p68 RNA helicase promotes glioma cell proliferation in vitro and in vivo via direct regulation of NF-κB transcription factor p50

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The DEAD-box RNA helicase p68 plays a very important role in early organ development and maturation. However, the role of p68 in glioma is unclear. In this study, we showed that p68 protein levels were significantly elevated in high-grade gliomas compared to low-grade gliomas and normal adjacent brain tissues. Importantly, the expression of p68 was significantly associated with poorer overall survival and enhanced resistance to treatment with radiotherapy plus temozolomide for glioma patients. Ectopic expression of p68 enhanced glioma cell proliferation both in vitro and in vivo. In contrast, knockdown of endogenous p68 prevented glioma cell proliferation. Using a tandem affinity purification assay, we found a new p68-binding protein, nuclear factor (NF)–κB p50. We found that p68 bound with the N-terminal of NF-κB p50, and the mutant of p68 lacking the p50-interaction domain failed to stimulate glioma cell proliferation and tumor growth. Moreover, p68 induced NF-κB p50 accumulation in the nucleus through release of NF-κB p50 from IkBα and increased NF-κB p50 target luciferase transcription activity. Knockdown of NF-κB p50 rescued the phenotypes induced by p68 both in vitro and in vivo. We concluded that p68 induces glioma tumor growth through binding with NF-κB p50, regulating NF-κB p50 nucleus accumulation and transcription activity.

Keywords: glioma, IkBα, NF-κB p50, p68.

The nuclear p68 RNA helicase (also called DDX5) is a prototypical member of the DEAD-box family of RNA helicases. As with other DEAD-box RNA helicase proteins, p68 is an ATPase that modulates pre-mRNA, rRNA, and micro RNA (miRNA) processing. Moreover, p68 plays a very important role in cell proliferation, neural development, and early organ development and maturation. The expression of p68 is upregulated in colorectal, prostate, and breast cancers and has been shown to correlate with tumor progression and transformation. Recently, p68 has been reported to interact with such transcription-related factors as RNA polymerase II, cAMP-response element-binding protein, p300, histone deacetylase 1, Smad3, and others. p68 also acts as a potent co-activator of estrogen receptor (ER)α, tumor suppressor p53, MyoD, and β-catenin.

The family of nuclear factor (NF)–κB has 5 cellular members: p105/p50 (NF-κB1), p100/p52 (NF-κB2), p65 (RelA), RelB, and c-Rel. Dominant among these cellular members of the NF-κB transcription factors is the p50/p65 heterodimer. Usually NF-κB complexes are localized in the cytoplasm, where they bind to IkB inhibitory proteins, including IkBα, IkBβ, and IkBε. Upon stimulation, IkB proteins are rapidly phosphorylated by IκB kinase α and β (IKKα and β) and degraded via the ubiquitin-proteasome pathway. NF-κB is then translocated into the nucleus and triggers transcription of various targeting genes critical to immune response, cell adhesion, differentiation, proliferation, angiogenesis, and apoptosis.

In the current study, we found that the expression of p68 was significantly associated with poorer overall survival and enhanced resistance to treatment with radiotherapy plus temozolomide (RT-TMZ) for glioma patients. Overexpression of p68 enhanced, and knockdown of p68 inhibited, glioma cell growth both in vitro and in vivo through binding with and regulating NF-κB p50.

Materials and Methods

Immunohistochemical Staining

Human glioma samples were obtained from the Second Affiliated Hospital of Harbin Medical University. The pathological grades of the tumors were defined according...
p68 cDNA, p68 short hair RNA (shRNA), and NF-κB p50 shRNA were purchased from Open Biosystems. Target sequences were 5'-GTGACTGGGTCTAATGAGT-3' for p68 shRNA, 5'-GCCCATACCTTCAATTCTG-3' for NF-κB p50 shRNA, and 5'-TGAGCACGGCCTAGTGTCTG-3' for the nonsense shRNA. Rabbit anti-p68 antibody (used for the Western blot, immunoprecipitation, and immunohistochemical assays) was purchased from Santa Cruz Biotechnology. Rabbit anti-p50 antibody (for the immunoblotting assay) was obtained via immunoblotting assay. Rabbit anti-IκBα antibody (for the immunoblotting assay) was purchased from Abcam. Mouse anti-α-tubulin antibody and rabbit anti-Sp1 antibody for immunoblotting assays and the NF-κB p50 inhibitor pyrrolidine dithiocarbamate were purchased from Sigma. Alexa Fluor-488 goat anti-rabbit IgG and DAPI (4',6-diamidino-2-phenylindole) were purchased from Invitrogen and MTT assay reagents from DingGuo Biotech.

**Cell Culture**

Human glioma cell lines H-4, HS-683, U-87, U-251, and U-343 were purchased from American Type Culture Collection (ATCC) and cultured according to manufacturer recommendations. HEK293 cells were purchased from ATCC and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C with 5% v/v CO2. U-251 and U-87 cells were transfected with the plasmids of full-length, wild-type p68 or the plasmic vector, with p68 shRNA or control (Ctrl) shRNA, or with the C-terminal of p68 or control vector, using Lipofectamine 2000 (Invitrogen).

Cells were then selected by using 0.8 mg/mL G418 (Gibco BRL) for 2 weeks, and then cell pools with stable overexpression or knockdown of p68 were obtained for the respective assays. For NF-κB p50 knockdown, Lipofectamine 2000 was used for transfection; after 48 h, cells were collected for the relevant experiments.

**Tandem Affinity Purification and Mass Spectrometry**

The tandem affinity purification (TAP) system was purchased from Stratagene (catalogue #240104). p68 was constructed into a pCTAP vector. Plain vector and p68/pCTAP plasmids were transfected into 1 × 10^6 HEK293 cells using Lipofectamine 2000. Cell lysates were collected after 48 h, and the TAP assay was performed according to the manufacturer’s instructions. The samples thus obtained were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. Unknown proteins were sent for identification by mass spectrometry.

**Immunoblotting, Immunoprecipitation, and Glutathione S-Transferase Pull-down**

For immunoblotting assays, samples were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and detected with the appropriate primary antibodies followed by horseradish peroxidase–conjugated goat anti-mouse or rabbit IgG. The blotting signals were detected using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology). Quantitative analyses of immunoblotting signals were obtained via densitometric analysis using LAS4000 Image Software (Fuji Film).

For immunoprecipitation, cells were lysed using RAPI buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 600 mM NP-40, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1 mM sodium orthovanadate) and incubated with 30 μL protein-A sepharose beads (GE Healthcare) and 1 μg of appropriate primary antibodies at 4°C overnight. Samples were washed 3 times with PBS and then analyzed by immunoblotting assay.

For the glutathione S-transferase (GST) pull-down assay, 3 μg GST–NF-κB p50, or GST control protein, was incubated with 3 μg His-p68, His-p68-N, or His-p68-C in 1 mL PBS with 0.1% BSA at 4°C for 2 h. Then 20 μL of glutathione beads (Sigma) were added and the samples were incubated for another 1 h at 4°C. The samples were washed 3 times with PBS and then analyzed by immunoblotting assay.

For subcellular fractionation, cytoplasmic and nuclear extracts were prepared using the Qproteome Cell Compartment Kit (Qiagen). Samples were then analyzed by immunoblotting assay.

**Immunofluorescent Staining**

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 in PBS at room temperature.
the temperature for 20 min. Samples were blocked with 1% BSA (Sigma) and incubated with the appropriate primary antibody at 37°C for 1 h. After extensive washing, they were incubated with Alexa Fluor-488 goat anti-rabbit IgG at 37°C for 1 h. Cells were then washed and mounted for observation under a scanning confocal microscope (TCS SP2, Leica).

Cell Proliferation

For the MTT assay, cells including all transfectants were grown in exponential phase and detached by trypsin treatment. Viable cells (2000 cells/mL) were plated onto 96-well tissue culture plates (100 μL complete medium/well) and cultured at 37°C in a 5% CO2 atmosphere. At different time points, MTT reagent was added (10 μL per well), and the cells were incubated at 37°C for 4 h. The reaction was stopped by the addition of 100 μL dimethyl sulfoxide, and the optical density was determined at 570 nm on a multiwell plate reader. For cell number counting, viable cells (1000 cells/mL) were plated onto 96-well tissue culture plates (100 μL complete medium/well) and cultured at 37°C in a 5% CO2 atmosphere. At different time points, cells were detached by trypsin and counted under the microscope. Data from 3 independent experiments were analyzed by Student’s t-test, and P < .05 was considered significant.

Xenograft Model of Tumor Growth

Cells were resuspended at 1 × 10⁷ cells/mL and a 0.1-mL aliquot of cell suspension was injected subcutaneously into athymic nude mice (n = 10). Tumor volume was measured at different time points. Tumor volumes were determined by external measurements and calculated according to V = [L × W²] × 0.52, where V = volume, L = length, and W = width. Data were analyzed by Student’s t-test, and P < .05 was considered significant.

Luciferase Reporter Assay

To evaluate NF-κB p50-dependent transcriptional activity, we performed the luciferase reporter assay with a luciferase reporter construct (Promega). Cells were transiently transfected in triplicate with one of these luciferase reporters and phosphorylated cytomegalovirus–β-galactosidase (Promega) using Lipofectamine 2000. Forty-eight hours after transfection, luciferase activity was determined using the Luciferase Assay System Kit (Promega). β-Galactosidase activity was determined using the Luminescent β-gal Detection Kit II (BD Clontech) as an internal control.

Statistical Analysis

Overall survival was reported in months and defined as the interval between the date of the surgery and the date of death or last follow-up. Overall survival curves were estimated by the Kaplan–Meier method, and the difference in survival was evaluated using the log-rank test. P-values of <0.05 and >0.01 were considered statistically significant and very significant, respectively. All computations were made using R 2.9.0 software (www.r-project.org).

Results

Overexpression of p68 in Human Glioma Tissues and Glioma Cell Lines

To determine the specific expression pattern of p68 protein in human glioma, immunohistochemical analysis was performed. Using the antibody for p68 staining, we examined tissue samples from 89 patients with a pathological diagnosis of glioma. Immunoreactivity for the p68 antigens was seen in 71.43% (35/49) of low-grade glioma tissues, 95% (38/40) of high-grade glioma tissues, and 4.35% (2/46) of adjacent normal tissues, and the staining of p68 was much stronger in high-grade glioma than in low-grade glioma (Fig. 1A). We then examined the expression of p68 in 6 human glioma cell lines using p68 antibodies. Expression of p68 was elevated in all 6 cell lines (Fig. 1B).

Expression of p68 Correlates with Shortened Survival and Enhanced Resistance to RT-TMZ

We further evaluated whether the p68 immunoreactivities correlated with overall survival in 138 patients with glioma. We found that p68 antigen inversely correlated with overall survival (Fig. 1C). We then investigated the role of p68 in mediating resistance to therapies using RT-TMZ. We found the 1-year survival rates to be 63.9% (39/61) for patients with negative p68 antigen and 47.5% (29/61) for patients with positive p68 antigen (P < .05, Fig. 1D). The p68-positive patients showed more resistance to RT-TMZ. These results highlighted the clinical importance of p68 in determining the prognosis for patients with glioma and also indicated a new target for glioma therapy.

p68 Enhances Glioma Cell Growth In Vitro and In Vivo

To investigate the biological role of p68 in glioma cells, we overexpressed p68 in human glioma U-251 cells and U-87 cells (Fig. 2A). We then tested the role of p68 in glioma cell proliferation using the MTT assay. The results showed a significant increase in the growth curve and indicated that cell proliferation was enhanced in vitro after transfection with p68 in both U-251 cells and U-87 cells (Fig. 2B). The cells were also counted, and U-251 cells and U-87 cells that overexpressed p68 had greater cell numbers in culture (Fig. 2C). We then examined whether p68 enhanced tumor growth in vivo. When tumor cells were injected subcutaneously into athymic nude mice, overexpression of p68 resulted in dramatically increased tumor volumes compared with vector
p68 Binds Directly with NF-κB p50

To investigate the mechanism by which p68 regulates glioma cell proliferation, we used the TAP system to map p68-binding proteins, and we found a new p68-binding protein of ~50 kDa, which was identified as having the same 3 segments as NF-κB p50 sequenced by mass spectrometry (Fig. 3A). We then verified the association between p68 and NF-κB p50 using the co-immunoprecipitation assay in glioma U-251 cells. p68 was immunoprecipitated with the anti-p68 antibody (α-p68) or control rabbit IgG and then immunoblotted for NF-κB p50 or p68. NF-κB p50 was co-immunoprecipitated with p68 antibody but not rabbit IgG (Fig. 3B). We next examined whether p68 interacted with NF-κB p50 directly using purified recombinant proteins with a His tag fused to p68 and GST fused to NF-κB p50. We found that GST–NF-κB p50, but not GST, bound directly to His–p68 (Fig. 3C). These biochemical studies clearly demonstrate that p68 binds directly to NF-κB p50.

N-Terminal of p68 Binds to NF-κB p50 and Is Necessary to Stimulate Glioma Cell Proliferation and Tumor Growth

We then mapped the domains of p68 involved in the interaction with NF-κB p50. N-terminal (aa1-387) and C-terminal (aa387-614) mutants of p68 were fused with a His tag and purified. We found that the N-terminal but not the C-terminal of p68 bound to NF-κB p50 following incubation with GST–NF-κB p50 (Fig. 3D). We then explored whether the N-terminal of p68 was necessary to stimulate glioma cell proliferation and tumor growth. When the C-terminal of p68 was transfected into U-251 cells, we found that this C-terminal, which lacks the p50-interaction domain, failed to stimulate both U-251 cell proliferation in vitro and tumor growth in vivo, as determined by the MTT assay (Fig. 3E), cell number counting (Fig. 3F), and xenograft model (Fig. 3G).

p68 Induces NF-κB p50 Dissociation from IκBa, Accumulation into Nucleus, and Enhanced NF-κB p50 Target Report Gene Transcription

IκBa inhibits activity of NF-κB p50 by acting with NF-κB p50 in the cytoplasm. We speculated that p68 might dissociate the IκBa/NF-κB p50 complex, thus transferring NF-κB p50 into the nucleus for transcriptional activation. To test this hypothesis, we assessed the interaction of endogenous NF-κB p50 with IκBa. Immunoblotting analysis of immunoprecipitated complexes from glioma U-251 cells with overexpression of p68 revealed that NF-κB p50 bound a lower level of IκBa compared to cells with vector control (Fig. 4A). We next tested whether overexpression of p68 altered the cellular distribution of NF-κB p50. Indeed, the expression of p68 induced the localization of NF-κB p50 from the cytoplasm to the nucleus, as observed by cellular fractionation followed by immunoblotting (Fig. 4B) and by immunofluorescent staining (Fig. 4C). α-Tubulin and Sp1 were used as the cytoplasmic and nuclear markers, respectively. We then used the luciferase reporter assay to explore whether p68 affects NF-κB p50 translocation activity. U-251 cells were co-transfected with a plasmid encoding the NF-κB p50 binding site luciferase reporter and system control plasmid encoding the β-galactosidase reporter. The results showed that ectopic expression of p68 induced an increase in luciferase activity (Fig. 4D).
expression of p68 increased NF-κB p50 target luciferase reporter activity (Fig. 4D). Consistently, when we knocked down p68 using shRNA (Fig. 4E), NF-κBp 5 0 bound more IκBα (Fig. 4F), which accumulated in the cytoplasm instead of the nucleus (Fig. 4G) and decreased NF-κB p50 target luciferase reporter transcription (Fig. 4H). Collectively, these data demonstrated that p68 dissociates the IκBα/NF-κB p50 complex, leads the NF-κB p50 to the nucleus, and increases NF-κB p50 target report gene transcription.

NF-κB p50 Inhibition Prevents p68-Induced Glioma Cell Proliferation

We next tested whether NF-κB p50 activation triggered by p68 might actually mediate glioma cell proliferation. We found that pretreatment of p68-overexpressing glioma U-251 cells with pyrrolidine dithiocarbamate (a chemical inhibitor of NF-κB p50), but not dimethyl sulfoxide control, inhibited cell proliferation (as shown by the MTT assay) (Fig. 5A). Using shRNA, we also knocked down endogenous NF-κB p50 in p68-overexpressing U-251 cells (Fig. 5B) and found (using the MTT assay) that knockdown of NF-κB p50 inhibited glioma cell proliferation in vitro. Furthermore, in vivo tumor growth was dramatically inhibited by NF-κB p50 shRNA compared with Ctrl shRNA in p68-overexpressing U-251 cells. These results demonstrated that NF-κB p50 activation is necessary in glioma cell proliferation induced by p68.

Discussion

The current study found that p68 enhances glioma cell proliferation in vitro and in vivo. We found a new p68-binding protein, NF-κB p50, and demonstrated that the N-terminal of p68 is necessary to bind with
p50 to stimulate glioma cell proliferation. p68 can induce NF-κB p50 release from IκBα and accumulation in the nucleus for activation of transcription activity. Inhibition of NF-κB p50 prevents glioma cell proliferation induced by p68. Remarkably, the p68 immunoreactivities positively correlated with increased pathological grade of gliomas and inversely correlated with overall survival in patients with a diagnosis of glioma, further underscoring the clinical significance of p68 in the pathogenesis, prognosis, and therapy of glioma.

Gliomas are the most frequent primary tumors arising in the brain. The highest grade glioma, glioblastoma, is one of the most aggressive human cancers and resists successful treatment. It is essential to investigate the mechanisms underlying the development and progression of gliomas. Our study showed that expression of p68 correlates with shortened survival and enhanced resistance to RT-TMZ, while the mutant of p68 lacking the p50-interaction domain failed to stimulate glioma cell proliferation both in vitro and in vivo. These results suggest that a drug targeting p68/p50 binding sites may be a potent adjunctive therapy in the treatment of glioma.

Several studies have demonstrated that p68 is ubiquitously expressed in tissues and that the expression of p68 is developmentally regulated. p68 has also been considered as a proto-oncoprotein, having been found to be overexpressed in colorectal tumors, where it bound with β-catenin and stimulated β-catenin–dependent transcription of target genes such as c-Myc and cyclin D1. Furthermore, downregulation of p68 in colorectal cancer cells reduced proliferation and tumor formation in nude mice. Similarly, p68 was reported to be overexpressed in breast tumors and to be a coactivator of ERα, regulating target gene transcription and enhancing breast tumorigenesis. p68 was also found to be overexpressed in prostate tumors and to be an androgen receptor coactivator that facilitates prostate tumorigenesis. In this study, we found that p68 was overexpressed in human glioma and regulated glioma cell proliferation both in vitro and in vivo, suggesting that p68 plays important roles in glioma. In addition to being an RNA helicase, p68 has been shown to act as a coregulator of several highly regulated transcription factors with important roles in cancer and/or development, such as ERα, cAMP-response element-binding.
Fig. 4. p68 induces NF-κB p50 release from IκBα, accumulation into nucleus, and activation of transcription activity. (A) Overexpression of p68 inhibited IκBα binding to NF-κB p50. NF-κB p50 was immunoprecipitated from the U-251/V or U251/p68 cell lysates followed by immunoblotting with the antibodies to IκBα and NF-κB p50. Sample loading controls are shown (5% input). (B) Cellular distribution of NF-κB p50 in U-251/V (V) and U-251/p68 (p68) cells by cell fraction sedimentation and immunoblotting with NF-κB p50 Ab. α-Tubulin was used as a cytoplasm loading control and Sp1 as a nucleus loading control. (C) Cellular distribution of NF-κB p50 in U-251/V (V) and U-251/p68 (p68) cells by immunofluorescent staining with NF-κB p50 Ab. DAPI was used for nucleus staining. Bar = 10 μm. (D) p68 increased NF-κB p50 target promoter activities, \( P < .05 \). (E) Knockdown of p68 enhanced IκBα binding to NF-κB p50. NF-κB p50 was immunoprecipitated from the U-251/Ctrl shRNA or U-251/p68 shRNA cell lysates followed by immunoblotting with the antibodies to IκBα and NF-κB p50. Sample loading controls are shown (5% input). (F) Cellular distribution of NF-κB p50 in U-251/Ctrl shRNA and U-251/p68 shRNA cells by cell fraction sedimentation and immunoblotting with NF-κB p50 antibody. α-Tubulin was used as a cytoplasm loading control and Sp1 as a nucleus loading control. (G) Cellular distribution of NF-κB p50 in U-251/Ctrl shRNA and U-251/p68 shRNA cells by immunofluorescent staining with NF-κB p50 antibody. DAPI was used for nucleus staining. Bar = 10 μm. (H) Knockdown of p68 decreased NF-κB p50 target promoter activities, \( P < .05 \). Results represent at least three separate experiments.

Fig. 5. Inhibition or knockdown of NF-κB p50 inhibits p68-induced glioma cell proliferation in vitro and in vivo. (A) In vitro growth of U-251/p68 cells treated with the NF-κB p50 inhibitor PDCT or with control DMSO was measured by the MTT assay. (B) NF-κB p50 was knocked down in U-251/p68 cells. U-251/p68 cells were transfected with control shRNA (Ctrl shRNA) or NF-κB p50 shRNA. NF-κB p50 levels were detected by immunostaining with NF-κB p50 antibody, with α-tubulin as the loading control. (C) In vitro growth of U-251/p68 cells treated with nonsense control shRNA (Ctrl shRNA) or NF-κB p50 shRNA, as measured by the MTT assay. (D) Average tumor volume in athymic nude mice subcutaneously inoculated with U-251/p68 treated with nonsense control shRNA (Ctrl shRNA) or NF-κB p50 shRNA cells. Representative tumors with mice inoculated with Ctrl RNA treated U-251/p68 cells or NF-κB p50 shRNA treated U-251/p68 cells are shown in the right panel.
protein/p300, MyoD, p53, AR, Runx2, β-catenin, and others.8–18 Herein, we demonstrated a new p68-binding protein, NF-κB p50, which is also a transcription factor. Given our findings and the results from others, we hypothesize that p68 may be a ubiquitous transcription factor binding partner.

The NF-κB transcription factor family regulates over 200 genes involved in cell function and inflammation. All 5 NF-κB subunits contain a conserved Rel homology domain responsible for nuclear localization and DNA binding.27 NF-κB p50 is produced from its precursor, p105, and forms dimers with itself and other NF-κB subunits. NF-κB family proteins have been detected in the nervous system and are involved in synaptic processes, neurotransmission, and neuroprotection. Furthermore, inducible NF-κB plays a crucial role in brain inflammation and neural stem cell proliferation.28–31 P50−/− mice have a lower learning ability and are sensitive to neurotoxins.32–34 Our results showed that p68 bound with NF-κB p50 and regulated NF-κB p50 nucleic accumulation in glioma cells. It may be that p68 plays important roles in learning and memory, neuron growth, and inflammation through regulation of NF-κB p50. Furthermore, NF-κB p50 is a major transcription regulator of immune response, and whether p68 plays important roles in immune response needs further study.

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R.W. and Z.J. contributed equally to this article.

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