Biochemical and Biophysical Research Communications 399 (2010) 300–306

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

I-κBα depletion by transglutaminase 2 and μ-calpain occurs in parallel with the ubiquitin–proteasome pathway

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ARTICLE INFO

Article history:
Received 7 July 2010
Available online 24 July 2010

Keywords:
Transglutaminase 2
I-κBα
NF-κB
μ-Calpain
Proteasome

ABSTRACT

Transglutaminase 2 (TGase2) is a calcium-dependent, cross-linking enzyme that catalyzes iso-peptide bond formation between peptide-bound lysine and glutamine residues. TGase 2 can activate NF-κB through the polymerization-mediated depletion of I-κBα without IKK activation. This NF-κB activation mechanism is associated with drug resistance in cancer cells. However, the polymers cannot be detected in cells, while TGase 2 over-expression depletes free I-κBα, which raises the question of how the polymerized I-κBα can be metabolized in cells. Among proteasome, lysosome and calpain systems, calpain inhibition was found to effectively increase the accumulation of I-κBα polymers in MCF7 cells transfected with TGase 2, and induced high levels of I-κBα polymers as well in MDA-MB-231 breast cancer cells that naturally express a high level of TGase 2. Inhibition of calpain also boosted the level of I-κBα polymers in HEK-293 cells in case of TGase 2 transfection either with I-κBα or I-κBα mutant (S32A, S36A). Interestingly, the combined inhibition of calpain and the proteasome resulted in an increased accumulation of both I-κBα polymers and I-κBα, concurrent with an inhibition of NF-κB activity in MDA-MB-231 cells. This suggests that μ-calpain proteasome-dependent I-κBα polymer degradation may contribute to cancer progression through constitutive NF-κB activation.

1. Introduction

The constitutive activation of nuclear factor (NF)-κB plays a key role in cancer cell proliferation, angiogenesis, metastasis, and drug resistance [1]. Ubiquitin–proteasome-dependent NF-κB activation is the most common pathway for signal-induced NF-κB activation [2]. However, most cancers with high constitutive NF-κB activity do not always depend on the I-κB kinase (IKK)-mediated processing of the inhibitor of NF-κB (I-κB) by the ubiquitin–proteasome pathway [3]. Therefore, understanding the mechanism of constitutive NF-κB activation in cancer cells is important.

Increased expression of transglutaminase 2 (EC 2.3.2.13, TGase 2) is closely associated with the pathogenesis of certain cancers [4]. However, the role of TGase 2 in oncogenesis remained unclear until a report from our group described the TGase 2-mediated constitutive activation of NF-κB [5]. Recent studies report that overexpression of TGase 2 is associated with constitutively activated NF-κB in several cancer cells including lung, brain, skin, breast, and pancreatic cancer cells [6,7] and breast tumors [8,9]. TGase 2 is a calcium-dependent cross-linking enzyme that catalyzes the formation of covalent iso-peptide bonds between peptide-bound lysine and glutamine residues [10]. In the canonical pathway, NF-κB is activated by I-κBα degradation via phosphorylation, ubiquitination and proteasome degradation [1], whereas TGase 2 causes I-κBα depletion via protein polymerization [11]. Increased TGase 2 expression has been reported in drug resistant cancer cells [12] and was shown to result in constitutive NF-κB activation [12–14]. The fate of polymerized I-κBα is unknown. Polymers that avoid degradation may accumulate and cause cytotoxicity, as in the case of protein deposits found in Lewy bodies, neurofibrillary tangles and nuclear inclusions [15]. Accumulation of polymerized I-κBα was not detected in cancer cells, although TGase 2 expression was constitutively increased concomitant with depletion of free I-κBα [16]. The processing of polymerized I-κBα could be mediated by a proteolytic pathway designed to allow rapid turnover of I-κBα. To test this theory, proteolytic degradation pathways were analyzed using pathway specific inhibitors, which showed that the μ-calpain system was required for the processing of polymerized I-κBα into fragments further metabolized by the proteasome. Interestingly, calpain inhibition reversed TGase 2-mediated NF-κB activation efficiently in addition to causing proteasome inhibition. This μ-calpain/proteasome pathway may be responsible for the rapid turnover of TGase 2-mediated I-κBα polymers in drug resistant cancer cells.

Abbreviations: TGase, transglutaminase; IKK, I-κBα kinase; NF-κB, nuclear factor-κB; I-κBα, inhibitory subunit of NF-κB; CTM, cystamine.

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doi:10.1016/j.bbrc.2010.07.078

2. Materials and methods

2.1. Antibodies and reagents

Anti-I-κBα and anti-phospho32/36-I-κBα antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA); anti-TGase 2 antibody was from NeoMarkers (clone CUB 7402; Fremont, USA); anti-β-actin from Abcam (Headquarters: Cambridge, UK); and anti-ubiquitin antibody and anti-calpain (H240) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000, Lipofectamine RNAiMAX transfection reagents, and the Stealth RNAi Negative Control were obtained from Invitrogen (Carlsbad, CA, USA); FITC-conjugated anti-rabbit and TRITC-conjugated anti-mouse IgGs were from Jackson ImmunoResearch (West Grove, PA, USA).

2.2. Cell culture and sub-cellular preparation

Human breast cancer cell lines including MCF7 and MDA231 were obtained from the American Type Culture Collection. Cells were grown in RPMI or DMEM (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone), 1 mM sodium pyruvate (Gibco-BRL, Grand Island, NY, USA), 10 mM HEPES and 100 U/ml penicillin–streptomycin (Gibco-BRL), and maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Transient transfection and inhibitor treatments

Experiments involving transient expression of TGase 2 were conducted using cDNAs encoding full-length human TGase 2 cloned into the pcDNA3.0 vector. Transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cells were seeded in a six-well plate at a density of 4×10^5 cells/well, 1 day prior to transfection. After 24 h and when cells had reached 50–60% confluence, the medium was removed and cells were washed with 2 ml Opti-MEM (Invitrogen, Carlsbad, CA), followed by the addition of a DNA–lipofectamine mixture (2 μg DNA and 3 μl lipofectamine reagent) to 1 ml of the Opti-MEM, and incubation for 6 h in 5% CO_2 at 37 °C. The transfection mixture was then removed and replaced with fresh culture medium for 24 h. Transfected cells were then treated with calpeptin (50 μM, CALBIOCHEM), Leupeptin (100 μM, Sigma), Chloroquine (100 μM, Sigma), or MG132 (10 μM, CALBIOCHEM) for 16 h.

2.4. μ-Calpain gene silencing by small interfering RNA

A small interfering RNA (siRNA) duplex targeting human μ-calpain, 5′-UAGAUGGUGGUGUUCCAUUUGCGGA-3′ (Invitrogen, Carlsbad, CA), was introduced into cells using Lipofectamine RNAiMAX, according to the manufacturer's instructions. At 48 h post-transfection, the cells were harvested and the cytosolic fraction was prepared for use in analyzing μ-calpain levels by Western blotting. Cells incubated with Lipofectamine RNAiMAX and universal negative siRNA (Invitrogen, Carlsbad, CA) were employed as the negative controls.

2.5. Western blotting

Western blotting was performed by following previously established methods, with the exception of I-κBα polymer samples, which were transferred to polyvinylidene difluoride membranes by the wet transfer method. The primary antibodies used in these studies were anti-I-κBα, anti-phospho-I-κBα, anti-TGase 2, anti-β-actin, and anti-calpain 1.

2.6. Immunoprecipitation

For immunoprecipitation assays, cytosolic fractions were prepared using the CelLytic™ NuCLEAR™ Extraction Kit (Sigma). After precleaning the lysate with A/G-agarose beads (PIERCE), the cytosolic fraction was mixed with lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP40) supplemented with a protease and phosphatase inhibitor cocktail (Sigma). The mixture was incubated overnight at 4 °C with diluted antibodies (1:100). A/G-agarose beads were then added and incubated for 3 h at 4 °C, after which the beads were washed four times with lysis buffer. The immune complex was released from the beads by boiling in 1.5× SDS sample buffer and then analyzed by Western blotting using the antibodies indicated.

2.7. Immunocytochemistry

I-κBα and TGase 2 were visualized by immunocytochemistry and confocal microscopy. Cells were seeded in two-well slide chambers (1×10^5 cells/well) 1 day before transfection. Cells were transfected with mock vector or TGase 2 for 24 h, and then treated with cystamine (1 mM, CTM) or calpeptin for 4 and 3 h, respectively. After medium removal, immunocytochemistry was done by the established method.

2.8. Isolation of the cytoplasmic fraction

Sample cytoplasmic fractions were prepared using the CelLytic™ NuCLEAR™ Extraction Kit (Sigma). Briefly, cells were harvested and resuspended in cellular lysis buffer (including DTT and protease inhibitors), and kept on ice for 15 min. After incubation on ice, 10% IGEPAL CA-630 solution (30 U/500 μl lysate) to a final concentration of 0.6% (6 μl/100 μl of mixture) was added and vortexed vigorously for 10 s, followed by centrifugation at 11,000 rpm, for 30 s. The resulting supernatant was saved as the cytosolic fraction.

2.9. Polymerization of I-κBα by human transglutaminase 2 and degradation by μ-calpain

Purified and concentrated 4 μg of human TGase 2 was incubated with human I-κBα (6 μg) at 37 °C for 2 h in reaction buffer (100 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10 mM $CaCl_2$, 5 mM DTT), and then treated with active μ-calpain (2 μg, μ-calpain, BioVision) at 37 °C for 4 h.

3. Result

3.1. TGase 2-induced I-κBα polymers do not accumulate in cancer cells

The levels of free I-κBα were reduced in the cytosolic fractions of TGase 2-transfected MCF7 and MDA-MB-231 breast cancer cells that naturally express a high level of TGase 2 (Fig. 1A). In addition, TGase 2 expression was highly increased in MDA-MB-231 cells that also exhibit constitutive NF-κB activation [6]. Detection of free I-κBα was correlated with the absence of I-κBα polymers. However, extended exposure times allowed detection of the dimer–trimer of I-κBα in MCF7/TGase 2 cells, while of I-κBα polymer was not detected in MCF7/TGase 2 cells (Fig. 1A). The decrease of free I-κBα was observed to be independent from I-κBα phosphorylation (Supplementary Fig. 1). Immunocytochemical staining of I-κBα and TGase 2 was performed in MCF7 cells with or without TGase 2 transfection (Fig. 1B). As shown in Fig. 1B, the level of TGase 2 expression showed an inverse correlation with the level of I-κBα in the cytosol. I-κBα shows almost stained pattern in cytosolic distribution in MCF7 cell, while I-κBα was only barely detected in