Negative Regulation of p53 by the Long Isoform of ErbB3 Binding Protein Ebp1 in Brain Tumors

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Abstract

The ErbB3 binding protein Ebp1 has been implicated in a number of human cancers. Ebp1 includes 2 isoforms, p48 and p42, that exhibit different cellular activities. Here we show that the larger p48 isoform is transforming and that it promotes cell growth, clonogenicity, and invasion in human glioblastoma (GBM). P48 overexpression in GBM cells facilitated tumorigenesis and enhanced tumor growth in mouse xenograft models. Human GBM tissues displayed elevated levels of p48 compared with surrounding normal tissues or low-grade tumors. Notably, p48 levels were inversely correlated with poor prognosis in GBM patients. We determined that p48 binds to the p53 E3 ligase HDM2, enhancing HDM2-p53 association and thereby promoting p53 polyubiquitination and degradation to reduce steady-state p53 levels and activity. Together, our findings suggest that p48 functions as an oncogene by promoting glioma tumorigenicity via interactions with HDM2 that contribute to p53 down-regulation. Cancer Res; 70(23); 9730–41. ©2010 AACR.

Introduction

Ebp1 is the human homologue of the mouse protein p38-2AG4 (1). The p38-2AG4, P42AG4, possesses 3 in-frame ATG codons and encodes 2 alternative spliced isoforms, p48 and p42, which differentially mediate PC12 cell survival and differentiation (2, 3). Recently, we showed that p48 Ebp1 prevents apoptotic cell death by interacting with nuclear Akt/PKB (protein kinase B) (2), (2), fitting with the observation that Ebp1 binds Bcl2 mRNA and contributes to Bcl2 overexpression (4). During the early stages of organ development in plant, stEBP1 promotes cell proliferation, affects the cell size threshold, and shortens the period of meristematic activity. In mitotic cells, Ebp1 enhances cell expansion (5). Furthermore, tamoxifen treatment of MCF-7 breast cancer cells dramatically decreases p48 Ebp1 transcription and, thus, protein levels. Breast cancer patients that express high level of P42AG4 have poor clinical outcomes, suggesting Ebp1 may promote aggressive tumor behavior (6).

Treatment with heregulin induces the nuclear translocation of p42 Ebp1 from the cytoplasm in AU565 breast cancer cells (7), correlating with our observation that p42 Ebp1 predominantly localizes to the cytoplasm and translocates to the nucleus upon growth factor stimulation (3). Ebp1 represses transcription of both E2F1 (8) and androgen receptor-mediated genes (9, 10). Collectively, these observations suggest that p42 Ebp1 acts as a potential tumor suppressor in various cancer cells, whereas p48 Ebp1 may function as an oncogene, promoting cell survival and proliferation.

Protein levels of tumor suppressor, p53, largely controlled by MDM2, a ubiquitin E3 ligase (11, 12), are the most important determinants of p53 function. In unstressed conditions, MDM2 promotes the p53 polyubiquitination, leading to proteosome-mediated degradation. Also, Akt-mediated phosphorylation of MDM2 regulates its nuclear translocation, thus stabilizing MDM2 and enhancing MDM2-dependent p53 degradation (13–15). Although the current regulatory model of MDM2 and p53 consists of an autoregulatory feedback loop, and MDM2-mediated p53 degradation depends on high cellular levels of MDM2 (11, 12, 16–18), the mechanisms regulating MDM2-p53 association are not well understood. It has been suggested that a cofactor may be required for MDM2-mediated p53 degradation, because low levels of MDM2 fail to facilitate polyubiquitination but rather facilitate monoubiquitination and nuclear export of p53 (17). In addition to interacting with and regulating p53, MDM2 binds to Rb, leading to Rb degradation and activation of E2F1 (19). MDM2 interacts with E2F1, enhancing its stability (20).

Although the 2 mRNA transcript expressions are comparable, in many mammalian cells, p48 is the predominant form...
and p42 Ebp1 is selectively degraded through ubiquitination and is barely detectable in malignant gliomas (3, 21). Despite expanding interest in the possible differential role of Ebp1 isoforms in tumorigenesis, little is known about the oncogenic potential of p48 Ebp1 in human cancers. Here, we show that p48 Ebp1 is highly expressed in various glioblastoma (GBM) cell lines and human malignant gliomas, and that it promotes cancer growth, migration, and cell survival. Using a mouse xenograft model, we demonstrated that p48 can induce malignant transformation and promote carcinogenesis. Glioma patients expressing high level of p48 have poor clinical outcomes, strongly suggesting a clinical relevance for p48. Moreover, overexpression of p48 leads to degradation of p53 by interacting with HDM2 protein (human homologue of MDM2), facilitating HDM2–p53 interaction.

Materials and Methods

Cell cultures

U87, T98G, and U138 cells (purchased from the American Type Culture Collection, during 2003 to 2007), and H1299 cells (provided by Dr. Jaewhan Song, Sungkyunkwan University, Korea) were maintained in medium A (Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 100 units of penicillin/streptomycin) at 37°C with 5% CO2. LNT-229 p53+/– and LNT-229 p53−/− cells (22) were a generous gift from Dr. Ulrike Naumann (Hertie-Institute for Clinical Brain Research, Tübingen, Germany). Cells were cultures in medium A with puromycin 2 μg/ml. All cell lines were authenticated by cell morphology monitoring, growth curve analysis, and mycoplasma detection using Mycoplasma detection kit (Roche) according to the ATCC cell line verification test recommendations in April 2010.

Proliferation, invasion, and colony formation assay

Cells were plated (5 × 10⁴ cells per 60-mm dish), and viable cell numbers were counted as indicated time. Invasion assays were performed as previously described (23) using Matrigel invasion assay Kit (BD bioscience, Inc.). For colony forming assay, cells were seed on 35-mm dishes in 0.35% bacto-agar (supplemented with complete medium) (Becton, Dickinson and Co., Franklin Lakes) and cultured for 4 weeks. The cells were fixed and stained with crystal violet, and then counted in random 5 fields. Each value from these indicated assays represents the mean ± SEM of triplicate measurements from 3 independent experiments.

Synthetic siRNA oligonucleotides

Si-Ebp1 (5′-UGAUAUAGUGUUCCUGCUUGU-3′), N-si-p48 (5′-CACGCAUGUAGUUGCUUGCUUGCUUGU-3′) and Si-HDM2 (5′-AUCACGAGAAUCAUGGAG-3′) were synthesized (Integrated DNA Technologies, Coralville, IA).

Animal experiments

All procedures were approved by the Sungkyunkwan University School of Medicine Institutional Animal Care and Use Committee. Briefly, 6- to 8-week-old nude mice were subjected for intracranial or subcutaneous injection with U87 or U138/p48, p42, mock cell. More detailed procedures are described in Supplementary Information.

Immunohistochemistry of tissue microarray

Microarray of 62 patients with GBMs tissue (24) was used for an immunohistochemistry with antibodies against Ebp1 (Upstate) and N-p48 (Abfrontier Co. Ltd., Seoul, Korea) as described previously (24). More detailed procedures are described in Supplementary Information.

Statistical analysis

Experimental data were analyzed for statistical significance by Mann–Whitney U test using SPSS software. The analysis of the relation between N-Ebp1 expression and overall survival was estimated by using the Kaplan–Meier method (version 11.0; SPSS Inc., Chicago, IL). The data were expressed as the means ± SEM. All P values less than 0.05 were considered significant.

Results

P48 Ebp1 stimulates cell growth, colony formation, and invasion in human glioma cells

Previous findings show that p48 Ebp1 is highly expressed in human cancer cells and enhances cell survival. Hence, we hypothesized that p48 Ebp1 may be oncogenic and that alteration of its expression and activation may contribute to cancer. To test this hypothesis, we established stably transfected GBM cell lines employing U87MG cells that express highest levels of endogenous Ebp1, and U138MG cells that express lowest levels of Ebp1 among tested glioma cell lines (Supplementary Fig. S1A and B), and this line is the only one that is not described by the ATCC as being tumorigenic in nude mice, we hypothesized that p48 Ebp1 might provoke cancer cell proliferation. In vitro invasion assay showed that p48 elicited a robust increase in invasion when compared with vector alone or p42 (Fig. 1B, left). Importantly, U138/p48 cells showed a greater than 9-fold increase in invasion compared with controls. Furthermore, in U87 cells, which express high levels of endogenous Ebp1 than U138 cells, exogenous p48 did not strongly stimulate invasion, suggesting that p48 overexpression is sufficient to induce tumor cell invasion. The relative proportion of endogenous Ebp1 to transfected GFP-p48 or p42 is shown in Figure 1B (right), supporting that overexpression of p48, promotes glioma cell growth and invasion. Moreover, overexpression of p48 promoted anchorage-independent growth in the soft agar, with larger and much more colonies formed than cells expressing the vector alone or p42 (Fig. 1C).

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To further define the role of p48 in oncogenic transformation, we employed NIH3T3 cells stably expressing GFP-p48, p42, and vector constructs (Supplementary Fig. S2C). The growth rate and saturation density analysis showed that p48 strongly stimulated NIH3T3 cells proliferation and reached a much higher saturation density than cells expressing p42 or control (Fig. 1D, top). Moreover, NIH3T3/p48 cells showed significantly enhanced colony formation and higher invasion in vitro (Fig. 1D, middle and bottom; Supplementary Fig. S2D), and tumor formation in nude mice compared with NIH3T3/p42 cells or control (Supplementary Fig. S2E). Therefore, our data indicate that stable expression of p48 induces cancerous growth, colony formation, and invasion of NIH3T3 cells.

**Figure 1.** p48 Ebp1 stimulates cell growth, clonogenicity, and invasion in human glioma cells. A, glioma cells expressing the indicated constructs were plated (5 × 10^4 cells per 60-mm dish) and the viable cells were counted at the indicated times. B, invasive cells were counted at a magnification of ×100 (left). The relative proportion of endogenous Ebp1 to transfected GFP-p48 or p42 stable cells was quantified (right). C, representative digital microscopic images of colony forming cells. Scale bar, 500 μm (top), 100 μm (middle). The colonies were counted at a magnification of ×40 (bottom). D, NIH3T3 cells stably expressing the indicated constructs were plated (1 × 10^5 cells per 60-mm dish) and cells were counted every 2 days (top). Colony formation assay (middle) and invasion assay (bottom). *, P < 0.05 versus vector control.

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**P48 Ebp1 silencing alters cell growth, colony formation, and invasion in human glioma cells**

To determine the physiological effect of p48 in tumor cells, we depleted p48 by N-terminal–specific p48-si-RNA (N-si-p48) or N-terminal–specific p48-sh-RNA (N-sh-p48) from glioma...
Figure 2. P48 Ebp1 silencing alters cell growth, colony formation, and invasion in human glioma cells. A, U87 cells were transfected as indicated. Apoptotic cells were detected by flow cytometry after staining for Annexin V (left). An MTT assay with cells treated with N-si-p48 as indicated. Colorimetric assay (middle top) and immunoblotting (middle bottom) were as shown. Colony formation (right top) and invasion assay (right bottom) were performed using scrambled RNA (SCR) or N-si-p48 (40 nmol/L) transfected U87 and U138 cells. A representative photomicrograph is provided in Supplementary Figure S3C and D.* P < 0.01 versus SCR control. B, U87 and U138 cells were transfected with SCR or si-Ebp1. After 18 hours, GFP-p48, p42, or vector control was transfected into Ebp1 knockdown cells and the viable cells were counted (left top) at the indicated times; or cells (5 x 10^3) were used in the colony formation assay (left bottom). Protein expression was evaluated by immunoblotting (right). C, U87 cells were transfected with SCR or N-si-p48 (80 nmol/L) and subsequently transfected with GFP-p42 or control vector. Cell proliferation assay, colony formation, and invasion analysis were performed. Cell lysates were subjected to immunoblotting. D, U87 cells were transfected with SCR or N-si-p48 before being treated with staurosoporine (STS; 100 nmol/L), etoposide (ETO; 100 μmol/L), or actinomycin D (ACT; 10 μg/mL) for 8 hours. Cells were stained with DAPI (100) and the condensed cells were counted (top). Image of nuclei condensation are provided in Supplementary Figure S3E. Genomic DNA fragmentation was shown (middle). Transfected cells were then exposed to 100 nmol/L of STS for 8 hours. The condensed nuclei were counted (bottom). CON, control.* P < 0.01 versus SCR control.
cells. Both methods yielded 70% to 80% decreases in Ebp1 levels, similar to sequence-specific p48 and p42 isoforms, and p42 expression was not affected by N-si-p48 (Supplementary Fig. S3A and B). Knockdown of p48 by N-si-p48 resulted in an 8-fold increase in apoptotic cells and a reduction in proliferating cells, which was supported by decreased proliferating cell nuclear antigen (PCNA) expression (Fig. 2A, left and middle). Moreover, p48 depletion inhibited anchorage-independent growth and invasion (Fig. 2A, right; Supplementary Fig. S3C and D). N-sh-p48 or si-Ebp1 produced the results similar to those of N-si-p48 (Supplementary Fig. S4A–C), implying that most of the Ebp1 in tumor cells may be p48.

To ascertain the contribution of p48 to the oncogenic properties of glioma cells, we removed endogenous Ebp1 by si-Ebp1 and then reintroduced GFP-p48 or -p42. Stepwise expression of p48 after depletion of endogenous Ebp1 increased cell proliferation, resulting in higher PCNA expression, and elevated the rate of colony formation whereas exogenous p42 expression did not increase cell proliferation and colony formation (Fig. 2B; Supplementary Fig. S4D). Moreover, when p48 was specifically depleted, overexpression of p42 did not enhance cell proliferation, soft agar growth, or invasion (Fig. 2C). Knockdown of p48 decreased resistance to drug-induced apoptosis, revealing increased condensed chromatin (Fig. 2D, top; Supplementary Fig. S3E) and DNA fragmentation (Fig. 2D, middle). Similar sensitivity to apoptosis was observed by si-Ebp1 (Supplementary Fig. S4E). Conversely, apoptosis resistance was notably increased in p48-expressing cells (Fig. 2D, bottom).

**P48 Ebp1 drives tumor formation in a mouse xenograft model**

In vivo function of p48 in GBM carcinogenesis was examined using glioma animal models. The mean tumor volume of mouse brain injected with U87/p48 cells was significantly larger (27.35 ± 6.11 mm³ (71% increase, P = 0.043)) than that in mice injected with vector controls (15.98 ± 2.84 mm³). As predicted, the average tumor volume for U87/p42 or U87/mock vector (top left). Tumor volumes were measured every 5 days (top right). U138 S.C tumor volumes were compared at 28 days (bottom). H&E, hematoxylin and eosin.
smaller (5.71 ± 1.54 mm³; 65% decrease, \( P = 0.005 \)) than that of control group (Fig. 3A, top). Representative brain tumors from nude mice are displayed in Fig. 3A (bottom). PCNA was more frequently expressed in mice injected with U87/p48 cells than control or p42-injected mice (Fig. 3B).

Importantly, tumor growth was detected at 28 days post-injection with U138/p48 cells only (Fig. 3C). No xenografts were formed in mice injected with U138/control or U138/p42 cells even at 60 days postinjection (data not shown). This phenomenon was confirmed by subcutaneous injection mice (each group \( n = 5 \)) (Fig. 3D, left). Mice injected with U138/p48 cells developed tumors 7 days postinjection and an increase in tumor growth was observed during later experimental periods (Fig. 3D, right). The average tumor volume measured 28 days postinjection was 68.75 ± 18.8 mm³ (Fig. 3D, bottom). Thus, p48 could induce the malignant conversion of the nontumorigenic U138 cells in mouse xenograft models.

**P48 expression is differentially regulated in tumor tissues and tumor grades**

This is done to determine whether \textit{in vivo} effects of p48 on tumorigenesis are relevant to patients with GBM. Notably, immunoblotting using different antibodies, namely anti-N-p48 (specific for p48) and anti-Ebp1 (detects both p48 and p42), revealed significant elevation of p48 protein in tumor tissues compared with adjacent neural tissue from the same patients (Fig. 4A, left), although p42 was slightly visible in adjacent brain tissues with the anti-Ebp1 antibody. In contrast, p48 mRNA levels in isolated tumor regions were comparable with those of normal tissue (Fig. 4A, right). These observations are consistent with the previous report demonstrating that p48 is the major isoform of Ebp1, and that p42 is undetectable in malignant human glioma tissues (21).

To determine the clinical relevance of p48 expression in human GBM, we conducted immunohistochemistry using the anti-N-p48 antibody and a tissue microarray containing 62 GBM surgical samples. According to the ratio of p48-positive cells, GBM patients were grouped into groups 1 (p48-positive cells < 25%, \( n = 27 \)), 2 (25% < p48-positive cells < 50%, \( n = 11 \)), 3 (50% < p48-positive GBM cells < 75%, \( n = 15 \)), and 4 (p48-positive GBM cells > 75%, \( n = 9 \)) (Fig. 4B). Consistent with the previous results, p48 expression (p48-positive cells > 25%) was detected in 35 of 62 (56.4%) samples. Specific expression of p48 in GBMs confirmed the specific tumor association of p48 (Fig. 4C; Supplementary Fig. S5A). Clinical implications of p48 expression were further investigated using the cohort of 62 GBM patients; overall survival and progression-free survival of p48-expressing patients were compared with patients who did not express p48.
between the patients who reveal less than 50% p48-positive cells (group 1 and 2, n=38) and more than 50% p48-positive cells (group 3 and 4, n=24). Importantly, the expression levels of p48 inversely correlated with the overall survival (Fig. 4D, left, P=0.021) and progression-free survival (Fig. 4D, right, P=0.022). The median survival lengths were 293 days (95% CI, 159–426 days) in p48-high patients (>50% p48-positive) and 512 days (95% CI, 300–724 days) in p48-low patients (<50% p48-positive), whereas the median times to recurrence were 144 days (95% CI, 68–220 days) in p48-high patients and 205 days (95% CI, 165–245 days) in p48-low patients. However, total Ebp1 expression levels versus p48 expression did not demonstrate the specific correlation (Supplementary Fig. 5B) in human patients tissues and the expression levels of Ebp1 were not associated with the overall survival and progression-free survival (Supplementary Fig. S5C). These data suggest that p48 expression contributes to tumor progression and poor prognosis.

**P48 Ebp1 controls p53 protein stability via ubiquitination-dependent proteasomal degradation**

Recent studies have shown that a defect in p53 function is an important cause of primary gliomas (25, 26). To understand the mechanisms underlying p48-mediated tumorigenicity of human glioma cells, we first considered whether p48 regulates the level of p53. Overexpression of p48 reduced p53 levels and its transactivational activity (measured by p21Waf1 protein) in U87 and but not in a p53-null H1299 (Fig. 5A, left). Knockdown of p48 increased the levels of endogenous p53 and p21Waf1 (Fig. 5A, right; Supplementary Fig. S6A). These effects were confirmed in LNT-229-p53-WT cells and LNT-229-p53-KD cells (Fig. 5B) (Supplementary Fig. S6B). These data suggest that the reduction in p53 levels may be regulated by the expression levels of p48.

We next evaluated p53 transcriptional ability using a p53-p21Waf1 luciferase reporter assay. Even low doses (0.2 μg) of p48 inhibited p53-dependent transcriptional activity by 50% (Fig. 5B, top, left). Introduction of p53 highly elevated the transactivation activity of a luciferase reporter gene, whereas addition of p48 inhibited p53-dependent transcriptional activity (Fig. 5B, top, right). Moreover, a marked reduction in the expression of several p53 transcriptions targets, p21 and Bax in the presence of p48 was observed (Fig. 5B, bottom, left). Conversely, depletion of p48 resulted in an increase in p21 mRNA and protein levels, and transcriptional activity of p53 (Fig. 5B, bottom, middle and right; Supplementary Fig. S6C and D). Thus, p48 expression negatively regulates the transcriptional activity of p53 in glioma cells.

In p48 overexpression condition, p53 mRNA levels were not altered (Supplementary Fig. S6E), although p53 protein levels were reduced (Fig. 5A), indicating that p48 regulates p53 at the posttranscriptional level. Indeed, the half-life of p53 was reduced in GFP-p48 cells versus control cells as measured after exposure of cells to cycloheximide (CHX) (Fig. 5C, top, and middle), whereas the p48-mediated p53 degradation was blocked by MG132 treatment (Fig. 5C, left, bottom). Moreover, cotransfection of GFP-p48 with HA-ubiquitin markedly enhanced p53 ubiquitination (Fig. 5C, right).

The above results demonstrate that p48 overexpression decreases p53 stability and activity, implying impairment of p53-mediated responses, such as apoptosis. Thus, we monitored γ-irradiation-driven apoptosis in U87/p48. These cells showed a more than a 50% reduction in apoptosis based on Annexin V staining and had less DNA fragmentation (Fig. 5D, top). As expected, the typical γ-irradiation-induced increase in p53 was inhibited by p48 overexpression (Fig. 5D, middle). Knockdown of p48 resulted in a reduction in the survival and demonstrated a higher rate of apoptosis in LNT-229-p53 WT cells whereas a limited effect was observed in LNT-229-p53-KD cells. Similar results were observed in HCT116 cells that express wild-type p53, and their isogenic clone that lacks p53 (27) (Supplementary Fig. S6F). Furthermore, knockdown of p48 in LNT-229-p53-WT cells enhanced drug-induced apoptosis than LNT-229-p53-KD cells (Supplementary Fig. S6G). Hence, these results suggest that p48 levels may modulate p53-mediated cellular responses.

**P48 is a cofactor for HDM2-mediated p53 degradation**

Because p48 does not contain a ubiquitin E3 ligase motif or activity, and p48 and p53 do not appear to interact directly, we hypothesized that p48 promotes HDM2-dependent p53 ubiquitination. Cotransfection of HDM2 and p48 into U87 cells enhanced downregulation of p53 in a dose-dependent manner and substantially enhanced the ubiquitination of p53, whereas knockdown of p48 decreased this effect (Fig. 6A), indicating that p48 affects the activity of HDM2.

Importantly, nutlin, which inhibits the interaction between HDM2 and p53 (28), reversed the effect of p48 on p53 levels (Fig. 6B). Silencing of HDM2 in U87 cells delayed p53 degradation whereas overexpression of p48 shortened the half-life of p53 (Fig. 6C, top and middle). Cotransfection of si-HDM2 with various amounts of p48 demonstrated that the effects of p48 on p53 levels are HDM2-dependent (Fig. 6C, bottom, left). Depletion of p48 prior to transfection with HDM2 blocked the HDM2-induced reduction in p53 levels (Fig. 6C, bottom, right). Thus, HDM2 is necessary for the regulation of p53 by p48 Ebp1. Moreover, a combination treatment of Nutlin3 and N-si-p48 led to an increased the protein levels of p53, inhibited proliferation, and enhanced apoptosis compared with that of p48 depletion alone or Nutlin3-only treatment (Fig. 6D).

**P48 regulates p53 through promoting the interaction between HDM2 and p53**

We demonstrated that endogenous HDM2 forms a complex with p48 (Fig. 7A, left). Importantly, HDM2 binds tightly to p48 but not binds to p42, revealing that mutants lacking the N-terminal domain (48–394) or amino acid 183 to 394 did not bind to HDM2 (Fig. 7A, middle and right).

Although we demonstrated a physical interaction between p48 and HDM2, p48 was not ubiquitinated by HDM2 (data not shown). The association between HDM2 and p53 was markedly enhanced by higher amounts of p48 whereas knockdown of p48 decreased the binding of HDM2 to p53 (Fig. 7B, left and middle). Moreover, HDM2–p53 interaction was enhanced in p48-expressing cells than control cells (Fig. 7B, right). These associations were supported by studies demonstrating that p48-mediated p53 ubiquitination correlated with p48 expression (Fig. 7B, right).

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Data suggest that p48 binds to HDM2 and promotes an HDM2–p53 interaction, thereby enhancing p53 degradation.

To ascertain the effect of p48 on p53, we transfected p48 into LNT-229-p53-WT/p53-KD cells. Overexpression of p48 obviously accelerated cell proliferation and colony formation in LNT229-p53-WT cells, whereas a limited change was observed in LNT-229-p53-KD cells compared with control cells (Fig. 7C), implying that p48 may exert oncogenic effect regulating p53, although we cannot rule out that p48 may have p53-independent effects.

Finally, we found that p48-low GBMs (<50% p48-positive cells) showed the intense immunoreactivity of p53 whereas p48-high GBMs (>50% p48-positive cells) showed only minimal staining of p53 (Fig. 7D, left). This was supported by immunoblotting with tissues of glioma patients. P53 was predominantly occurred in adjacent neural tissue compared with tumor tissues, whereas the expression levels of p48 were reversed (Fig. 7D, right). Thus, the expression levels of p48 in human gliomas contribute to the alteration of p53.

Discussion

Because Ebp1 was originally identified as an ErbB3 binding protein, it has been studied in breast and prostate cancer cells and is considered a tumor suppressor, as it inhibits cell proliferation and induces differentiation. Most of the research on Ebp1 has focused on events that are mediated by p42. However, the evidence is now compelling that 2 isoforms of Ebp1 exist, and this study provides evidence that p48 is one of them. P48 binds to HDM2 and promotes an HDM2–p53 interaction, thereby enhancing p53 degradation. Overexpression of p48 in LNT-229-p53-WT cells leads to accelerated cell proliferation and colony formation, whereas in LNT-229-p53-KD cells, a limited change was observed. This suggests that p48 may exert oncogenic effects regulating p53, although further studies are needed to confirm its independent effects.

Immunohistochemical analysis of human glioma tissues revealed that p48-low GBMs showed intense p53 staining, whereas p48-high GBMs showed minimal p53 staining. These findings were supported by immunoblotting results, further confirming the role of p48 in p53 regulation. The expression levels of p48 in human gliomas contribute to the alteration of p53, indicating a potential therapeutic target for glioma treatment.
Ebp1 proteins exist, and perhaps 2 isoforms possess different cellular functions. Interestingly, p42 was shown to be degraded following ubiquitination, thus leading to its absence in cancer cells, whereas p48 was strongly expressed in cancer cells (21). In this study, we found that p48 is highly expressed in glioma cells and cancer tissues from glioma patients. Overexpression of p48 in glioma cells promoted cell proliferation, colony formation, and invasion whereas specific ablation of p48 led to substantial attenuation of these effects. Moreover, we demonstrated that p48 induced malignant transformation of nontumorigenic glioma cells.

Figure 6. P48 is a cofactor for HDM2-mediated p53 degradation. A, U87 cells, transfected with myc-HDM2 with or without GFP-p48, was examined by immunoblotting (top). Lysates from U87 cells, transfected as indicated and treated with MG132 (10 μmol/L), were immunoprecipitated and immunoblotted as shown (bottom). B, U87 cells, transfected as shown, treated 10 μmol/L of Nutlin3 for 12 hours, and were immunoblotted as shown.

C, U87 cells, transfected as indicated with SCR or si-HDM2, were treated with CHX on the indicated times. Immunoblotting was done as indicated (top). Differences in p53 protein levels between p48 and mock-transfected cells were quantified (middle). U87 cells, silenced with si-HDM2 and transfected GFP-p48 as indicated or silenced with SCR or N-si-p48, were transfected with myc-HDM2 or control and were detected by immunoblotting as shown (bottom). D, cells were silenced with control or N-si-p48 and then exposed to Nutlin3 (3 μmol/L). Immunoblotting was done as indicated (top). Differences in p53 protein levels between N-si-p48 and SCR-transfected cells were quantified (middle). The relative fold changes of proliferating (MTT assay) and apoptotic cells (Annexin V–positive; bottom) are presented as bar graphs.
Figure 7. P48 regulates p53 through promoting the interaction between HDM2 and p53. A, U87 (500 μg) cell lysate was immunoprecipitated and immunoblotted as indicated (left). U87 cell lysates, transfected as indicated, were immunoprecipitated and immunoblotted as shown (middle). U87 cells were cotransfected with GFP-p48 fragments and myc-HDM2. Cell lysate was immunoprecipitated and immunoblotted as indicated (right). B, U87 cells were transfected with GFP p48 or control (left), silenced with SCR or N-si-p48 (middle), or cotransfected with HA-p53 and myc-HDM2 (right). Immunoprecipitation and immunoblotting were done as indicated. C, LNT-229-p53-WT /p53-KD cells were transfected with control or GFP-p48 (3 μg) and immunoblotted as shown (left). Transfected cells were subjected for MTT assay (middle) or cultured in soft agar (right). D, GBM patients [p48-low expression (Pt#1 and Pt#2) and -high expression (Pt#3 and Pt#4)] tissues were stained with anti-N-p48 or anti-p53 antibody (left and middle). Scale bar, 100 μm. Immunoblotting was performed using anti-p53 and N-p48 antibodies (right) from dissected GBM and adjacent normal tissue of the same patient.
U138 glioma cells and that p48 expression was associated with poor prognosis in human patients, implying for its clinical relevance. Notably, we demonstrated that p48 associated with HDM2 and promoted the interaction between HDM2 and p53, reducing p53 levels and impairing p53-mediated responses. Conceivably, p48 acts as a cofactor of HDM2, promoting the binding of HDM2 and p53, thereby facilitating p53 polyubiquitination and degradation in human cancers.

How would p48 lead to tumorigenicity? Because p53 is a prominent tumor suppressor in gliomas, we assumed that p48 might accelerate tumor progression by attenuating p53 activity. Indeed, we found that p48 expression not only downregulated p53 transcriptional activity, but also markedly decreased protein levels (Fig. 5A and B). The N-terminal fragment of p48 interacted directly with HDM2 (Fig. 7A and B) but not with p53, and enhanced HDM2 function toward p53 (Fig. 6A), resulting in downregulation of p53. The crystal structure of p42 is missing one and a half helices at the N terminus of p48 (29); this structural difference between the 2 isoforms may account for the ability of p48, but not of p42, to interact with HDM2. It is generally accepted that tumor suppressors are frequently degraded in tumors, and low levels of HDM2 barely polyubiquitinate p53, indicating that there may be a cofactor that aids in p53 ubiquitination. Our studies add a new layer of complexity to the regulation of p53 in cancers, where p48 mediates the HDM2–p53 interaction and HDM2-dependent degradation. Although it is possible that tumor progression and p48 expression are independently related to degradation of p53 in cancer cells, our findings suggest that p48 expression can contribute to tumorigenicity. Previous studies demonstrated that Akt binds to and phosphorylates HDM2, promoting its nuclear translocation (13, 14, 30), and our previous work demonstrates that p48 binds to nuclear Akt and enhances Akt kinase activity (2, 3). Thus, we cannot rule out the possibility that p48 accelerates Akt-mediated HDM2 phosphorylation and consequently prevents HDM2 self-ubiquitination. Alternatively, HDM2 was shown to interact with both Rb and E2F1, resulting in E2F1 activation, it is possible that the association of HDM2 and p48 in the nucleus might sequester hyperphosphorylated Rb into the nucleolus and release E2F1 from its inhibition, promoting cell proliferation, because hyperphosphorylated Rb is imported into the nucleolus in S or G2 phase and we detected nucleolar residency of p48 and an interaction between p48 and Rb (data not shown).

Given the role of apoptosis in successful anticancer therapy, it is crucial to understand the mechanism underlying tumor cell susceptibility and resistance to cell death and, in particular, to p53-mediated apoptosis. Ablation of p48 from glioma cells induced cell death (Fig. 2D) and overexpression of p48 dramatically enhanced tumor cell resistance to IR exposure and suppressed p53 accumulation, thereby overcoming cell death signaling (Fig. 5D). The antiapoptotic nature of p48 is consistent with its proproliferation and tumor-promoting abilities.

Why distinct isoforms play opposite roles in regulating cancer progression and the mechanisms responsible for upregulation of p48 levels in gliomas remain to be determined. We noticed that the promoter of PA2G4 contains several p53-binding motifs; indicate that p53 may regulate the transcription of p48 or transrepression of p42 depending on the amount of p53 present in the cell. It is tempting to speculate that cancer cells are poised to translate p48 depending on the amount of p53 in the cell under certain conditions, rendering cells resistant to apoptosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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