Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 255 (2007) 117–126
CANCER Letters
www.elsevier.com/locate/canlet

Zinc-induced downregulation of Notch signaling is associated with cytoplasmic retention of Notch1-IC and RBP-Jk via PI3k–Akt signaling pathway

Sang-Hyun Baek [a], Mi-Yeon Kim [a], Jung-Soon Mo [a], Eun-Jung Ann [a], Kyu Shik Lee [a], Ji-Hye Park [a], Jin-Young Kim [a], Mi-Sun Seo [a], Eui-Ju Choi [b], Hee-Sae Park [a,*]

[a] *Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, 300, Yongbong-dong, Buk-ku, Gwangju 500-757, Republic of Korea*
[b] *National Creative Research Initiative Center for Cell Death, Graduate School of Biotechnology, Korea University, Seoul 136-701, Republic of Korea*

Received 18 December 2006; received in revised form 4 April 2007; accepted 10 April 2007

Abstract

The Notch signaling pathway appears to perform an important function in the determination of cell fate and in differentiation, in a wide variety of organisms and cell types. In this study, we provide evidence that the inactivation of Notch signaling by zinc is achieved via a PI3K–Akt-dependent, cytoplasmic retention of Notch1-IC and RBP-Jk. Extracellular zinc has been determined to inhibit constitutive active mutants of both Notch1 (ΔEN1) and Notch1-IC-mediated transcription. However, in such cases, neither the cleavage pattern of Notch nor the protein stability of Notch1-IC and RBP-Jk was found to have significantly changed. With regard to the modulation of Notch signaling, zinc appears to exert a significant negative influence on the binding occurring between Notch1 and RBP-Jk, both *in vivo* and *in vitro*. The zinc-induced inhibition of Notch signaling can be rescued via pretreatment with wortmannin or LY294002, both of which are specific PI3K signaling pathway inhibitors. Furthermore, we ascertained that zinc triggers the cytoplasmic retention of Notch1-IC and RBP-Jk, and that cytoplasmic retention could be rescued via treatment with wortmannin. Overall, we have determined that an important relationship exists between zinc and the Notch1 signaling pathway, and that this relationship is intimately involved with the cytoplasmic retention of Notch and RBP-Jk.

Keywords: Notch intracellular domain; RBP-Jk; Zinc; Phosphatidylinositol 3-kinase; Akt; Cytoplasmic retention; Wortmannin; LY294002

1. Introduction

The Notch signaling pathway is an evolutionarily conserved intracellular signaling mechanism, which is known to play key roles in a variety of phenomena,

* Corresponding author. Tel.: +82 62 530 0021; fax: +82 62 530 2199.
E-mail address: proteome@jnu.ac.kr (H.-S. Park).

0304-3835/$ - see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2007.04.002

including the determination of cell fate, differentiation, adult cell self-renewal, cancer, neurodegenerative diseases, wound healing, and inflammation [1–9]. Upon the receipt of extracellular signals mediated via the binding of the specific ligands, Jagged and Delta, the Notch intracellular domain is released via proteolytic cleavages [10–15]. This process triggers the γ-secretase-dependent proteolytic release of the Notch intracellular domain (Notch1-IC) from the membrane, and induces the nuclear translocation of NotchIC, thereby resulting in the formation of a complex with the CSL family (CBF1/RBP-Jk/KBF2 in mammals, Su(H) in *Drosophila* and *Xenopus*, and Lag2 in *Caenorhabditis elegans*), which regulates the transcription of a variety of downstream target genes, including Hes1, Hes5, and Notch itself [16–19]. Subsequent to the transcriptional regulation of the target genes, Notch1-IC is degraded within the nucleus by the ubiquitin–proteosome system, with the aid of Fbw7, an E3 ligase for Notch1-IC ubiquitination [20–22].

Zinc has been the subject of a great deal of study for decades, as it is fundamental to both the structure and function of a host of proteins [23–26]. The maintenance of discrete subcellular pools of zinc contributes to a number of crucial biological processes, including gene expression, DNA synthesis, enzymatic catalysis, hormonal storage and release, neurotransmission, and memory [23–26]. Recent studies have revealed that zinc stored within glutamatergic vesicles is released into the extracellular space in response to several stimuli [27–30]. Zinc has also been implicated in the mechanism underlying neuronal cell death in various acute brain injury models; the physiological roles of zinc release are, however, not yet understood in detail [31–35]. Otherwise, zinc is believed to play a potential antioxidant role, exerting a preventive effect against cell death [36–40]. Zinc, in its anti-apoptotic capacity, has been determined to activate both phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT) pathways [41–43]. Kinases including phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT) have been demonstrated to modulate cell survival [44–47]. AKT phosphorylates and inhibits the activity of glycogen synthase kinase-3β (GSK-3β), inducing the activation of β-catenin/Lef-1 and AP-1 transcription factors [48,49]. GSK-3β, a serine/threonine kinase and a component of the Wnt/wingless signaling cascade, modulates Notch1 signaling via the direct phosphorylation of Notch1-IC, and active GSK-3β protects Notch1-IC against proteosomal degradation [50,51].

In this study, we have assessed the signal crosstalk occurring between zinc and Notch1 signaling. We determined that the transcriptional activity of Notch1 was inhibited by treatment with zinc, and then by the binding of the suppressed Notch1-IC to RBP-Jk. Interestingly, the downregulation of Notch1-IC transcriptional activity in the presence of zinc was found to be the consequence of the cytoplasmic retention of Notch1-IC and RBP-Jk via the activation of the PI3K–AKT signaling pathway. Collectively, our findings appear to indicate that zinc functions as a negative regulator of Notch1 signaling, via the PI3K–AKT signaling pathway.

2. Materials and methods

2.1. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). For plasmid DNA transfection, the cells were plated at a density of 2×10^6 cells/100-mm dish, grown overnight, and transfected with the appropriate expression vectors in the presence of the indicated plasmid DNA combinations, via the calcium phosphate method [52].

2.2. Transcription reporter assay

After 48 h of transfection, the cells were lysed in chemiluminescent lysis buffer (18.3% of 1 M K_2HPO_4, 1.7% of 1 M KH_2PO_4, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT)), then assayed for luciferase activity using a luciferase assay kit (Promega) [53]. The activity of the luciferase reporter protein within the transfected cells was normalized in accordance with the β-galactosidase activity detected in the same cells.

2.3. Western blot analysis

After the 48 h transfection, the cultured HEK293 cells were harvested and lysed in RIPA buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM DTT, and 2 μg/ml leupeptin and aprotinin) for 30 min. The cell lysates were subjected to 20 min of centrifugation at 12,000*g* at 4 °C. The resultant soluble fraction was then boiled in Laemmli buffer and subjected to SDS–PAGE. After gel electrophoresis, the separated proteins were transferred via electroblotting onto