Biochemical and Biophysical Research Communications 450 (2014) 1656–1661

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Constitutive NF-κB activation and tumor-growth promotion by Romo1-mediated reactive oxygen species production

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ARTICLE INFO

Article history:
Received 1 July 2014
Available online 17 July 2014

Keywords:
Reactive oxygen species
Nuclear factor-kappa B
Tumor growth
Hepatocellular carcinoma
Romo1
Oxidative stress

ABSTRACT

Deregulation of nuclear factor-κB (NF-κB) and related pathways contribute to tumor cell proliferation and invasion. Mechanisms for constitutive NF-κB activation are not fully explained; however, the underlying defects appear to generate and maintain pro-oxidative conditions. In hepatocellular carcinoma (HCC) tissues, up-regulation of reactive oxygen species modulator 1 (Romo1) correlates positively with tumor size. In the present study, we showed that Romo1 expression is required to maintain constitutive nuclear DNA-binding activity of NF-κB and transcriptional activity through constitutive IκBα phosphorylation. Overexpression of Romo1 promoted p65 nuclear translocation and DNA-binding activity. We also show that Romo1 depletion suppressed anchorage-independent colony formation by HCC cells and suppressed tumor growth *in vivo*. Based on these findings, Romo1 may be a principal regulatory factor in the maintenance of constitutive NF-κB activation in tumor cells. In the interest of anti-proliferative treatments for cancer, Romo1 may also present a productive target for drug development.

1. Introduction

Nuclear factor-kappa B (NF-κB) is a redox-sensitive transcription factor that regulates expression of various genes involved in cell proliferation and inflammation [1,2]. NF-κB is activated in primary tumors induced by carcinogens [3]. Deregulation of NF-κB contributes to the proliferation, resistance to apoptosis, angiogenesis, invasiveness and metastasis of various tumor cell types [4,5]. It may promote tumor development by favoring cell survival rather than by tumor initiation [6]. NF-κB may also inhibit cell death in response to diverse apoptotic stimuli such as tumor necrosis factor (TNF)-α and chemotherapeutic drugs through up-regulation of anti-apoptotic genes [7].

NF-κB activation promotes tumor cell survival by regulating genes such as cyclin D1 that govern cell cycle progression [1]. Inhibition of constitutive NF-κB activity may significantly suppress tumor formation and growth after xenotransplantation of malignant cells into severe combined immunodeficient (SCID) mice [8–10]. Some findings suggest that constitutive activation of NF-κB results from deregulated IκB kinase (IKK) [11,12], which promotes IκBα phosphorylation.

A variety of pro-inflammatory stresses may activate the reactive oxygen species modulator 1 (Romo1), which stimulates cellular reactive oxygen species (ROS) production. Such stressors include phorbol 12-myristate 13-acetate (PMA) and 5-FU [13–15], and intracellular proinflammatory signaling factors such as TNF-α and c-Myc [16,17]. ROS production plays regulatory roles in c-Myc turnover and TNF-α signaling. Romo1 overexpression is observed in most cancer cell lines and hepatic tumor tissues [13,15] and Romo1 up-regulation correlates positively with ROS levels in cancer cells and with tumor progression [14,15]. Mitochondrial ROS may induce NF-κB-dependent gene transactivation [18,19]; however, ROS may either activate or inhibit NF-κB in a cell type-specific manner [20]. Persistent NF-κB activation is found in many cancer cell types [1,3,9,21–23] and ROS concentrations are also relatively high in cancer cells [14,24,25]. Therefore, we investigated the correlation between the expression of Romo1 and NF-κB activation. Our results, presented here, indicate that Romo1 expression leads to an increase in NF-κB activity, which promotes tumor cell growth.

2. Materials and methods

2.1. Cell culture and reagents

Human hepatocarcinoma cells (Huh-7), breast cancer cells (MDA-MB-231, T47D and MCF-7), cervical carcinoma cells (HeLa), prostate cancer cells (DU145), human embryonic kidney (HEK) 293 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-Invitrogen, Grand Island, NY). All media contained 10% heat-inactivated FBS (Gibco-Invitrogen), sodium bicarbonate

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http://dx.doi.org/10.1016/j.bbrc.2014.07.059
0006-291X/

(2 mg/ml; Sigma–Aldrich, St Louis, MO), penicillin (100 units/ml), and streptomycin (100 μg/ml; Gibco-Invitrogen) and were grown in 5% CO_2 at 37 °C. N-acetyl-L-cysteine (NAC), myxothiazol and N-carbobenzoxy-1-leucinyl-1-leucinyl-1-norleucinal (MG132) were obtained from Sigma–Aldrich. MitoSOX was obtained from Molecular Probes (Eugene, OR). Rabbit polyclonal anti-phospho-p65 (Ser^{536}) and anti-IκBα, and mouse polyclonal anti-phospho-IκBα ($Ser^{32/36}$) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Mouse polyclonal anti-p65, antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-cytosol-specific-β-actin, anti-FLAG (M2) and anti-HA antibodies were from Sigma–Aldrich. Mouse monoclonal antibody against Romo1 has been described previously [16].

2.2. siRNA transfection

Romo1 double-stranded small interfering (siRNA) sequences were described previously [17,26]. Control and *Romo1* siRNA sequences were purchased from Bioneer (Taejon, Republic of Korea). The cells (3×10^5) were seeded into 60-mm plates and then transfected with *Romo1* siRNA using Lipofectamine™ (Gibco-Invitrogen).

2.3. shRNA and lentivirus

The shRNA construct in pLKO.1-puro targeting Romo1 sequence was purchased from Sigma–Aldrich. *Romo1* shRNA in the pLKO.1-puro vector was co-transfected with an expression vector containing the *gag*/*pol* and *vsvg* genes into 293 TN cells. Lentivirus was harvested at 48 h after transfection and 8 μg/ml of polybrene was added. Huh-7 and MDA-MB-231 cells were infected with lentivirus and stable transfectant cells were selected in 250 ng/ml of puromycin for 7 days.

2.4. Promoter activity assay

Cells (2×10^5) were seeded into 6-well dishes and then co-transfected with 2 μg of a NF-κB-specific promoter-reporter consisting of three tandem κB sites upstream of a luciferase construct and 0.5 μg of a *β-galactosidase* plasmid using Lipofectamine reagent (Gibco-Invitrogen). After 24 h of transfection, the cells were lysed, and luciferase and β-galactosidase activities were assayed according to the manufacturer's instructions (Promega). Luciferase activity was normalized to the β-galactosidase activity.

2.5. Preparation of cellular extracts and electrophoretic mobility shift assay

The NE-PER® Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, Rockford, IL) was used to perform nuclear and cytosolic extraction according to the manufacturer's instructions. The electrophoretic mobility shift assay (EMSA) for NF-κB was performed using the Gelshift™ Chemiluminescent EMSA Kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions. The biotin 3′end-labeled double-stranded NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was obtained from Bioneer (Taejon, Republic of Korea). For suppression of the complex formation, nuclear proteins were pre-incubated with antibody against p65 (1 μg) for 30 min at 37 °C before the addition of labeled oligonucleotide.

2.6. Western blotting and immunofluorescence assay

The Western blotting and immunofluorescence assay were described previously [16].

2.7. In vitro colony-forming assay

Cells (1×10^4) were mixed with medium containing 0.4% agar and seeded onto medium containing 0.6% agar. Complete medium was added onto the top layer every 3 days. Colonies were stained with 0.5 mg/ml nitroblue tetrazolium and counted after 14 days.

2.8. Tumorigenicity assay

Male 5-weeks-old athymic BALB/c nu/nu mice were obtained from Hanlim Animal Center (Suwon, Republic of Korea). Mice ($n = 6$/group) were subcutaneously injected with 5×10^6 cells in a volume of 200 μl PBS. The size of the tumor was measured every 2 days and tumor volumes were estimated as width^2 × length × 0.52.

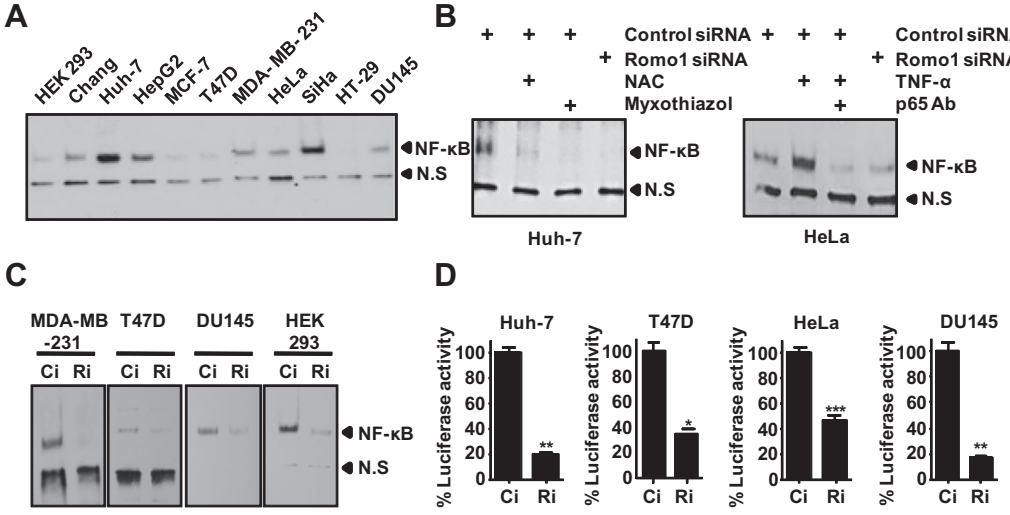

Fig. 1. Constitutive activation of NF-κB by Romo1 expression. (A) EMSA was performed using nuclear extracts from various cell lines. (B) Huh-7 cells were transfected with *Romo1* siRNA for 48 h and then incubated with NAC (20 mM) or myxothiazol (1 μM) for 4 h. HeLa cells were transfected with *Romo1* siRNA and then incubated with TNF-α (20 ng/ml) or anti-p65 antibody; EMSA was performed using nuclear extracts from these cells. (C) EMSA was performed using nuclear extracts from MDA-MB-231, T47D, DU145 and HEK 293 cells transfected with *Romo1* siRNA. (D) Huh-7, T47D, HeLa and DU145 cells were co-transfected with *Romo1* siRNA, NF-κB luciferase reporter gene and *β-galactosidase* gene. Cells were lysed and luciferase activity was measured. Data represent the mean (SE) of at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control siRNA by two-way analysis of variance (ANOVA). Ci, control siRNA; Ri, *Romo1* siRNA; N.S, non-specific signal.