

## An N-terminal Region of Mot-2 Binds to p53 *In Vitro*

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### Abstract

The mouse mot-2 protein was earlier shown to bind to the tumor suppressor protein, p53. The mot-2 binding site of p53 was mapped to C-terminal amino acid residues 312–352, which includes the cytoplasmic sequestration domain. In the present study, we have found that both mot-1 and mot-2 bind to p53 *in vitro*. By using His-tagged deletion mutant proteins, the p53-binding domain of mot-2 was mapped to its N-terminal amino acid residues 253–282, which are identical in mot-1 and mot-2 proteins. Some peptides containing the p53-binding region of mot-2 were able to compete with the full-length protein for p53 binding. The data provided rationale for *in vitro* binding of mot-1 and mot-2 proteins to p53 and supported the conclusion that inability of mot-1 protein to bind p53 *in vivo* depends on secondary structure or its binding to other cellular factors. Most interestingly, the p53-binding region of mot-2 was common to its MKT-077, a cationic dye that exhibits antitumor activity, binding region. Therefore it is most likely that MKT-077-induced nuclear translocation and restoration of wild-type p53 function in transformed cells takes place by a competition mechanism. *Neoplasia* (2001) 3, 110–114.

**Keywords:** mot, p53, binding domain, MKT-077.

### Introduction

Mot-2 is an hsp70 family member that was shown to bind to p53 [1]. Its overexpression results in malignant transformation of NIH 3T3 cells [2], growth advantage and attenuation of differentiation HL-60 promyelocytic leukemia cells [3], and extension of life span of normal human fibroblasts [4]. Mouse mot-1 protein differs from mot-2 by two amino acids in the C-terminus; it has pancytoplasmic cellular distribution, causes senescence in NIH 3T3 cells and is allelic to mot-2 [5–8]. Mot-2 binds to and represses p53 activity [1]. Mot-1 neither colocalizes nor coprecipitates nor affects p53 function [Refs. [1, 9]; unpublished observations]. In contrast to mouse cells, human cells have only one kind of mortalin protein that has activity similar to mouse mot-2 [2] and is therefore called hmot-2. It has been suggested that there are at least two mechanisms operating for differential distribu-

tions of the mot protein. One is by distinct cDNAs, mot-1 and mot-2 found in mouse, and the other by yet undefined protein modifications or cellular factors found in mouse and human cells.

Mot-2 was also identified as PBP74, mtHSP70, and GRP75 and has been assigned roles in antigen processing, *in vivo* nephrotoxicity, and radioresistance [10]. In different studies, it has been localized to mitochondria, ER, plasma membrane, and cytoplasmic vesicles [11–13]. Expression level of mot-2/mthsp70/GRP75 level correlates with muscle activity [14], mitochondrial activity, and biogenesis [15], and is induced by low levels of ionizing radiations [16], glucose deprivation [17], calcium ionophore [18], and ozone [19]. Whereas the expression of mot-2/mthsp70/GRP75 is upregulated in human transformed and tumor cell [2,20,21], it decreased during induction of differentiation in HL-60 promyelocytic leukemia cells. Mot-2-overexpressing derivatives were markedly impaired for induction of differentiation [3].

The yeast homologue of mot-2/mthsp70/GRP75, SSC1p, is essential for cell viability [22] and has indispensable functions in mitochondrial import [23,24]. It binds to Tim-44, an inner mitochondrial membrane anchor, and is an essential component of mitochondrial import machinery [24,25]. Mutations in Tim-44 that result in inefficient recruitment of mthsp70/SSC1 are lethal in *Saccharomyces cerevisiae* [25,26]. Based on the studies in yeast, at least three kinds of activities can be hypothesized for mot-2/mthsp70/GRP75. These include (i) unfolding of proteins outside mitochondria, (ii) unidirectional translocation across mitochondrial membranes that is initiated by membrane potential  $M\Psi$ , and (iii) completion of import by acting as an ATP-driven motor. It is also required for degradation of misfolded peptides by m-AAA and PIM1 proteases in mitochondria [27]. There is some evidence to suggest that it cooperates with mthsp60 and CPN 10 chaperonins for folding of imported proteins to functionally competent forms in mitochondria and for yet undefined roles of mthsp60 at extra-mitochondrial sites [28]. It is likely but not shown so far that

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mitochondrial importer and chaperonin functions of mot-2/mthsp70 as predicted above contribute to the phenotypes described above. We have shown that inactivation of wild-type p53 by mot-2 mediates life span extension and malignant transformation phenotypes of normal human (MRC-5) and immortal murine (NIH 3T3) cells, respectively [1,2,4]. Most recently, we have shown that MKT-077, a water-soluble delocalized lipophilic cationic dye that exhibits significant antitumor activity binds to mot-2. It results in abrogation of cytoplasmic sequestration and reactivation of wild-type p53 function in transformed cells [29].

The present study was undertaken to map the domain of mot-2 that is responsible for its interaction with p53. Both mot-1 and mot-2 coimmunoprecipitated with p53 in *in vitro* pull-down assays. Mortalin residues 253–282 that are identical in mot-1 and mot-2 were found critical for its binding to p53. Based on these data, the inability of mot-1 to bind p53 *in vivo* can be attributed to either some other mot-1 binding cellular factors or the secondary structure of the proteins. The p53-binding region of mot defined in this study overlapped with the MKT-077-binding region of mot [29]. MKT-077 was shown to abrogate mot-2–p53 interactions and cytoplasmic sequestration of p53 resulting in reactivation of wild-type p53 function and growth arrest of transformed cells [29]. Binding of p53 and MKT-077 to the same region of mot elucidates a competitive mechanism for abrogation of cytoplasmic retention of p53 by MKT-077 [29].

## Materials and Methods

### Cell Culture

Monkey kidney cells (COS 7) were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum.

### In Vitro Pull-Down Assays

Murine p53 (pSP65/p53, a kind gift from Dr. Antony Braithwaite) [30] was transcribed (Transprobe T kit, Pharmacia) and translated *in vitro* using rabbit reticulocyte lysate (Stratagene, La Jolla, CA) supplemented with L-[<sup>35</sup>S]methionine for 1 h. The translation products were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography using a phosphorimager. *In vitro* translated p53 protein was immunoprecipitated with anti-p53 antibodies (PAb421 and PAb 1620, Calbiochem; PAb122, Boehringer Mannheim) and was used for *in vitro* binding assays as below.

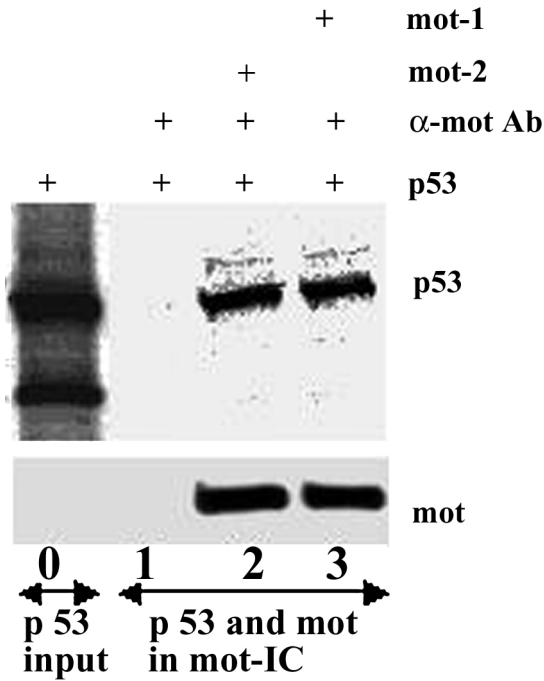
For preparation of recombinant mortalins, the ORF of mortalin cDNA and its various deletions were amplified by polymerase chain reaction (PCR) with sense and antisense primers with *Bam*HI and *Sal*I sites, respectively. These were then cloned into pQE30 vector (Qiagen) to yield His-tagged proteins that were purified as described earlier [31]. The purity of preparations was examined by SDS-PAGE.

Aliquots of the purified protein were stored at –20°C in small volumes to avoid repeated freeze–thaw cycles. Purified recombinant His-tagged mortalin proteins (0.5–1 µg) were mixed with *in vitro* translated <sup>35</sup>S-labeled wild-type p53 in the presence of either 1–2 mg bovine serum albumin or COS 7 cell lysate (400 µg protein) in 400 µl Nonidet P-40 lysis buffer [4]. The mixture was incubated with an anti-mortalin (mthsp70, Affinity Bioreagents) or anti-p53 (CM-1, Novocastra) antibody at 4°C for 1–2 h. Immuno-complexes were separated by incubation with protein–A/G sepharose (Gibco) (20 µl) for 30 min followed by centrifugation. After heating at 95°C in SDS sample buffer, proteins were resolved on SDS-PAGE, transferred to nitrocellulose membrane by semidry transfer and Western blotted with the indicated antibodies and detected by ECL-chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). For competition assays, the indicated amounts of the competitor peptide were incubated with COS 7 lysates for 30 min at 4°C following which His-tagged mortalins were added. Anti-p53 antibody was added 30 min after the addition of mortalin proteins. COS 7 cells transfected with expression plasmids (Invitrogen, San Diego, CA) encoding various V5-tagged mortalin deletion mutants were used for p53 immunoprecipitation. The detection of coprecipitating mortalin was detected by anti-V5 tag antibody (Invitrogen).

## Results and Discussion

### In Vitro Binding of mot-1 to p53

*In vitro* translated <sup>35</sup>S-labeled murine p53 was mixed with recombinant His-tagged mot-1 and mot-2 proteins and was immunoprecipitated from the mixture with anti-mortalin antibody. Immunoprecipitated mortalins and p53 were detected by Western blotting with anti-His tag antibody and autoradiography, respectively. As shown in Figure 1, p53 was immunoprecipitated along with mot-2 as well as mot-1 proteins. Controls such as incubation of p53 alone with anti-mortalin antibody or mortalin–p53 mixture with control isotype matched IgG did not result in precipitation of p53. In a reciprocal assay, glutathione S-transferase (GST)–wild-type p53 fusion protein (Santa Cruz Biotechnology, Santa Cruz, CA) or GST alone was incubated with either recombinant His-tagged mot-1 or mot-2 protein. Proteins were pulled down using glutathione–sepharose and coprecipitating mortalins were detected with anti-mortalin or anti-His antibodies. p53 was detected with anti-GST antibodies (Santa Cruz Biotechnology). Both mot-1 and mot-2 were seen to coimmunoprecipitate with p53 protein (data not shown). This suggested that the region harboring the two amino acid differences between mot-1 and mot-2 may not be involved in its binding to p53 *in vitro*. These data showed that in contrast to the *in vivo* situation in which only mot-2 was seen to affect p53 function both mot-1 and mot-2 bind to p53 protein *in vitro*.



**Figure 1.** *In vitro* binding of *mot-1* and *mot-2* to *p53*. *In vitro* translated  $^{35}\text{S}$ -labeled murine *p53* (53-kDa band) and a small-sized (43-kDa) product (lane 0) were mixed with His-tagged *mot-2* (lane 2) or *mot-1* (lane 3) recombinant proteins and were immunoprecipitated with anti-mortalin antibodies. Immunoprecipitated *mot-1* and *mot-2* were detected by Western blotting with anti-His tag antibody and coimmunoprecipitated *p53* was detected by autoradiography. *p53*, but not its carboxy-terminal truncated form coprecipitated with both *mot-1* and *mot-2* (lanes 2–3).

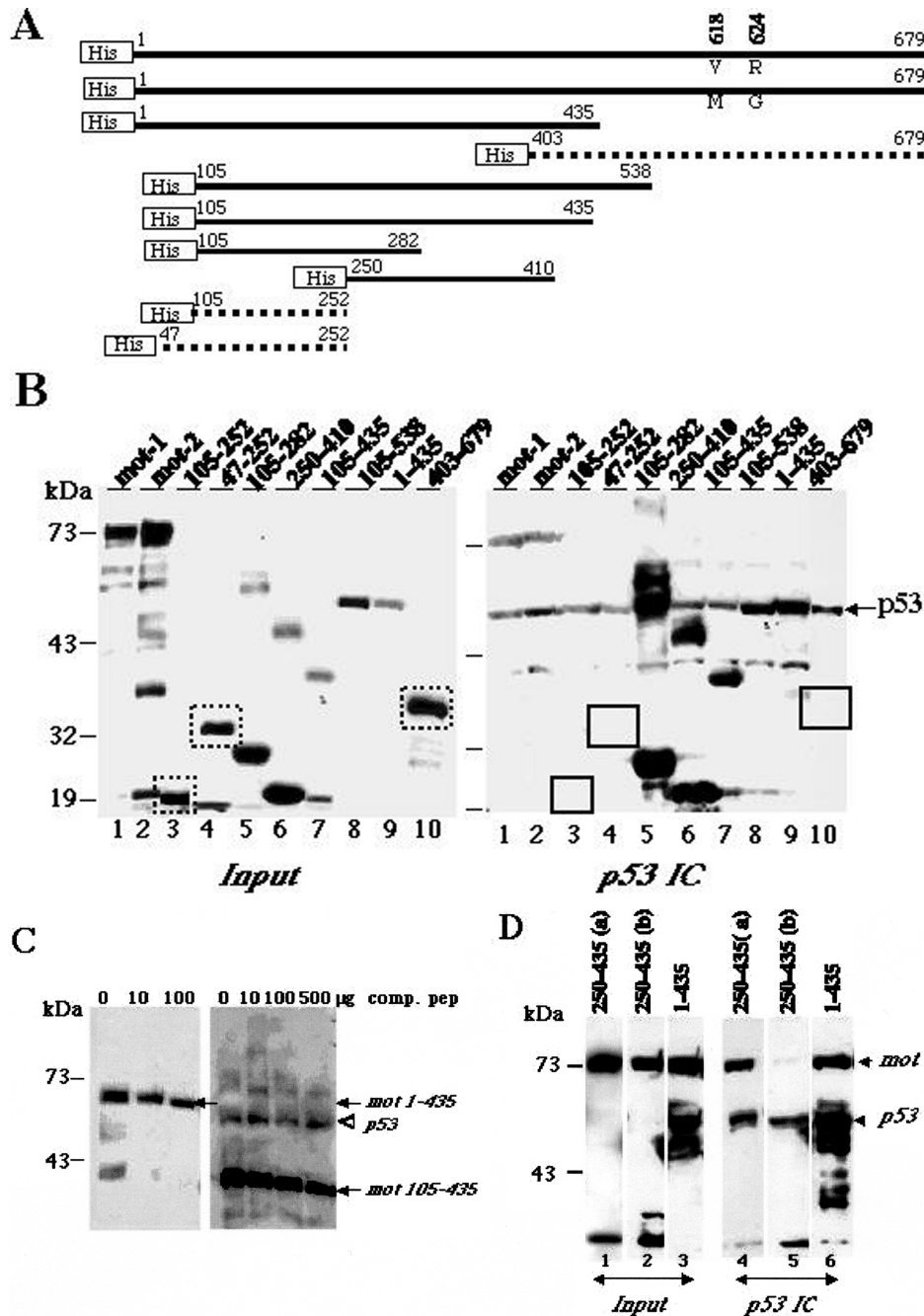
#### *p53* Interacting Domain of Mortalin

The above data led us to define *p53*-binding domain of *mot-2*. Various His-tagged deletion mutants of *mot* protein (Figure 2A) were expressed in bacteria and were purified as described in Materials and Methods. These were detected as expected size bands by Western blotting with anti-His tag antibody; some of the constructs also gave low- and high-molecular-weight bands (most likely the degraded and aggregated products, respectively). COS 7 cell lysate (400  $\mu\text{g}$ ) that provided a complex mixture of proteins with wild-type *p53* for pull-down assays, was mixed with 0.5–1  $\mu\text{g}$  of bacterially derived recombinant 6 X His-tagged full-length or variously deleted mortalins. *p53* immunocomplexes from the mixture were analyzed for coprecipitated recombinant mortalins by Western blotting with anti-His tag antibody (Clontech, Palo Alto, CA). As seen in Figure 2B, *mot* 403–679 a. a. residues of mortalin did not show any coimmunoprecipitation with *p53*. Mortalin fragments 105–538 a. a., 105–435 a. a., 105–282 a. a. and 250–410 a. a. showed binding to *p53* to various extents. Interestingly, two of the fragments spanning 47–252 a. a. and 105–252 a. a. did not show any binding. The extra bands of high molecular weight in lanes 5 and 6 (Figure 2B) most likely are the aggregated proteins that reacted to anti-His antibody and were coprecipitated with *p53*. Similarly, the unexpected low molecular weight bands in lanes 1, 2, 7, and 10 (Figure 2B) are degraded by-

products and did not show appreciable coprecipitation with *p53*. These data implied that the mortalin amino acid residues 253–282 are critically required for its binding to *p53* (Figure 2, A and B). These regions are identical in *mot-1* and *mot-2*, in agreement with the data that both proteins bind to *p53 in vitro* (Figure 1).

A peptide corresponding to mortalin amino acid residues 253–282 was next used to compete out the binding of recombinant His-tagged mortalin to *p53*. Increasing amounts of the competitor peptide were incubated with COS 7 lysates before the addition of His-tagged mortalin proteins, *mot* 1–435, 105–435 and 105–538 (not shown) that contained *p53*-binding region and did not show either the degradation or aggregation (based on data in Figure 2B). It resulted in decreased amounts of coimmunoprecipitated His-tagged *mots* (Figure 2C). The detection of immunoprecipitated *p53* along with *mot* 1–435 a. a. and *mot* 105–538 a. a. (not shown) was obscured because of the very similar sizes of *p53* and these mortalin deletion mutants. Western detection of *p53* on the same blot with 105–435 a. a. ruled out the possibility that the decreasing amount of immunoprecipitated *mot* protein was due to a decreased amount of immunoprecipitated *p53* (Figure 2C). Although the peptide corresponding to 253–282 a. a. residues was able to compete out binding of larger mortalin fragments, the large amount of peptide required suggested that its competition effect is weak. Such inefficiency of the peptide may be due to its binding-incompetent secondary or tertiary structures. Therefore, we next expressed V5-tagged mortalin deletion mutants expressing amino acid residues 250–435, 252–679 (not shown) and 1–435 in COS 7 cells. Their presence in *p53* immunocomplexes was analyzed by Western blotting with anti-V5 tag antibody (Figure 2D). Each of the mortalin deletion mutants was detected in the *p53* immunocomplexes (as a single band in the case of a. a. 250–435, and as multiple bands (degraded products) in the case of a. a. 1–435 indicating their binding to *p53*. Notably, only a negligible amounts of endogenous mortalin was coprecipitated with *p53* from lysates expressing mortalin residues 250–435 (Figure 2D, lane 5) demonstrating that this region can compete efficiently with full-length *mot* for its binding to *p53*. Furthermore, competition effect was seen only in lysates that expressed higher amount of the 250–435 protein (compare lanes 1 and 2 for expression of *mot* 250–435 and lanes 4 and 5 for *mot* in *p53* immunocomplexes, respectively). The other two constructs (252–679 and 1–435 a. a.) that also contained *p53*-binding residues as defined by *in vitro* pull-down assays did not compete out the binding of full-length mortalin for the reasons that remain undefined in this study. Nevertheless, these data defined mortalin residues 253–282 as essential for its binding to *p53* and residues 250–435 as an efficient competitor of the binding of full-length mortalin to *p53*. Because the residues 253–282 are identical in *mot-1* and *mot-2* proteins it implies that there are yet unknown *in vivo* factor(s) that prevent mouse *mot-1* from binding to *p53*.

Most interestingly, the *p53*-binding region of *mot* (residues 253–282) defined in this study overlapped with the



**Figure 2.** Mapping the p53 binding domain of the mot. (A) Schematic representation of His-tagged mortalin deletion mutants. The two amino acids (residues 618 and 624) that differ between mot-1 and mot-2 are indicated. The deletion mutants that did not bind to p53 are shown as dotted lines. (B) His-tagged mortalin deletion mutants were added to COS 7 cell lysates and were detected by Western blotting with anti-His tag antibody (input (10%), left panel). p53 immunocomplexes (p53 IC) were analyzed for coprecipitated mortalin by Western blotting with anti-His tag antibody (right panel). The bands corresponding to the deletion mutants that did not show binding are boxed in the input as well as p53 immunocomplexes panels. Immunoprecipitated p53 was detected by probing the same membrane with anti-p53 (monoclonal) antibody, indicated by an arrowhead. Extra bands of high molecular weight in lanes 5 and 6 most likely are the aggregated proteins that also coprecipitated with p53. Low-molecular-weight bands in lanes 1, 2, 7, and 10 are the degraded products and did not coprecipitate with p53. (C) Addition of increasing amounts of a peptide (comp. pep) corresponding to a. a. residues 253–282 of mortalin reduced the amount of mot 1–435 a. a. and 105–435 a. a. p53 was immunoprecipitated by a polyclonal anti-p53 antibody (CM-1, Novocastra). Coimmunoprecipitated recombinant mortalin was detected by Western blotting with anti-His tag antibody (indicated by arrows). Immunoprecipitated p53 along with mot 105–435 is shown by an arrow. (D) A high level of expression of mot 250–435 a. a. prevents binding of p53 to endogenous mortalin. COS 7 cells were transfected with expression constructs encoding V5-tagged mortalin deletion mutant proteins spanning residues 250–435 (a — 5  $\mu$ g DNA and b — 15  $\mu$ g DNA per 10-cm dish) and 1–435 (15  $\mu$ g DNA), and the lysates were immunoprecipitated with an anti-p53 antibody (CM-1, Novocastra). p53 immunocomplexes (lanes 4–6) and input protein (lanes 1–3, 10% of the amount of lysate used for immunoprecipitation) were Western blotted with anti-mortalin and anti-V5 tag antibodies to detect endogenous mot (indicated by arrow) and V5-tagged mot fragments, respectively. The p53 immunocomplexes (IC) were also Western blotted with a monoclonal anti-p53 antibody (Pab 421) (indicated by arrowhead) to detect immunoprecipitated p53. Immunoprecipitated p53 and mot 1–435 protein are overlapped in lane 6. Note that only negligible amount of endogenous mot was coprecipitated with p53 from cells that were transfected with a high amount of DNA (lanes 2 and 5).

region that is defined as MKT-077-binding region (residues 252–310) [29]. We have shown that MKT-077 binds to these residues of mot. Treatment of transformed cells with increasing concentrations of MKT-077 resulted in abrogation of mot-p53 complexes and concurrent nuclear translocation and transcriptional activation of p53 [29]. The present study has shown that p53 and MKT-077 bind to the same region of mot, and therefore MKT-077 could compete out mot-p53 interactions resulting in abrogation of the cytoplasmic retention of p53.

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