the strongest inhibition in three independent experiments. B, mortalin binding characteristics, subcellular localization, and p53 activation function of various deletion mutants of p53 are summarized.

mortalin-nonbinding fragment was neutral (Fig. 3B). We also performed Western analysis on nuclear fractions of p53 transfectants. The data confirmed that the p53 peptides such as 312–352 and 323–352 result in an increase in nuclear p53 (Fig. 3C). Similar to the data shown in Fig. 2B, peptide 323–390 was stronger than 312–352 and 323–390 in its p53-translocating function. Taken together, these data clearly showed that the small p53 peptides such as 323–352 and 323–390 that co-localize with mortalin (Fig. 3, D and E, and data not shown) competed with endogenous p53, disrupted mortalin-p53 binding, and resulted in translocation of the endogenous p53 protein to the nucleus as was expected in our model (Fig. 1C). Similar results were obtained in both U2OS and MCF7 cells. The data showed that the stabilization and activation of p53 function by p53 peptides involve nuclear translocation of endogenous p53.

Sequence analysis of mortalin did not reveal any homology to the E3 family of ubiquitin-protein isopeptide ligases; it does not contain any of the three known motifs (U-box, Ring finger, and HECT) present in three families of E3 ligases. Therefore, it is likely that the cytoplasmic retention of p53 by mortalin enhances its degradation by the MDM2-mediated proteasome degradation pathway. Disruption of mortalin-p53 interactions in the cytoplasm by p53-peptides, as demonstrated above, caused translocation of endogenous p53 to the nucleus and thus protected it against the degradation in the cytoplasm (Fig. 1C) as supported by an increase in the level of endogenous p53 and its activity (Fig. 2, A, B, and D). Furthermore, similar results were obtained (data not shown) when cells were transfected with MDM2 expression construct (driven by the MDM-2 promoter, a kind gift from Dr. Uri Alon, Weizmann Institute of Science, Rehovot, Israel) along with the p53 peptides such as 323–352 and 323–337 (but not 312–352), implying that the nuclear translocation of p53 caused by these p53 peptides (323–352 and 323–337) was effective in protecting it from MDM2-mediated degradation in the cytoplasm. Because nuclear translocation of endogenous p53 caused by the peptide 312–352 was disrupted by overexpression of MDM2, it is suggested that the peptide 312–352 is a weaker competitor than 323–352 or 323–337 in translocating the endogenous p53 to the nucleus and activating it. Of note, the data in Fig. 2B showing a higher level of endogenous p53 in cells transfected with peptide 323–390 than with 312–352 or 312–390 supported this finding. Taken together, these results suggest the following two points. (i) The p53 residues 312–322, which localize in the nucleus when present along with cytoplasmically localizing residues such as 323–352, disrupt their activity as a p53-binding antagonist to mortalin. (ii) The p53 peptide-induced activation of endogenous p53 in this study takes place by bypassing the MDM2 mediating degradation, at least in part.

**Activation of Wild Type p53 Function**—We next investigated whether the activation of endogenous wild type p53 affects the growth of cells. MCF7 and U2OS cells transfected with cytoplasmically localizing p53 peptides showed delayed growth as compared with the vectortransfected cells. Of note, peptides 312–352 and 323–337 were strongly inhibitory (data not shown). Colony-forming assays were also performed in U2OS cells. Cells were transfected with p53 deletion mutants and selected by co-transfection of a puromycin resistance marker plasmid. The selected cells were subjected to colony-forming assays (Fig. 4A). Of note, whereas mortalin-binding p53 peptides (312–352 and 323–337) caused strong inhibition (53 and 76%, respectively), mortalinin-binding p53 peptides (312–322, 337–352, and 352–390) had only a weak effect (<20%) on the colony-forming efficiency of cells. Taken together, these data showed that the cytoplasmically localizing deletion mutants of p53 displaced endogenous p53 from mortalin-p53 complexes, resulting in its nuclear translocation and activation of growth arrest function.

Functional inactivation of p53 is a very common characteristic of tumors (37, 38), and the mechanisms whereby this occurs include the following three main categories: (i) mutations of p53 that abrogate its DNA binding or transcriptional activation functions; (ii) abnormal expression of p53-interacting proteins, e.g. MDM2, that result in accelerated degradation of the wild type or stability of mutant p53; and (iii) nuclear exclusion of wild type p53 (22). Although the first two categories have been investigated intensively in the last decade, the third remains poorly understood. The functional domains of p53 include an amino terminus transactivation domain, a sequence-specific DNA-binding domain, a carboxyl terminus oligomerization/tetramerization domain, and a regulatory domain (39). The intracellular localization of p53 has been shown to be determined by nuclear localization signals, single amino acid residues such as Leu<sup>205</sup>, Arg<sup>300</sup>, and Ser<sup>315</sup>, nuclear export signals (18, 40), and its interactions with other proteins including MDM2 and some of the Hsp70 family members (18, 20, 31, 41). There are three nuclear translocation signals (NLS I, 313–322 aa; NLS II, 369–375 aa; and NLS III, 379–384 aa) at the carboxyl terminus of p53. As expected, the deletion mutants that retained NLS localized in the
nucleus (Fig. 4f). On the other hand, amino acid residues 323–337 by themselves or with extended C terminus (323–390 and 323–352 aa) localized almost exclusively in the cytoplasm; the deletion mutants lacking amino acid residues 323–337, e.g. 337–390 and 337–352 aa) localized in the nucleus.

In vivo activation of p53 by small molecule antagonists of MDM2 that inhibit the interactions of p53 and MDM2 was shown (42). Mutant mice with an allele coding for carboxy-terminal p53 fragment showed enhanced resistance to spontaneous tumors and early onset of aging phenotypes (43), providing evidence for the overactivation of wild type p53 function by carboxy-terminal p53. We have reported here that the overexpression of mortalin-binding, cytoplasmically localizing carboxy-terminal p53 peptides harboring amino acid residues 323–337 disrupts mortalin-p53 complexes, resulting in nuclear translocation and significant functional activation of wild type p53. These findings may be adopted for therapeutics of tumors with wild type but inactive p53.

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