Biochemical and Biophysical Research Communications 413 (2011) 282–287

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Loss of DLK expression in WI-38 human diploid fibroblasts induces a senescent-like proliferation arrest

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ARTICLE INFO

Article history:
Received 13 August 2011
Available online 27 August 2011

Keywords:
DLK protein kinase
Cell proliferation
Cell cycle progression
Senescence
WI-38 cells

ABSTRACT

DLK, a serine/threonine kinase that functions as an upstream activator of the mitogen-activated protein kinase (MAPK) pathways, has been shown to play a role in development, cell differentiation, apoptosis and neuronal response to injury. Interestingly, recent studies have shown that DLK may also be required for cell proliferation, although little is known about its specific functions. To start addressing this issue, we studied how DLK expression is modulated during cell cycle progression and what effect DLK depletion has on cell proliferation in WI-38 fibroblasts. Our results indicate that DLK protein levels are low in serum-starved cells, but that serum addition markedly stimulated it. Moreover, RNA interference experiments demonstrate that DLK is required for ERK activity, expression of the cell cycle regulator cyclin D1 and proliferation of WI-38 cells. DLK-depleted cells also show a senescent phenotype as revealed by senescence-associated galactosidase activity and up-regulation of the senescence pathway proteins p53 and p21. Consistent with a role for p53 in this response, inhibition of p53 expression by RNA interference significantly alleviated senescence induced by DLK knockdown. Together, these findings indicate that DLK participates in cell proliferation and/or survival, at least in part, by modulating the expression of cell cycle regulatory proteins.

1. Introduction

DLK is a serine/threonine kinase that acts as an upstream activator of the MAPKs, among which the extracellular signal-regulated kinases (ERKs), p38 kinases and c-Jun N-terminal kinases (JNKs) are best characterized in mammals [1]. It is expressed in a tissue-specific manner and targeted deletion of the *DLK* gene in mice results in perinatal death with defects in brain development [2]. Recent analyses of mice, flies and worms bearing loss of functions mutations in either DLK or its invertebrate orthologs also demonstrated that this enzyme plays a central role in the neuronal response to injury, being required for axon regeneration or degeneration depending on the species [3,4]. Additionally, a number of studies carried out over the last few years have provided support for pro-differentiation and pro-apoptotic functions of DLK [5,6].

More recently, a novel functional role for DLK in the response of cells to growth factors has emerged from RNAi-based experiments demonstrating that DLK knockdown in mouse fibroblasts substantially reduced platelet-derived growth factor (PDGF)-stimulated activation of the ERK signaling pathway [7]. As abundantly documented in the literature, this pathway plays a crucial role in cell proliferation control by relaying mitogenic signals from activated receptors to downstream effectors in the cytoplasm and the nucleus [8]. Ultimately, ERK signaling leads to the induction of factors involved in cell proliferation, including cyclin D1, which promotes G1 to S phase transition [8]. Loss of ERK function, on the other hand, causes G1 arrest and premature senescence in fibroblasts [9–12], suggesting that ERK is critical for cell cycle progression.

Because these data raise the possibility that DLK might play a role in cell cycle control, the aim of the present study was to investigate whether DLK is required for cell proliferation. Using WI-38 human diploid fibroblasts as a model, we show that DLK expression is modulated during cell cycle progression and that DLK depletion by RNA interference causes inhibition of ERK activity concomitantly with down-regulation of cyclin D1 expression, G1 growth arrest and senescence. Thus, DLK appears to be important for proper cell proliferation and/or cell survival.

2. Materials and methods

2.1. Antibodies

The polyclonal antiserum used for detection of DLK was described previously [13]. The cyclin D1 (catalogue number sc-718) polyclonal, p21 (catalogue number sc-397) polyclonal and p53 (catalogue number sc-126) monoclonal antibodies were purchased

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doi:10.1016/j.bbrc.2011.08.086

from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The phospho-Rb (catalogue number 9307) polyclonal antibody and the monoclonal antibodies against the total or phosphorylated, activated forms of ERK (catalogue numbers 9102 and 9106), JNK (catalogue numbers 9252 and 9255), p38 (catalogue numbers 9212 and 9215) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). The polyclonal antibody against γ-actin was from Sigma–Aldrich Canada Ltd. (Oakville, Ontario).

2.2. Cell culture

Human WI-38 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. When indicated, cells were serum-starved by incubation in DMEM containing 0.1% FBS for 4 days and then cultured in DMEM supplemented with 10% FBS for 12 or 24 h.

2.3. Preparation of cell lysates and immunoblotting

Cells were lysed for 60 min at 4 °C in lysis buffer (50 mM Tris–HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin). Lysates were clarified by centrifugation (12,000 × g for 10 min at 4 °C) and the concentration of total protein in the supernatant fraction was quantified by the modified Bradford protein assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada). For immunoblotting, equal amounts of proteins were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics, Laval, Québec, Canada) using a semidry transfer apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Membranes were incubated overnight on a rotating plate at 4 °C in a solution containing 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20 (TBS-T) supplemented with 5% skim milk powder (w/v) and the primary antibody. Membranes were washed two times in TBS-T before incubation in a solution containing TBS-T, 5% skim milk powder (w/v) and the secondary horseradish peroxidase-conjugated antibodies on a rotating plate for 1 h at room temperature. Membranes were washed two more times in TBS-T before immunoreactive bands were detected by enhanced chemiluminescence (ECL Plus Western blotting kit, Amersham Pharmacia Biotech, Inc.).

2.4. Lentivirus production and infection of WI-38 cells

293T cells grown in DMEM supplemented with 10% (v/v) FBS and antibiotics were cotransfected with the envelope protein expressing vector pMD2.G and the packaging protein expressing vector psPAX2, (kindly provided by Dr. Didier Trono University of Geneva Medical School, Geneva, Switzerland) and with either the transfer pLKO.1 empty lentiviral vector [14] (Addgene, Cambridge, MA, USA, plasmid 8453), the pLKO.1-based lentiviral mouse DLK shRNA vector (clone TRCN0000022570, Open Biosystems, Huntsville, AL, USA) as a control, the pLKO.1-based lentiviral human DLK shRNA vectors (clones TRCN0000001000 [Sh-hDLK1] and TRCN0000001001 [Sh-hDLK2], Open Biosystems) or the pLKO.1-based human p53 shRNA vector [15] (Addgene, plasmid 19119) using FuGENE 6 reagent (Roche Diagnostics, Laval, Québec, Canada). At 72 h post-transfection, the culture medium containing lentiviruses was harvested, treated with polybrene (8 μg/ml) and filtered. Subconfluent WI-38 cells were incubated with viral supernatants for 24 h, washed twice with phosphate-buffered saline (PBS) and incubated with normal medium for the indicated times. Then, the cells were harvested and processed for further analyses.

2.5. Cell proliferation

To determine the effects of DLK knockdown on proliferation, WI-38 (5×10^4) cells were seeded in 100 mm dishes and infected 24 h later with either the pLKO.1 empty lentiviral vector or the lentivirus expressing human or mouse DLK shRNA. On day 1, 3 or 5 after infection, cells were harvested with trypsin and the number of cells was determined using a hemocytometer.

2.6. Cell cycle analysis

WI-38 cells were collected by trypsin digestion and stained with propidium iodide. DNA content per cell was determined by flow cytometry (FACScalibur; BD Biosciences) and used to estimate the proportion of cells in each phase of the cell cycle.

2.7. β-Galactosidase staining

Senescence of WI-38 cells was evaluated by β-galactosidase staining as described previously [16]. Cell counts were made on five random fields of at least 200 cells.

3. Results

In an attempt to elucidate the role of DLK in cell proliferation, we first examined its expression during cell cycle progression using the WI-38 human diploid fibroblast cell line as a model. WI-38 cells were made quiescent by serum starvation for 4 days in medium containing 0.1% fetal bovine serum (FBS) and then released from quiescence by treating them with 10% FBS for 6, 12 or 24 h. Our results indicated that DLK protein levels are down-regulated in response to serum starvation and substantially up-regulated upon serum readdition (Fig. 1A), showing that DLK expression is modulated by exit and entry into the cell cycle. The accumulation of DLK became detectable 6 h after serum readdition to starved cells, which coincides well with the increase in expression of cyclin D1, a critical positive regulator of G1 progression [17]. DLK recovery also precedes the increase in retinoblastoma protein (pRb) phosphorylation and the entry of cells into S phase (Fig. 1A and B).

To more directly address the role of DLK in cell cycle progression, WI-38 cells were depleted of endogenous DLK by RNA interference. Knockdown of DLK was accomplished by infecting WI-38 cells with lentiviral vectors (numbered Sh-hDLK1 and -2) carrying short hairpin RNAs (shRNA) that target distinct regions of human DLK mRNA. Western blotting (Fig. 2A) showed that a substantial knockdown of DLK was achieved in cells infected with both lentiviral vectors encoding human DLK shRNAs. In contrast, infection with either an empty lentivirus or a lentivirus expressing a mouse DLK shRNA (Sh-mDLK), with a few nucleotide mismatches compared to human DLK, was without effect. Using the Sh-hDLK2 lentiviral construct for subsequent studies, we first examined the consequences of DLK knockdown on the MAPK signaling pathways. To do so, the activity of JNK, ERK and p38 was measured in DLK-depleted and control cells by Western blotting using antibodies specific to the phosphorylated, activated forms of these proteins. Our results showed no difference between DLK-depleted and control cells in the levels of JNK phosphorylation and basal p38 phosphorylation was undetectable in any of these conditions (Fig. 2B). However, in DLK-depleted cells, we noted a substantial decrease in ERK phosphorylation level relative to control (Fig. 2B), indicating that ERK activity in WI-38 cells is dependent on DLK.

Given the known requirement of ERK for cell cycle progression, cell proliferation and survival [8], we measured the effects of DLK depletion on cell proliferation 1, 3 or 5 days after infection. Cells in-