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TRIB3 enhances cell viability during glucose deprivation in HEK293-derived cells by upregulating IGFBP2, a novel nutrient deficiency survival factor

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ABSTRACT

Glucose deprivation occurs in several human diseases, including infarctions and solid tumors, and leads to cell death. In this article, we investigate the role of the pseudokinase Tribbles homolog 3 (TRIB3) in the cellular stress response to glucose starvation using cell lines derived from HEK293, which is highly glycolytic under standard conditions. Our results show that *TRIB3* mRNA and protein levels are strongly upregulated in glucose-deprived cells via the induction of activating transcription factor 4 (ATF4) by the endoplasmic reticulum (ER) stress sensor kinase PERK. Cell survival in glucose-deficient conditions is enhanced by *TRIB3* overexpression and reduced by *TRIB3* knockdown. Genome-wide gene expression profiling uncovered approximately 40 glucose deprivation-responsive genes that are affected by TRIB3, including several genes involved in signaling processes and metabolism. Based on transcription factor motif analysis, the majority of TRIB3-downregulated genes are target genes of ATF4, which TRIB3 is known to inhibit. The gene most substantially upregulated by TRIB3 is insulin-like growth factor binding protein 2 (*IGFBP2*). IGFBP2 mRNA and protein levels are downregulated in cells subjected to glucose deprivation, and reduced *IGFBP2* expression aggravates cell death during glucose deficiency, while overexpression of *IGFBP2* prolongs cell survival. Moreover, IGFBP2 silencing abrogates the pro-survival effect of TRIB3. Since TRIB3 augments IGFBP2 expression in glucose-starved cells, the data indicate that IGFBP2 contributes to the attenuation of cell death by TRIB3. These results implicate TRIB3 and IGFBP2, both of which are known to be overexpressed in several types of cancers, as pro-survival modulators of cell viability in nutrient-deficient microenvironments.

1. Introduction

Glucose deprivation is a cell death-inducing condition that occurs during diseases such as cerebral and myocardial infarctions, due to a blockage of blood flow, and in the central regions of solid tumors, due to insufficient vascularization. When having the opportunity, malignant cells tend to consume glucose at a particularly high rate and predominantly generate energy from glycolysis, which is followed by lactate production even in aerobic conditions (the Warburg effect) [1]. This elevation of glucose utilization is readily exploited in the diagnostic imaging of tumors using ^{18}F-fluorodeoxyglucose positron emission tomography [1] and is thought to contribute to the depletion of glucose in the tumor interior [2]. Oncogenic activation of proliferative signaling appears to sensitize cells to glucose deprivation [3], and the inhibition of glycolysis forms the basis of several cancer treatment strategies investigated at the pre-clinical as well as the clinical level, with compounds such as 2-deoxyglucose, 5-thioglucose and 3-bromopyruvate being applied to target different steps of glycolysis [3]. Thus, the cellular responses to glucose deficiency are relevant to tumor growth as well as anti-cancer therapy.

TRIB3 (also known as *TRB3*, *NIPK*, *SKIP3*, and *SINK*) is a gene that is overexpressed in many types of tumors, such as breast, colon, esophagus and lung tumors [4,5], and high *TRIB3* mRNA level is associated with poor prognosis in colorectal and breast cancer patients [6,7]. In terms of protein structure, TRIB3 is considered to be a pseudokinase, since it contains a kinase-like domain that is predicted to be catalytically inactive due to amino acid substitutions [8]. TRIB3 interacts with a number of different proteins, including cellular stress-associated transcription factors (ATF4, CHOP, C/EBPβ, and NF-κB) and protein kinases (Akt/PKB, MEK1, MKK7, and MLK3) [9], and appears to regulate stress-induced cell death by multiple context-dependent mechanisms,

Abbreviations: ATF4, activating transcription factor 4; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; IGF, insulin-like growth factor; IGFBP2, IGF binding protein 2; TCA, trichloroacetic acid; Tet, tetracycline; TRIB3, Tribbles homolog 3.

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as opposing effects of TRIB3 on cell viability have been reported for different cell types and stress situations (for example, a pro-survival function in [7,10–14] and a pro-death function in [15–17]). Further investigation of the biology of TRIB3 may increase the comprehension of its dichotomous effects on cell viability and help to bring TRIB3 closer to clinical applications.

IGFBP2 is one of the six members of the insulin-like growth factor (IGF) binding protein family, which bind circulating IGF-I or IGF-II, thereby limiting the bioavailability of IGF and increasing IGF half-life in plasma [18]. By modulating IGF activity, IGFBPs can affect cell functions that are mediated by the IGF receptor; for example, overexpression of IGFBP2 in cell culture can inhibit IGF-induced cell proliferation in low-serum medium [19]. Additionally, IGFBPs also have IGF-independent functions, which may be exerted intracellularly as well as extracellularly. In an IGF-independent manner, IGFBP2 is able to inhibit apoptosis by repressing the expression of procaspase-3 [20] and increase cell motility by interacting with integrin α5 and activating integrin signaling [21]. *IGFBP2* is overexpressed in multiple malignancies, including colorectal, breast, gastric, pancreatic and ovarian cancer, leukemia and glioma [22–28]. Moreover, a high in situ level of IGFBP2 is associated with poor prognosis in glioma and leukemia patients [29,30], and a high serum level of IGFBP2 is a biomarker for poor prognosis in colorectal cancer and glioma patients [31,32].

In the current work, we study the expression and function of human TRIB3 during glucose deprivation using cells lines derived from HEK293, which in standard growth conditions exhibits a highly glycolytic ("glucose-addicted") metabolic phenotype that is comparable to tumor cells, consuming glucose at a rate 7-fold greater than that of the next most utilized carbon source, glutamine, and converting approximately 80% of the intracellular pyruvate pool into lactate [33]. We determine the effect of TRIB3 on glucose-starved cell survival, and perform genome-wide gene expression profiling to shed light on TRIB3-induced changes to the glucose deprivation transcriptional response. Based on the results of transcriptional profiling, further experiments are performed to determine the effect of IGFBP2 on glucose deprivation-induced cell death and to uncover the relationship between IGFBP2 and TRIB3 in the cellular response to glucose deficiency.

2. Materials and methods

2.1. Cell culture and treatment

T-REx-293 cells (HEK293-derived cell line stably expressing tetracycline (Tet) repressor protein) were obtained from Invitrogen. TRIB3-293 and Vector-293 cells were created by stable transfection of T-REx-293 cells with a Tet-inducible human *TRIB3* expression construct and the corresponding empty vector, respectively [10]. Cells were grown in IMDM supplemented with 10% FCS and 1× penicillin/streptomycin (all purchased from PAA) in an atmosphere of 5% CO_2 at 37 °C.

For glucose deprivation experiments, cells were seeded onto poly-L-lysine-coated tissue culture plates. All cells were placed in fresh growth medium 24 h before glucose withdrawal. Tet-regulated gene expression was activated 24 h prior to the start of glucose starvation by replacing the culture medium with fresh medium supplemented with 1 μg/ml Tet. Cell confluency at the onset of glucose deprivation was approximately 75%. To induce glucose deprivation, cells were washed three times with PBS and incubated in glucose-free DMEM (without sodium pyruvate; Gibco) supplemented with 10% dialyzed FCS (Sigma-Aldrich) and 1× penicillin/streptomycin (PAA). For Tet-treated cells, the glucose-free medium was additionally supplemented with 1 μg/ml Tet. To obtain glucose-containing (complete) control medium, D-glucose was added to the glucose-free medium at a final concentration of 4.5 g/l. Wortmannin (Calbiochem) and tunicamycin (Sigma-Aldrich) were used at 1 μM and 2.5 μg/ml concentrations, respectively, in the glucose-free or glucose-containing experimental treatment medium, respectively. To prepare methionine-deficient growth medium, methionine-free DMEM was obtained from BioSera and supplemented with dialyzed FCS and antibiotics as described above for the preparation of glucose-free medium. To inhibit PERK (EIF2AK3) activity, cells were pre-incubated with the indicated concentration of PERK inhibitor GSK2606414 (Axon Medchem) for 45 min in fresh growth medium before the application of the indicated experimental treatment medium supplemented with the indicated concentration of PERK inhibitor.

2.2. Transfection of siRNA and plasmid DNA

Transfections were performed 24 h prior to glucose withdrawal, using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's reverse transfection protocol. During transfection, cells were incubated in Opti-MEM I reduced-serum medium (Invitrogen) supplemented with 3% FCS (unless specified otherwise) on poly-L-lysine-coated tissue culture plates. For plasmid transfection, 500 ng of DNA was used per 24-well plate well (containing 500 μl of growth medium), and for siRNA transfection, the concentration of siRNA was 80 nM (unless specified otherwise). For both siRNA and plasmid DNA transfection, 3 μl of transfection reagent was used per milliliter of culture medium. The siRNAs targeting *TRIB3* mRNA and *ATF4* mRNA have been described previously [10], and the sequence of the siRNA targeting *IGFBP2* mRNA was 5′-CCUCAAACAGUGCAAGAUGdTdT-3′ (sense strand shown; based on [34]). The AllStars Negative Control siRNA (Qiagen) was used as a non-targeting negative control siRNA. The IGFBP2 and BiP overexpression plasmids used in this study are described below.

2.3. Plasmid construction

To generate the human IGFBP2 expression plasmid IGFBP2-pCG, the *IGFBP2* coding sequence was PCR-amplified from HEK293 cell line cDNA using the primers 5′-GCGTCTAGACCATGCTGCCGAGAGTGGGCT-3′ and 5′-GCGGGTACCTACTGCATCCGCTGGGTGTG-3′ (sense and anti-sense, with *Xba*I and *Acc*65I restriction sites underlined, respectively) and cloned into the pCG vector [35]. The human BiP expression plasmid BiP-pCG was constructed by the same approach, using the primers 5′-GCGTCTAGACCATGAAGCTCTCCCTGGTGGC-3′ and 5′-GCGGGTACCACT AGCAGATCAGTGTCTACAACTCA-3′ (sense and anti-sense, respectively). Sequencing confirmed that the *IGFBP2* and *BiP* coding sequences in the respective plasmids correspond to the reference mRNA sequences (NCBI RefSeq accession codes NM_000597.2 and NM_005347.4, respectively).

2.4. Real-time PCR quantification of gene expression

For RT-qPCR, total RNA was extracted from cells using the TRIzol reagent (Invitrogen) according to the manufacturer's recommendations and quantified spectrophotometrically using NanoDrop 1000 (Thermo Scientific). Total RNA was treated with DNase I and used for first strand cDNA synthesis as described previously [11,36]. Real-time PCR was performed as reported previously [11] and ribosomal protein L7a (*RPL7A*) mRNA was used as the endogenous reference for expression level normalization. The sequences of the primers used for RT-qPCR are as follows: *TRIB3* mRNA: described in [37], *TRIB3* unspliced pre-mRNA: described in [38], *IGFBP2* mRNA: 5′-AAGCATGGCCTGTACAACCT-3′ (sense) and 5′-GGGTTCACACACCAGCACT-3′ (anti-sense), *ATF4* mRNA: 5′-CCCT GGCCAAGGAGATCCAGTACCT-3′ (sense) and 5′-ACATTGACGCTCCTGA CTATCCTCAACT-3′ (anti-sense), and *RPL7A* mRNA: described in [37].

2.5. Trypan blue exclusion assay

To determine cell viability, the trypan blue dye exclusion method was used as described previously [10]. In each independent experiment, viable and non-viable (dye-accumulating) cells were counted from two

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