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Distinct functions of the dual leucine zipper kinase depending on its subcellular localization

Manuel Wallbach [a,1,2], Jorge Duque Escobar [b,1], Rohollah Babaeikelishomi [a,b,1,3], Marie-Jeannette Stahnke [a], Roland Blume [a], Sabine Schröder [b], Jenny Kruegel [c], Kathrin Maedler [d], Oliver Kluth [e], Ralph H. Kehlenbach [f], Nicolai Miosge [c], Elke Oetjen [a,b,g,*]

[a] Department of Pharmacology, University of Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany
[b] Department of Clinical Pharmacology and Toxicology, University Medical Center Hamburg Eppendorf, Martinistr. 52, 20246 Hamburg, Germany
[c] Department of Prothetics, Faculty of Medicine, Georg-August-University, GZMB, Göttingen, Humboldtallee 23, 37073 Göttingen, Germany
[d] Center for Biomolecular Interactions Bremen, Leobener Str. Im NW2, 28359 Bremen, Germany
[e] German Institute of Human Nutrition Potsdam-Rehbrücke, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany
[f] Department of Molecular Biology, Faculty of Medicine, Georg-August-University, GZMB, Göttingen, Humboldtallee 23, 37073 Göttingen, Germany
[g] Institute of Pharmacy, University of Hamburg, Bundesstr. 45, 20146 Hamburg, Germany

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ABSTRACT

The dual leucine zipper kinase DLK induces β-cell apoptosis by inhibiting the transcriptional activity conferred by the β-cell protective transcription factor cAMP response element binding protein CREB. This action might contribute to β-cell loss and ultimately diabetes. Within its kinase domain DLK shares high homology with the mixed lineage kinase (MLK) 3, which is activated by tumor necrosis factor (TNF) α and interleukin (IL)-1β, known prediabetic signals. In the present study, the regulation of DLK in β-cells by these cytokines was investigated. Both, TNFα and IL-1β induced the nuclear translocation of DLK. Mutations within a putative nuclear localization signal (NLS) prevented basal and cytokine-induced nuclear localization of DLK and binding to the importin receptor importin α, thereby demonstrating a functional NLS within DLK. DLK NLS mutants were catalytically active as they phosphorylated their down-stream kinase c-Jun N-terminal kinase to the same extent as DLK wild-type but did neither inhibit CREB-dependent gene transcription nor transcription conferred by the promoter of the anti-apoptotic protein BCL-xL. In addition, the β-cell apoptosis-inducing effect of DLK was severely diminished by mutation of its NLS. In a murine model of prediabetes, enhanced nuclear DLK was found. These data demonstrate that DLK exerts distinct functions, depending on its subcellular localization and thus provide a novel level of regulating DLK action. Furthermore, the prevention of the nuclear localization of DLK as induced by prediabetic signals with consecutive suppression of β-cell apoptosis might constitute a novel target in the therapy of diabetes mellitus.

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1. Introduction

Loss of functional pancreatic β-cell mass is the underlying cause for the pathogenesis of diabetes mellitus type 1 and type 2 [1–4]. Proinflammatory cytokines like tumor necrosis factor (TNF) α and interleukin (IL)-1β, gluco- and lipotoxicity have been implicated in β-cell toxicity and ultimately the loss of functional β-cell mass [2,4,5]. Previous studies showed that overexpressed dual leucine zipper kinase (DLK) induces neuronal and β-cell apoptosis [6,7]. DLK belongs to the class of the mixed lineage kinases (MLK) and its catalytic domain shares high homology with the catalytic domain of MLK3 [8,9]. Both, TNFα and IL-1β stimulate MLK3 kinase activity at least in part dependent on the activation of Rac/Cdc42 and the interaction with the Rac/Cdc42 interaction site (CRIB) within MLK3 [10–12].

DLK is expressed in many and diverse tissues like brain and the peripheral nervous system, β-cells and primary islets [8,13,14]. Mice lacking DLK die perinatally, exhibiting impaired axon growth and neuronal radial migration, absence of the anterior commissure and reduced apoptosis in multiple neuronal populations during development [15–17].

Abbreviations: CBP, CREB binding protein; CRE, cAMP responsive element; CREB, CRE binding protein; CRTC, cAMP regulated transcriptional coactivator; DLK, Dual leucine zipper kinase; IL-1β, Interleukin-1β; JIP/IB-1, JNK interacting protein/islet-brain-1; JNK, c-Jun N-terminal kinase; LZK, Leucine zipper kinase; MAPK, Mitogen-activated protein kinase; MBP, Maltose binding protein; MLK, Mixed lineage kinase; NES, Nuclear export signal; NLS, Nuclear localization signal; TNFα, Tumor necrosis factor α.

* Corresponding author at: Department of Clinical Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany.
E-mail address: e.oetjen@uke.uni-hamburg.de (E. Oetjen).

[1] M.W., J.D.E. and R.B. contributed equally.
[2] Present address: Department of Nephrology and Rheumatology, University of Göttingen, Göttingen.
[3] Present address: DKFZ Heidelberg.

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Acting as a mitogen-activated protein kinase kinase kinase (MAP3K) it is generally assumed that DLK exerts its effects through phosphorylation of the dual specificity kinases MKK4/7 and MKK3/6 resulting in the activation of the MAPKs JNK (c-Jun N-terminal kinase) and p38 [6,9,15,18–21]. DLK activity is at least in part regulated by signals triggering its oligomerization [20,22] and by its association with the scaffold protein JIP/IB-1 (JNK interacting protein/islet brain-1): Under basal conditions, monomeric, inactive, unphosphorylated DLK is bound to JIP/IB-1. Phosphorylation of JIP/IB-1 on tyrosine residues by the Src family kinases strengthens the association between DLK and the scaffold protein, thereby keeping DLK in its inactive state [23]. Phosphorylation of JIP/IB-1 on Thr-103 by JNK results in the dissociation of DLK from JIP/IB-1. DLK then homodimerizes via its leucine zipper domain and becomes catalytically active presumably after its autophosphorylation [24–26]. Moreover, JNK-induced phosphorylation of DLK on diverse sites suppresses its ubiquitination [27]. Thus, signals activating JNK may be amplified by the induction of DLK activity and stability, thereby generating a feed-forward loop. In addition, inhibition of the calcium/calmodulin dependent phosphatase calcineurin increased the phosphorylation state of DLK and its catalytic activity, suggesting that calcineurin decreases DLK activity, presumably by impairing its autophosphorylation [7,13,28].

In the β-cell line HIT DLK decreased β-cell function by inhibiting the transcriptional activity of the β-cell specific transcription factor MafA [29]. Furthermore, DLK reduced the transcriptional activity conferred by the cAMP response element binding protein CREB [13,30]. Considering the important role of CREB for the maintenance of β-cell function and mass, this action might well contribute to the loss of β-cell mass and ultimately the development of diabetes mellitus [31–37]. It is however unknown, whether DLK is regulated by the prediabetic cytokines TNFα and IL-1β.

2. Material and methods

2.1. Cell culture and plasmids

The insulin producing pancreatic β-cell line HIT-T15 [38,39] was grown in RPMI 1640 medium supplemented with 10% FCS, 5% horse serum, penicillin and streptomycin (100 μg/ml). The expression vectors for flag epitope tagged DLK wild-type, DLK K185A, DLK PP and for fusion proteins consisting of the DNA binding domain of the yeast transcription factor GAL4 and CREB or CBP (full length) have been described before [13,28,38]. The luciferase reporter genes under control of four copies of the CRE of the rat somatostatin promoter (4×SomCRE), under control of five copies of the DNA binding site for the yeast transcription factor GAL4 and under control of the BCL-xL (from −664 to +617) have been described elsewhere (pG5E1BLuc) [13,40]. The expression vectors for flag epitope tagged DLK NLS1, DLK NLS2 and DLK NES were generated by primerless PCR. The mutations of aa 186 and 188 from lysine and arginine, respectively, to alanine were introduced by 5′-GTGAAGGCGGTTGCAGAT CTCAAGG-3′ as 5′-primer and 5′-CCTTGAGATCTGCAACCGCCTTCAC-3′ as 3′-primer. The mutations of aa 196 lysine, aa 199 arginine, and aa 200 lysine to alanine were introduced using 5′-CTGACA TCGCGCATCTTGCAGCGCTGAAGCACCCCAAC-3′ as 5′-primer and 5′-GCTTCAGCGCTGCAAGATGCGCGATGTCAGTCTCC-3′ as 3′-primer. The nucleotides corresponding to the mutations are underlined. As outside primers served 5′-CATCTAGAGTTCGAGCTGATGAGG-3′ as 5′-primer and 5′-GCGCAGAATTCCATAAGGATGCAG-3′ as 3′-primer. The mutations of aa 481 leucine, aa 483 leucine and aa 485 leucine to alanine were introduced using 5′-CTCATGGCGCAAGCAGAAGCGAAAGAGAGGG-3′ as 5′-primer and 5′-TTTCGCTTCTGCTTGCGCCATGAG-3′ as 3′-primer. The nucleotides corresponding to the mutations are underlined. As outside primers served 5′-ACTGGGTCACCTGAGAGTCGAGCA-3′ as 5′-primer and 5′-TGCATGCTCGAGTCATGGAGGAAGGGAGGC-3′ as 3′-primer. The expression vectors for MBP-DLK wild-type, MBP-DLK NLS1, MBP-DLK NLS2, and MBP-DLK PP encompassing the aa from 140 to 518 of DLK or its mutants were generated by PCR using 5′-AAGGGATCCTCCACAG AACACAAG-3′ as forward and 5′-AAGTGTCGACCTTCACCACATCGTC-3′ as reverse primer and DLK wild-type or the respective mutants as templates. The PCR fragments were cloned into the BamH I and Sal I sites of pMalC2×. All constructs were verified by sequencing.

2.2. Transient transfection assays

For studying CRE-directed gene transcription, the luciferase reporter gene 4×SomCRE, for studying CREB- and CBP-directed transcription, the luciferase reporter gene pG5E1BLuc (2 μg/6 cm-dish) was transiently transfected by the DEAE-Dextran method [41]. Expression vectors for GAL4–CREB, DLK wild-type, DLK K185A, DLK NLS1, DLK NLS2 and DLK NES (2 μg/6 cm-dish, each) were cotransfected as indicated. To balance the amount of DNA, pbluescript (Stratagene, La Jolla, CA, USA) was cotransfected. To check for the transfection efficiency, a GFP reporter fusion gene under control of the cytomegalovirus promoter (GFP-tpz) (0.75 μg/6 cm-dish) was cotransfected. Cells were treated with KCl leading to membrane depolarization as indicated and harvested 48 h after transfection. Reporter gene activities were determined as described [13]. For immunoblot analysis and for immunocytochemistry, the expression vectors for DLK and its mutants were transiently transfected by metafectene (Biontex, Munich), according to the manufacturer's protocol.

2.3. Immunocytochemistry

HIT cells were cultured on coverslips. When indicated, cells were transiently transfected by the metafectene method and treated with TNFα or IL-1β. After 48 h, cells were washed twice with PBS, fixed in 100% methanol (−20 °C), washed three times in PBS, treated with 0.1% fresh sodium borohydride dissolved in PBS and with blocking buffer (10% horse serum, 1% BSA in PBS). For the detection of transfected DLK or its mutants, the murine monoclonal antibody M2 against the flag epitope was used (Sigma, Taufkirchen; Germany) (dilution 1:50) overnight at 4 °C. TRITC-anti-mouse antibody (Invitrogen, Karlsruhe, Germany) was used as secondary antibody. For the detection of endogenous DLK, the DLK antibody against the C-terminal 223 aa of DLK was used [8,42]. The fluorophore-labeled anti-rabbit antibody Alexa Fluor 488 (Invitrogen, Karlsruhe, Germany) (1:50 dilution) served as secondary antibody. For the detection of apoptotic cells, cells were stained with an antibody against cleaved caspase-3 (Cell Signaling, Danvers, MA, USA) (1:50 dilution) overnight. Nuclei were stained by DAPI in the embedding solution. Cells were counted by DAPI stain and cells with nuclear DLK localisation were expressed as percentage of all cells examined. For the determination of apoptosis, cleaved caspase-3 positive-cells were counted and expressed as percentage of cells expressing flag epitope tagged DLK or its mutants. Images were captured by Zeiss Axiovert 200 and Zeiss Axiovert 200 M microscopes complemented with laser module. In each group, approximately 300 cells were counted manually.

2.4. Immunohistochemistry

Pancreata from mice (strain C57BL/6J) fed either normal or high fat/high sucrose diet were processed as described elsewhere [43]. For a quantitative estimate of the nuclear localization of DLK, 10 to 15 β-cells each in 3 to 4 islets from slides of the pancreata of mice with normal or high fat diet were evaluated. The nuclear relative intensity of the DLK representing signal was divided by the relative intensity of the DAPI signal and multiplied by 100. In each group approximately 50 cells were evaluated. Pancreata from NZO mice were processed as described [44]. Slides were stained with the antibody against DLK (1:200), with the secondary TRITC labeled anti-rabbit antibody (1:400) and with DAPI for the detection of nuclei. Images were captured with a LSM 710 Zeiss microscope. All animal studies were conducted

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