Biochemical and Biophysical Research Communications 420 (2012) 651–655

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

SKP1-CULLIN1-F-box (SCF)-mediated DRG2 degradation facilitated chemotherapeutic drugs induced apoptosis in hepatocellular carcinoma cells

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ARTICLE INFO

Article history:
Received 6 March 2012
Available online 17 March 2012

Keywords:
DRG2
SKP1-CULLIN1-F-box
Chemotherapeutic drugs
Hepatocellular carcinoma cells

ABSTRACT

Developmentally regulated GTP-binding protein 2 (DRG2), an evolutionarily conserved member of the DRG subfamily in the GTP-binding protein, is thought to play an essential role in the control of cell growth and differentiation. However, the role of DRG2 in hepatocellular carcinoma cells is largely unknown. Here, we show that DRG2 is down-regulated during chemotherapeutic drug induced apoptosis in four hepatocellular carcinoma cell lines. We further provided evidence that DRG2 was a substrate of a SKP1-CULLIN1-F-box E3 ligase complex and inhibition the function of Cullin1 prevented the degradation of DRG2 during apoptosis. Moreover, over-expression of DRG2 inhibited doxorubicin induced apoptosis in hepatocellular carcinoma cells. Taken together, these results demonstrate that regulated degradation of DRG2 has a role in chemotherapeutic drug induced hepatocellular carcinoma cells apoptosis.

1. Introduction

Hepatocellular carcinoma is the fifth most common cancer and the fourth leading cause of cancer-related mortality worldwide [1]. Although surgical management has been wildly used to against hepatocellular carcinoma, the incidence of this disease continues to rise year by year [2]. Apoptosis is an important physiological process of cell death and occurs during tissue remodeling, immune regulation, and tumor regression. Most of the chemotherapeutic drugs cause cancer cells death by inducing apoptotic death pathways [3]. Apoptosis is a highly regulated cell suicide process that is mediated through two central pathways: the extrinsic pathway involving death receptors and the intrinsic pathway involving the mitochondria/endoplasmic reticulum [4]. When stimulated, these two pathways lead to the release of cytochrome c from the mitochondria and to the activation of caspase 3 and cell death [4].

Fundamental cellular functions such as cell growth, differentiation and apoptosis are accomplished by large, multi-protein and highly precisely regulated molecular machines. Numerous reports have clearly shown that E3 ubiquitin ligases have essential roles in cell death controls by targeting key apoptosis-regulated proteins for destruction [5–9]. E3 ubiquitin ligases have been classified into three groups: the single-subunit RING-FINGER type, the multisubunit RING-finger type and the HECT-domain type. Most of the multi-subunit RING-finger type of E3 ligases contains a Cullin protein including Cul1, Cul2, Cul3, Cul4, Cul5 and Cul7 [10]. Many structural and functional details have been described for the most well-characterized mammalian Cullin-dependent ligase—the SKP1-CULLIN1-F-box (SCF) ligase [11–13]. In this ligase, Cul1 functions as a molecular scaffold that simultaneously interacts at the amino terminus with the crucial adaptor subunit Skp1 which recruits one of many F-box proteins and at the carboxyl terminus with a RING-finger protein Rbx1 and a specific E2 enzyme such as Ubc3, Ubc4 or Ubc5. Each F-box protein appears to be matched with a discrete number of specific substrates through a protein–protein interaction domain [14].

Developmentally regulated GTP-binding proteins (DRG) are an evolutionally conserved novel GTP binding protein. These proteins harbor the five characteristic motifs, G1–G5, that are believed to interact with GTP [15]. Apart from these motifs, they do not display significant similarity with the well-characterized G-proteins and therefore, they constitute a new subfamily within the superfamily of GTP-binding proteins [16]. There are at least two distinct members, DRG1 and DRG2, and they are widely expressed in human and mouse tissues and show a very similar distribution pattern [15], which suggests the similar functions of these two DRGs. Mouse DRG1 expression was highly regulated during embryonic development and over-expression of mouse DRG1 together with c-myc and ras was found to stimulate cell transformation in fibroblast [17]. Fish DRG2 was increased by rhabdovirus infection and human DRG2 was down-regulated in fibroblasts transformed by SV40 [18]. Over-expression of DRG2 increased G2/M phase cells and decreased sensitivity to nocodazole-induced apoptosis in human T cells [19]. However, whether DRG2 plays a role in hepatocellular carcinoma is completely unknown.

Here, we show that DRG2 was ubiquitinated and degradated by a SCF complex during apoptosis in hepatocellular carcinoma cell

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.03.058

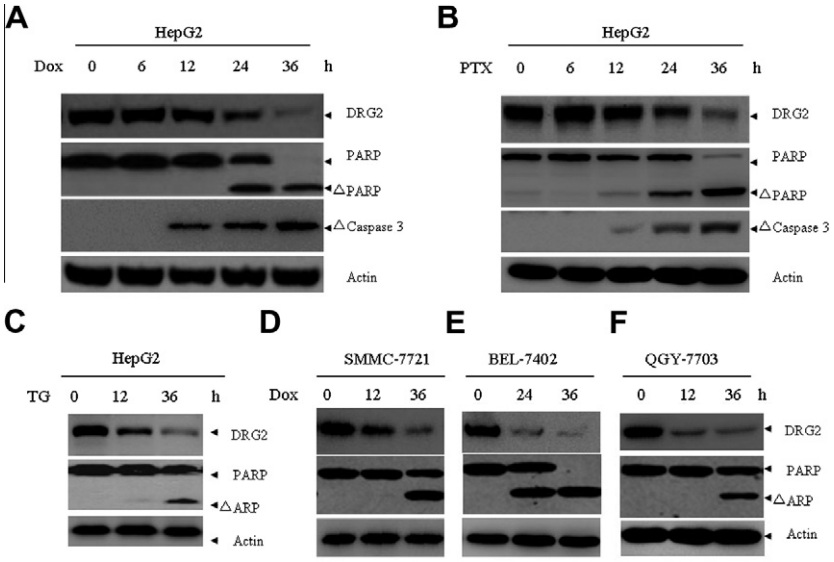

Fig. 1. DRG2 was down-regulated during apoptosis in hepatocellular carcinoma cell lines. (A) HepG2 cells were treated with Dox (1 μg/ml) for indicated times and the indicated proteins were detected by western blot. (B and C) HepG2 cells were treated with PTX (1 μM) (B) or TG (1 mM) (C) for indicated times and the indicated proteins were detected by western blot. (D–F) SMMC-7721 (D), BEL-7402 (E) and QGY-7703 (F) cells were treated with Dox for indicated times and the indicated proteins were detected by western blot.

lines. Inhibition of SCF by dominant Cullin1 prevented the degradation of DRG2 during apoptosis. Over-expressing of DRG2 in hepatocellular carcinoma cells inhibited doxorubicin induced apoptosis. Our data might shed new insight on the role of DRG2 in hepatocellular carcinoma cells apoptosis and the post-translational regulation of DRG2.

2. Materials and methods

2.1. Cell culture and drugs

293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD). Hepatocellular carcinoma cell lines including HepG2, SMMC-7721, BEL-7402, and QGY-7703 (from Cell Bank of Shanghai Institutes of Biological Sciences, Shanghai, China) were cultured in RPMI-1640 medium (Sigma, St Louis, MI) supplemented with 10% fetal calf serum. All these cells were cultured in a 5% CO_2/95% air at 37 °C. DMSO and drugs in this work were purchased from sigma.

2.2. Antibodies

Antibodies were obtained from the following sources: anti-DRG2 (BD Biosciences, USA), anti-cleaved caspase-3 (Cell Signaling, USA), anti-poly-ADP ribose polymerase (PARP), anti-Flag M2 (Sigma, USA), anti-HA, Cul1, Skp1 (Santa Cruz Biotech, USA) and anti-actin (Calbiochem, Germany),

2.3. Plasmids and transfection

All the plasmids of this work are purchased from Addgene. All the transient transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. RNA isolation and real-time PCR analysis

Total RNA was isolated from tissues or cells using TRIzol (Invitrogen) according to the manufacturer's instructions. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on LightCycler 480 (Roche, Switzerland). The primers used were available upon request.

2.5. Western blotting

Protein extracts were equally loaded on 10% SDS–PAGE, electrophoresed, and transferred to nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK). After blocking with 5% nonfat milk in PBS, the membranes were incubated with the indicated primary antibodies and followed by horseradish peroxidase (HRP)-linked secondary antibodies (Cell signaling). The signals were detected by chemiluminescence phototope-HRP kit (Pierce Biotechnology, Rockford, USA) according to manufacturer's instructions.

2.6. Immunoprecipitation

Cells were lysed in 6 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5% Nonidet P40, Roche complete EDTA-free protease inhibitor cocktail) for 20 min with gentle rocking at 4 °C. Lysates were cleared using centrifugation (13,000 rpm, 10 min), the supernatant was subjected to immunoprecipitation (IP) with 20 μl of anti-DRG2 antibody for 4 h at 4 °C and 50 μl protein A/G beads (Santa Cruz Biotech, USA) were added and incubated overnight at 4 °C with gentle inversion. Beads containing immune complexes were washed with 1 ml ice cold lysis buffer four times followed by three 1 ml Tris Buffered Saline (TBS) washes. Precipitates were denatured in Laemmli (gel loading) buffer at 95 °C for 5 min.

2.7. Cycloheximide inhibition test

HepG2 cells were treated with Dox in the presence or absence of 10 μg/ml cycloheximide (CHX, Sigma–Aldrich) for the indicated time points. The expression of DRG2 protein was measured by western blots with b-actin as loading control.