Merlin Neutralizes the Inhibitory Effect of Mdm2 on p53*

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The stability of p53 tumor suppressor is regulated by Mdm2 via the ubiquitination and proteasome-mediated proteolysis pathway. The c-Abl and PTEN tumor suppressors are known to stabilize p53 by blocking the Mdm2-mediated p53 degradation. This study investigated the correlation between p53 and merlin, a neurofibromatosis 2 (NF2)-related tumor suppressor, in association with the Mdm2 function. The results showed that merlin increased the p53 stability by inhibiting the Mdm2-mediated degradation of p53, which accompanied the increase in the p53-dependent transcriptional activity. The stabilization of p53 by merlin appeared to be accomplished through Mdm2 degradation, and the N-terminal region of merlin was responsible for this novel activity. This study also showed that overexpression of merlin-induced apoptosis of cells depending preferentially on p53 in response to the serum starvation or a chemotherapeutic agent. These results suggest that merlin could be a positive regulator of p53 in terms of tumor suppressor activity, and provide the promising therapeutic means for treating tumors with non-functional merlin or Mdm2 overexpression.

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§ The abbreviations used are: NF2, neurofibromatosis type 2; CNS, central nervous system; CHAPS, 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonic acid; moi, multiplicity of infection; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HEK, human embryonic kidney.

MATERIALS AND METHODS

Plasmids—The expression plasmids for the NF-κB p65 subunit (pcDNA-p65), merlin (pcDNA-NF2), (26) GAL4 DBD-fused p53 (pSG-p53) (27), and intact p53 (pcDNA-p53) (29) were described previously. The plasmids for the NF2 deletion mutants (M1, M2, M3, and M4) were also described previously (26). The p21 promoter-luciferase reporter plasmid (p21-Luc) is constructed by inserting p21 promoter region from plasmid (pFA2-Elk1), and the luciferase reporters with the GAL4 DNA binding sites (pFR-Luc) and the NF-κB response elements (pNF-κB-Luc) were pur-
chased from Stratagene. The human Mdm2 expression plasmid (pcDNA-Mdm2) was kindly provided from Dr. Carl Maki (Harvard University). The expression plasmid for the full-length Mdm2 was constructed by cloning the PCR fragment in pcDNA3.1 (Invitrogen, San Diego, CA) using the EcoRRI/SalI sites.

**Cell Culture, Transient Transfection, and Reporter Assay**—The NIH3T3 cells on tissue culture plates (U57MG, U373, and A172) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. Human lung carcinoma cell lines (H358 and H460) and human osteosarcoma cell lines (U2OS and SAOS-2) were maintained in RPMI 1640 containing 10% fetal bovine serum and antibiotics. The Gene Porter2 transfection reagent (Gene Therapy System, Inc.) was used to transfect the plasmids according to the manufacturer’s instruction. For the reporter assay, 3 × 10⁴ NIH3T3 cells were plated in a 35-mm plate. After 16 h, the cells were transfected with 0.2 µg of reporter plasmids and combinations of expression plasmids. Total amount of the transfectant DNA was kept same between samples using the pcDNA3.1 plasmid. The cells were harvested after 24 h, and the luciferase activity was measured. An internal control for the transfection efficiency could not be used because merlin influences the expression of most of the commercial promoters. Instead, the experiments were repeated three times and the results were statistically analyzed.

**Establishment of Merlin-expressing Stable Cell Lines (NIH3T3-NF2)**—The NIH3T3 cells were transfected with pcDNA-NF2. After 48 h, the cells were split at a 1:10 ratio and cultured in the presence of 400 µg/ml G418. After 21 days in the selective medium, the individual G418-resistant colonies were isolated, and merlin expression was confirmed by Western analysis using anti-merlin antibodies.

**Adenoviruses**—The adenovirus expressing merlin (Ad-NF2) was kindly provided by Dr. Jeun (Catholic University of Korea, Korea). The adenovirus expressing green fluorescent protein (Ad-GFP) was purchased from Neurogenex Inc. (Korea). The viruses were propagated in the human embryonic kidney 293 (HEK293) cell line, and the viral titers were determined by the limiting-dilution bioassay in HEK293 cells.

**Preparation of Meningiomas Samples**—Human meningioma biopsy samples (neoplastic regions of the meninges) were obtained from the department of neurosurgery, St. Mary’s hospital at the Catholic University of Korea. The tissues were suspended in 1 ml of a lysis buffer containing 35 mM Tris, 7 mM urea, 2 mM thiourea, 4% CHAPS, 65 mM dithiothreitol (1,4-dithioerythritol), and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 µg of each peptatin A, chymostatin, and leupeptin, and antipain). The suspension was homogenized for ~30 s and centrifuged at 13,000 rpm for 30 min. The supernatant was further centrifuged at 15,000 rpm for 60 min at 4°C to remove the undissolved materials. The protein content in the supernatant was measured using the Bradford assay. Thirty micrograms of each sample were used for the Western blotting analysis.

**Antibodies and Western Analysis**—The antibodies for merlin, p53, and Mdm2 (Santa Cruz Biotechnology, Inc.), β-actin (Sigma, Inc.), and p21 (Calbiochem) were obtained from commercial sources. The cells were rinsed with phosphate-buffered saline, and lysed for 30 min on ice in a radiolabeled precipitation buffer-B buffer (0.5% Nonidet P40, 20 mM Tris, pH 8.0, 50 mM NaCl, 50 mM NaF, 100 µM Na3VO4, 1 mM dithiothreitol, 50 µg/ml phenylmethylsulfonfluoride). The insoluble material was removed by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was then subjected to the SDS-PAGE and Western blotting analysis. After transferring the proteins, the membranes were blocked with 5% skim milk in phosphate-buffered saline containing 0.05% Tween 20. This was followed by the sequential incubation with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies. The signals were detected using ECL.

**Analysis of DNA Fragmentation**—The cells (1 × 10⁶) were plated in 100 mm dishes. After 24 h, the stable NIH3T3-NF2 or NIH3T3-pcDNA3.1 cells were directly starved with serum in a medium containing 10% fetal bovine serum and antibiotics. The human lung carcinoma (H358 and H460) and human osteosarcoma (U2OS and SAOS-2) cell lines were first infected with either Ad-GFP or Ad-NF2 at a dose of 100 moi for 2 h, after which the virus was washed off and replaced with the fresh medium. After 24 h, these cells were also starved with serum as above.

**Expression of Merlin and p53 in Human Brain Tumors**—It was recently reported that the cooperation between the NF2 and p53 mutations are important in tumor development in mice (9). The c-Ab or PTEN tumor suppressor was known to elevate both the steady state level and the transcriptional activity of p53 (22, 23). Therefore, it was hypothesized that the function of merlin and p53 are also directly related, as in the case of c-Ab and PTEN. In this study, the levels of p53 and merlin were first examined in three glioma cell lines (A172, U373, and U87MG) and the tissues from 13 meningiomas. As shown in Fig. 1A, both p53 and merlin were highly expressed in the U373 cells only, and neither was detected in the A172 or U87MG cells. In the case of the meningiomas tissues, the expression levels of both merlin and p53 were examined in the 13 frozen samples. As shown in Fig. 1B, the meningiomas tissues that strongly expressed merlin also showed a high p53 expression level (lanes 6–9 and 11–13). Overall, these results suggest that the levels of p53, and merlin might be related to each other in many glioma cell lines and brain tumor samples.

**Merlin Stabilizes p53 Protein and Activates Its Transcriptional Activity**—To assess the direct correlation between the p53 and merlin levels, this study first focused on the effect of merlin on the p53 levels as suggested from the cases of c-Ab.
Merlin Down-regulates Mdm2

Merlin Inhibits the Mdm2-mediated Degradation of p53—

The stabilization of p53 by merlin raises an intriguing possibility if merlin rescues p53 from Mdm2-mediated degradation. This was assessed by examining the effect of merlin on the p53 degradation caused by the ectopic expression of Mdm2 in NIH3T3 cells. As expected, the level of overexpressed GAL4-DBD-p53 was reduced considerably by Mdm2, which was recovered by the co-expression of merlin in a dose-dependent manner (Fig. 3A). Taken together, these results suggest that merlin activates p53 via enhancing its stability.

Merlin Stabilizes p53 via Down-regulating Mdm2 Levels—

From the finding that merlin neutralizes Mdm2-promoted p53 degradation, we examined if the merlin effect on p53 stability is Mdm2-dependent. A plasmid containing an antisense sequence of human Mdm2 cDNA (pcDNA-As-Mdm2) was constructed to neutralize the Mdm2 expression in human lung carcinoma H460 cells with wild-type p53. The cells were transfected with the pcDNA-As-Mdm2 and the expression plasmids for merlin and p53, and the levels of p53, Mdm2, and merlin were examined by Western blotting (Fig. 5A). As expected, As-Mdm2 alone clearly reduced the endogenous Mdm2 level and induced the level of co-expressed p53. Interestingly, the expression of merlin alone also resulted in the significant reduction of the endogenous Mdm2 level even without As-Mdm2, while it induced the p53 level. Co-expression of merlin and As-Mdm2 showed no additive effect on the p53 stability, when compared with the effects of each. This result suggests that the effect of merlin on p53 stability is Mdm2-dependent.

The merlin effect was further examined on the overexpressed Mdm2 under the CMV promoter, to assess if it occurred at the transcriptional level or at the protein level. As shown in Fig. 5B, co-expression of merlin significantly reduced the level of overexpressed Mdm2 in NIH3T3 cells. This result suggests that merlin induces the destabilization of Mdm2, because merlin did not affect the CMV promoter-derived gene expression (data not shown).

Merlin and p53 on Cell Growth and Apoptosis—

Based on the above results, it was expected that merlin might induce the growth arrest and/or apoptosis of cells preferentially in cooperation with p53. Stable cell lines expressing merlin (NIH3T3-p53

and PTEN. NIH3T3 cells stably expressing merlin (NIH3T3-NF2-1) or the control cell line (NIH3T3-pcDNA3.1) were constructed. NIH3T3 cells were used because the tumor suppressor activity of merlin is well known in this cell line. The levels of p53 and p21/Waf1/Cip1, a transcriptional target of p53, were measured using Western blotting. As shown in Fig. 7A, merlin overexpression resulted in an increase in the endogenous levels of p53 and p21. This merlin effect on p53 and p21 levels was confirmed using a transient expression of p53 and merlin under the CMV promoter. Consistent with the result using the stable cell lines, overexpression of merlin resulted in an increase in the cellular levels of co-expressed p53 and endogenous p21 in a dose-dependent manner (Fig. 2B). No effect of merlin was shown on the CMV promoter (data not shown). These results suggest that merlin could induce the accumulation of transcriptionally active p53.

The effect of merlin on the p53 activity was further assessed by the reporter assays performed in the co-expression system using p21 promoter (p21-Luc). The p21 promoter activity was increased considerably by the co-expression of p53 and merlin depending on the amount of merlin protein (Fig. 2C, left). Under similar conditions, the activity of the NF-κB p65 subunit was reduced by merlin (Fig. 2C, right) as reported previously (26).

To exclude the possibility that merlin activates p53 directly by enhancing its DNA binding activity, for instance, the GAL4 DNA binding domain (DBD)-fused p53 (GAL4-DBD-p53) was constructed and tested for the merlin effect in NIH3T3 cells. As shown in Fig. 3B, merlin induced transcriptional activity of GAL4-DBD-p53 (left), while it reduced the activity of GAL4-DBD-Elk1 (pFA2-Elk1) (right) on the GAL4 DBD responsive reporter (pFR-Luc) in a dose-dependent manner. The GAL4-DBD-p53 protein level was also increased along with the increase in the amount of merlin (Fig. 3A). Taken together, these results suggest that merlin activates p53 via enhancing its stability.

Merlin Inhibits the Mdm2-mediated Degradation of p53—

The stabilization of p53 by merlin raises an intriguing possibility if merlin rescues p53 from Mdm2-mediated degradation. This was assessed by examining the effect of merlin on the p53 degradation caused by the ectopic expression of Mdm2 in NIH3T3 cells. As expected, the level of overexpressed GAL4-DBD-p53 was reduced considerably by Mdm2, which was recovered by the co-expression of merlin in a dose-dependent manner (Fig. 3A). The functional assessment of this phenomenon in the GAL4-DBD-p53-dependent reporter system also showed that merlin relieves the inhibitory effect of Mdm2 on the p53 transcriptional activity in a dose-dependent manner (Fig. 3B).

Merlin Stabilizes p53 via Down-regulating Mdm2 Levels—

From the finding that merlin neutralizes Mdm2-promoted p53 degradation, we examined if the merlin effect on p53 stability is Mdm2-dependent. A plasmid containing an antisense sequence of human Mdm2 cDNA (pcDNA-As-Mdm2) was constructed to neutralize the Mdm2 expression in human lung carcinoma H460 cells with wild-type p53. The cells were transfected with the pcDNA-As-Mdm2 and the expression plasmids for merlin and p53, and the levels of p53, Mdm2, and merlin were examined by Western blotting (Fig. 5A). As expected, As-Mdm2 alone clearly reduced the endogenous Mdm2 level and induced the level of co-expressed p53. Interestingly, the expression of merlin alone also resulted in the significant reduction of the endogenous Mdm2 level even without As-Mdm2, while it induced the p53 level. Co-expression of merlin and As-Mdm2 showed no additive effect on the p53 stability, when compared with the effects of each. This result suggests that the effect of merlin on p53 stability is Mdm2-dependent.

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Merlin and p53 on Cell Growth and Apoptosis—

Based on the above results, it was expected that merlin might induce the growth arrest and/or apoptosis of cells preferentially in cooperation with p53. Stable cell lines expressing merlin (NIH3T3-
NF2-1 and NIH3T3-NF2-2) or a control cell line (NIH3T3-pcDNA3.1) were starved of serum for 72 h, and the level of cell apoptosis was examined by a DNA fragmentation assay. The control cell line showed no sign of apoptosis, but the typical DNA ladders observed in apoptosis were detected in the two merlin-expressing cells (Fig. 7A). In order to clearly show whether this merlin-induced apoptosis is dependent on p53 protein, the DNA fragmentation assays were performed in p53-positive and p53-negative cells using merlin-expressing adenovirus (Ad-NF2). The human lung carcinoma cells, H460 (p53-positive) and H358 (p53-negative), infected with Ad-NF2 or Ad-GFP were starved of serum for 72 h, and the level of cell apoptosis was examined by a DNA fragmentation assay. No sign of apoptosis was observed in H358 cells without active p53 after infection with Ad-GFP or Ad-NF2 (Fig. 7B, lines 3 and 4). However, H460 cells infected with Ad-NF2 showed the typical DNA ladders observed in apoptosis, while the Ad-GFP infection showed just a minor effect (Fig. 7B, lines 1 and 2). Similar results were observed in the human osteosarcoma cells with (U2OS) or without (SAOS-2) p53 (data not shown). Overall, these results suggest that merlin could induce apoptosis preferentially in cells with wild-type p53.

To assess the effect of merlin on cell growth after DNA damage, the stable cell lines expressing merlin or the Ad-NF2-
infected human lung carcinoma cells were treated with a DNA-damaging agent, doxorubicin, for 48 h and the viability of the cells were determined using an MTT assay. As shown in Fig. 7C, stable expression of merlin aggravated the adverse effect of doxorubicin on the growth of NIH3T3 cells. The viability of human lung carcinoma cells with wild-type p53 (H460 cells) was more sensitive than that of the cells without p53 (H358 cells) in response to the increasing amount of doxorubicin (Fig. 7D). Infection of Ad-NF2 further reduced the viability of H460 cells by a greater extent than that of H358 cells. We also observed similar results with the osteosarcoma cell lines, U2OS (p53-positive cell line) and SAOS-2 (p53-negative cell line) (data not shown). These results suggest that merlin induces apoptosis in response to exogenous stresses, at least in part, through p53 activation.

**DISCUSSION**

Previously, Nf2 and p53 double mutant mice showed a higher level of tumor induction than each of the single mutant mice (9). In addition, two tumor suppressors, c-Abl and PTEN increased the stability and activity of p53 leading to an increase in the apoptosis rate. This indicates that cooperation between p53 and other tumor suppressors (C-Abl, PTEN, and merlin) would be important for the inhibition of tumor development.

In this study, it was first found that there was a strong correlation between the p53 and merlin levels in many brain tumor samples (Fig. 1). Therefore, this study further investigated the functional cooperation between p53 and merlin. The results showed that merlin induced the accumulation of p53 by destabilizing Mdm2, a key regulator of p53 degradation, which increased the p53 transcriptional activity (Figs. 2–5). The N-terminal region of merlin (1–130) was responsible for this novel activity (Fig. 6), which also supports the specificity of merlin effect on p53 and Mdm2. The activation of p53 by merlin appears to further potentiate apoptosis of cells in response to exogenous stresses (Fig. 7).

The p53 activation by merlin is likely to depend mainly on the p53 stabilization at the protein level in our results. Although it may not be completely excluded, merlin does not appear to directly activate the p53 promoter at the transcriptional level or induce p53 transcriptional activity based on the results using the GAL4-DBD fused p53 under the CMV promoter.

The mechanism of merlin-induced Mdm2 degradation is not yet clear. However, the transcriptional repression of Mdm2 expression by merlin could be also excluded in this overexpression system. The fact that merlin activates p53, a key activator of Mdm2 expression, also limits this possibility. Therefore, merlin may directly regulate the stability of Mdm2 or restrict it in the cytoplasm for degradation as the case of PTEN (23). In the case of c-Abl, it is known to stabilize and activate p53 without Mdm2 degradation (22). The direct phosphorylation of Mdm2 by c-Abl at Tyr394 was reported to inhibit the ubiquitination and nuclear export of p53 by Mdm2 (24, 25).

Mdm2 overexpression has been observed in 5–10% of all human tumors by gene amplification, increased transcription or enhanced translation. Moreover, Mdm2 was reported to immortalize and transform both primary and cultured cells (32, 33), and induce tumor development in transgenic mice (14). Therefore, neutralizing the inhibitory effect of Mdm2 might be
an efficient and common defense mechanism of cells against the development of tumors. In addition to the cases of merlin, c-Abl and PTEN shown here, the p19<sup>Arf</sup> tumor suppressor and adenoviral oncoprotein, E1A, were also reported to stabilize and activate p53 by inhibiting Mdm2 activity (34–36). The p19<sup>Arf</sup> binds and sequesters Mdm2 in the nucleolus (39). E1A binds p300 thereby inhibiting both Mdm2 expression (35) and the interaction between Mdm2 and p300, which is required for p53 degradation (36). It is yet to be determined if merlin employs p19<sup>Arf</sup>, p300 or some other means to neutralize the Mdm2 activity. However, p19<sup>Arf</sup> is unlikely to be the case for merlin, because p19<sup>Arf</sup> locus is mutated in the NIH3T3 cell lines.

It has recently been reported that merlin overexpression induced apoptosis in the schwannoma cells, but its mechanism was not suggested (37). This study suggests that the activation of p53 by merlin could be one of the mechanisms by which merlin induces apoptosis. However, the experimental settings in this report may have a confusing point for this conclusion. Although the glioma cell lines, the frozen meningioma samples and the stable NIH3T3 cell lines having high level of merlin and p53 are thought not to proliferate well, but they still did in our experiment. The reason for this is not clear at this time. However, our result on the functional cooperation between p53 and merlin would explain the cellular response to the tumorigenic signals, and may not be exactly mimic the physiology of established cell lines. It is likely that those cell lines have somehow adapted for survival probably via inactivating or increasing the threshold for the growth arrest or apoptotic signals. For example, the glioma cell lines are all negative for PTEN tumor suppressor, and the meningiomas may have inactive p53 like the U373 glioma cell line (28). In the case of stable NIH3T3-NF2 cells, its growth rate was somewhat slower than that of NIH3T3-pcDNA3.1 control cells, but it was not significant. Besides, no apoptosis was observed in stable NIH3T3-NF2 cells or by the transient expression of merlin in NIH3T3 cells under the normal culture condition. This result is in the same line with that of Schulze et al. (37) showing that transduction of merlin alone in primary schwannoma cells for 7–8 day induced only a moderate level of apoptosis. Therefore, we think that high level expression of merlin or the secondary
insults such as serum deprivation and chemotoxic treatments might be required for the merlin-mediated and p53-dependent apoptosis of cells.

If merlin can efficiently induce p53-mediated apoptosis, the activation or supply of merlin as a therapy may accelerate the apoptotic cell death of tumors with non-functional merlin or Mdm2 overexpression. In the case of PTEN, its overexpression protected p53 from Mdm2-mediated degradation and sensitized the U87MG glioma cell lines to chemotherapy (23). In conclusion, these results suggest a new implication for the interplay between the merlin and p53 tumor suppressors in tumor development. The molecular mechanism underlying their interplay remains to be determined.

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