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Mustard NPR1, a mammalian IκB homologue inhibits NF-κB activation in human GBM cell lines

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ABSTRACT

NF-κB activity is tightly regulated by IκB class of proteins. IκB proteins possess ankyrin repeats for binding to and inhibiting NF-κB. The regulatory protein, NPR1 from *Brassica juncea* possesses ankyrin repeats with sequence similarity to IκBα subgroup. Therefore, we examined whether stably expressed BjNPR1 could function as IκB in inhibiting NF-κB in human glioblastoma cell lines. We observed that BjNPR1 bound to NF-κB and inhibited its nuclear translocation. Further, BjNPR1 expression down-regulated the NF-κB target genes iNOS, Cox-2, c-Myc and cyclin D1 and reduced the proliferation rate of U373 cells. Finally, BjNPR1 decreased the levels of pERK, pJNK and PKCα and increased the Caspase-3 and Caspase-8 activities. These results suggested that inhibition of NF-κB activation by BjNPR1 can be a promising therapy in NF-κB dependent pathologies.

Introduction

NF-κB is a family of dimeric transcription factors that play vital roles in regulation of inflammation, immune responses, proliferation and apoptosis in various human pathologies [1,2]. There are five mammalian NF-κB family members: RelA/p65, RelB, c-Rel, p50/p105 and p52/p100, which possess a highly conserved 300 amino acid long N-terminal Rel homology domain (RHD) containing the dimerization, nuclear localization, and DNA-binding domains. Three of the family members, RelA, c-Rel, and RelB, have a transactivation domain at the C-terminus. The RHD possesses a nuclear-localization sequence (NLS) at its C-terminus, which is rendered inactive in unstimulated cells through binding of specific NF-κB inhibitors known as inhibitor of κB (IκB) proteins. NF-κB1/p105 and NF-κB2/p100 are inactive precursors of the p50 and p52 localized in the cytoplasm and their proteolytic processing removes C-terminal inhibitory domains allowing the proteins to enter the nucleus. P50 and p52 usually form homodimers or heterodimers with one of the three proteins that have transactivation domain [2,3]. In unstimulated cells, most NF-κB/Rel dimers are bound to IκB and retained in cytoplasm. IκB gene family includes seven known mammalian members, IκBα, IκBβ, IκBε, IκBγ, bcl-3, and the precursor Rel proteins p100 and p105. The IκBs are characterized by the presence of multiple ankyrin repeats, which are 33 amino acid sequences that mediate interaction with NF-κB via the RHD [2]. The expression of IκB proteins is tightly regulated by signals that degrade IκB. In the classical NF-κB signaling pathway, IκB proteins are phosphorylated by an activated IκB kinase (IKK) complex followed by polyubiquitination and degradation by 26S proteasome releasing free NF-κB dimers [4].

All IκB class of proteins have ankyrin repeats for binding to and inhibiting NF-κB translocation. The most important ankyrin proteins that regulate the transcription in disease response pathways include the IκBs in animals and NPR1 proteins in plants [5]. Ankyrin repeat containing proteins are conserved among plants, animals and protozoan kingdoms participating in protein–protein and protein–DNA interactions [6]. NPR1 is a key regulatory protein in systemic acquired resistance (SAR) pathway in plants and possesses ankyrin repeats with sequence homology to the mammalian NF-κB regulator IκBα [7,8]. NF-κB signal transduction pathway is crucial for immune functions, inflammatory responses, apoptosis and tumorigenesis in mammals [1,9,10]. The SAR response in plants is functionally analogous to inflammatory response of animals in that normal resistance processes after first pathogen encounter are potentiated leading to enhanced disease resistance. Furthermore, inactivation of both pathways leads to enhanced susceptibility to bacterial, viral, and fungal pathogens [8,11].

Previous studies with *Arabidopsis NPR1* (*AtNPR1*) showed significant similarity to several important structural domains of IκBα

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throughout the entire length of the protein, including ankyrin domains, two N-terminal serines, a pair of lysines, and an acidic C-terminus [5,8].

Understanding the mechanisms that regulate NF-κB activity is critical for developing therapeutic strategies for many human diseases [2,12,13]. Several other IκB-like ankyrin repeat containing NF-κB binding proteins have been reported to modulate nuclear NF-κB transcriptional activity on a subset of genes [14,15]. When overexpressed, they also prevented nuclear localization of NF-κB [16–19]. We have cloned *Brassica juncea NPR1* (*BjNPR1*) earlier [20]. Its amino acid sequence has similarity with IκBα proteins. These observations tempted us to study the functional role of NPR1 in human tumor cells.

In the present study, we investigated whether BjNPR1 could function like the mammalian IκB. We observed that BjNPR1 interacted with and inhibited NF-κB nuclear translocation suppressing its target gene expression. Further, it down-regulated the pERK, pJNK and PKCα expression and enhanced the Caspase-3 and Caspase-8 activities.

Materials and methods

Reagents. RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin and geneticin (G418) were purchased from Gibco BRL (California, USA). TNF-α, Trypsin–EDTA, protease inhibitor cocktail, Caspase-3 and Caspase-8 substrates (Ac-DEVD-AFC, Ac-IETD-AMC, respectively) were purchased from Sigma Chemicals (St. Louis, USA). p50, p65, pERK, pJNK, p38, iNOS, Cox-2, cyclin D1, c-Myc, cleaved-Caspase-3, cleaved-PARP, HDAC-1, Lamin B2, GAPDH and β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Enzyme immunoassay kit for measurement of NF-κB and Lipofectamine™ 2000 were obtained from Invitrogen Inc., (CA, USA). All secondary antibodies were purchased from Genei Pvt. Ltd. (Bangalore, India). Mammalian expression vector pCDNA3.0 was purchased from Upstate Biotech (USA) and Ready-To-Glow™ NF-κB secreted Luciferase Reporter Systems and IκBα Dominant-Negative Vector Set were purchased from Clontech (USA), and pRL-TK vector was purchased from Promega (USA).

Cell culture and stable transfection. Human U373 glioblastoma cell line was obtained from National Centre for Cell Science, Pune, India. Cells were grown in RPMI 1640 media supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in humidified atmosphere with 5% CO_2 at 37 °C. For transfection studies, 1×10^5 U373 cells were transfected with 5 μg of linearized pcDNA-BjNPR1 and pcDNA plasmids using lipofectamine. After 48 h, cells were grown in culture media containing 500 μM G418 for selecting stable transfectants, which were used for further analysis.

Transient transfection and luciferase assays. To examine TNF-α induced NF-κB reporter activity, U373 and U373-NPR1 cells were seeded in 24-well plates and transfected with 1 μg of pNF-κB-MetLuc2-Reporter, pMetLuc2-Control and 0.5 μg of pRL-TK (for normalization of transfection efficiency) vectors by using lipofectamine 2000. For IκBα inhibited NF-κB assays, cells were transfected with 1 μg of pCMV-IκBαM, 1 μg of pNF-κB-MetLuc2-Reporter and 0.5 μg of pRL-TK vectors. After 24 h, cells were stimulated with 10 ng/ml of TNF-α. The cell culture medium was harvested after 24 h and subjected to metridia luciferase activity according to the manufacturers protocol (Clontech). Luciferase activities were normalized with renilla luciferase activity.

Preparation of cytosolic and nuclear extracts. Control (U373) and transfected (U373-BjNPR1) cells were grown in 100 mm culture plates and stimulated with TNF-α for various time periods. Cells were then harvested and washed in ice-cold PBS, lysed in 400 μl of cold buffer A [Hepes 10 mmol/L, pH 7.9, KCl 10 mmol/L, 1 mM EDTA, phenylmethanesulphonylfluoride (PMSF) 1 mmol/L, 1 mM EGTA, dithiothreitol (DTT) 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L and pepstatin A 1 mg/L]. After 15 min incubation on ice, 0.1% NP-40 was added to the lysates and the tubes were vigorously rocked for 1 min and centrifuged (20,800*g*, 5 min) at 4 °C. The supernatant was collected as cytosolic fraction. The nuclear pellets were washed once with cold buffer A, then resuspended in 50 μl of cold buffer B (Hepes 20 mmol/L, pH 7.9, NaCl 420 mmol/L, edetic acid 0.1 mmol/L, egtazic acid 0.1 mmol/L, PMSF 1 mmol/L, DTT 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L and pepstatin A 1 mg/L) and vigorously rocked for 30 min at 4 °C followed by centrifugation at 20,800*g* for 5 min. The supernatant was collected as nuclear fraction. The protein concentration was determined according to the Bradford method.

Western blotting. Cytosolic and nuclear proteins from U373 and U373-BjNPR1 cells were subjected to electrophoresis on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes, which were blocked with 5% non-fat dry milk in Tris buffered saline (TBS) (10 mM Tris (pH 7.5), 150 mM NaCl) for 1 h at room temperature and incubated with primary antibody at 4 °C for 12–16 h. Membranes were incubated with respective secondary antibody for 1–2 h. Blots were washed with TBS and TBST (TBS containing 0.1% Tween 20) before and after incubation with secondary antibodies. Immunoreactivity was detected using western blot detection reagents.

Isolation of RNA and Northern blotting. Total cellular RNA was extracted from U373 and U373-BjNPR1 cells using TRI Reagent™ (Sigma–Aldrich, USA). Purified total RNA was dissolved in DEPC treated water and stored at −80 °C. For northern blotting, 20 μg of total RNA was electrophoresed on 1% denaturing formaldehyde gel and transferred onto Hybond N^+ membrane. PCR amplified *BjNPR1* was used as hybridization probe prepared using Prime-a-Gene radiolabeling kit (Promega Corporation, USA).

Determination of NF-κB levels. NF-κB levels were measured in cytosolic and nuclear extracts by using colorimetric enzyme immunoassay (EIA) kit.

Caspase-3 and Caspase-8 activity assay. Caspase-3 and Caspase-8 activities were measured in U373 and U373-BjNPR1 cell lysates using synthetic fluorogenic substrates (Ac-DEVD-AFC for Caspase-3, Ac-IETD-AMC for Caspase-8). Briefly, cells were washed with PBS and lysed in the lysis buffer (100 mM NaCl, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM EDTA, 50 mM Hepes, 0.1% CHAPS, 0.1 mM DTT, 100 μM PMSF). Then, 50 μg of the protein and 8 μM of the substrate were added to 1 ml of assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% (w/v) CHAPS and 10% sucrose) and incubated for 1 h at 37 °C. Measurements were made on a spectrofluorimeter with an λ_{ex} of 380 nm and a λ_{em} of 460 nm for Caspase-8 and with a λ_{ex} of 400 nm and λ_{em} of 480–520 nm for Caspase-3.

Co-immunoprecipitation. Cytosolic extracts from U373-BjNPR1 cells were immunoprecipitated with BjNPR1 antibody and the immune complex was probed with p50 and p65 antibodies. Briefly, cytosolic proteins were pre-cleared using protein A-agarose and incubated with 1 μg of BjNPR1 primary antibody overnight at 4 °C. After the addition of 20 μl of protein A-agarose, samples were further incubated for 2–3 h at room temperature. The immune complex was washed thrice with a wash buffer containing 20 mM Hepes (pH 7.4), 500 mM NaCl, and 10 mM $MgCl_2$ and suspended in 40 μl of rinse buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, and 10 mM $MgCl_2$. The beads were resuspended in SDS sample buffer and subjected to Western blot analysis.

Immunofluorescence. U373-BjNPR1 cells were seeded onto glass cover slips in 12-well plates. After reaching confluence, cells were washed with PBS and fixed with 4% paraformaldehyde followed by permeabilization with 0.2% Triton X-100 and washing with PBS. Cells were incubated in a cocktail of two primary antibodies