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TRB3 is a PI 3-kinase dependent indicator for nutrient starvation

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Abstract

We have identified TRB3, a human homologue of *Drosophila tribbles*, as a novel transcriptional target of phosphatidylinositol (PI) 3-kinase. TRB3 expression is remarkably reduced in prostate cancer PC-3 cells after inhibition of PI 3-kinase. TRB3 expression is furthermore controlled by nutrient supplies: Both the lack of glucose or amino acids results in a substantial increase in TRB3 protein levels in a PI 3-kinase-dependent manner. This increase is reversed by the addition of fresh nutrients. Stress stimuli, such as osmotic stress, hypoxia or serum starvation do not affect TRB3 expression. Thus, TRB3 may function as a nutrient sensor. Inhibition of TRB3 expression has no effect on growth of PC-3 cells under regular growth conditions. However, in the absence of glucose overexpression of TRB3 in PC-3 cells can interfere with apoptosis and restore growth on extracellular matrix. Taken together, our data point to an important role of TRB3 in sensing reduced nutrient supplies and in providing survival signals during these periods.

Keywords: TRB3; PI 3-kinase; Starvation; Nutrition status; Tribbles

1. Introduction

The ability of cells to react appropriately to the nutritional environment is a critical determinant of cellular behavior for unicellular microorganism, but also for cells in metazoa (for review see Ref. [1]). Processes such as growth, proliferation, development, migration and other responses are therefore also influenced by the nutrient status. Consequently, stress signals induced in response to starvation conditions must be integrated into coherent cellular responses by key intracellular regulators. In particular solid tumors often encounter stress conditions, such as glucose-, amino acid-starvation and/or hypoxia when reaching a critical size [2]. The susceptibility of tumor cells to stress and apoptotic stimuli depends on the balance between pro-apoptotic and survival signals. Activation of PI 3-kinase or its downstream effector, the protein–serine/threonine kinase Akt was shown to be a major signaling pathway that promotes survival and interferes with apoptosis [3,4]. Chronic upregulation of the PI 3-kinase pathway (e.g. in cells with mutational inactivation of the tumor suppressor PTEN) has been shown to protect tumor cells against various apoptotic stimuli and therefore contributes to tumorigenesis and metastasis [5]. As described previously we have identified novel effector genes of the PI 3-kinase pathway by using an approach that is based on three-dimensional (3D) cell culture systems [6]. Hereby, we compared the gene expression profile between cells, in which PI 3-kinase was switched on versus cells, in which it was turned off (for details see Refs. [6–8]). Among the mRNA species that exhibited a strong differential expression pattern was TRB3 (tribbles 3), the mammalian homologue of *Drosophila tribbles* [9–11]. TRB3 has also been designated as NIPK (neuronal cell death-inducible putative protein kinase), SINK and SKIP3 [12–14]. TRB3 has been suggested to function as negative modulator of insulin dependent activation of Akt in a liver cell starvation model [15]. Furthermore, it has been suggested that the tribbles protein family is involved in the regulation of the MAP kinase pathway [16]. TRB3 has also been reported to be upregulated in response to hypoxia [13] and to interact with the activating transcription factor 4 (ATF4) [13,17].

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Taken together these data suggest that TRB3 has diverse functional roles in several pathways dependent on the cellular context.

Here we report that TRB3 is expressed in a PI 3-kinase-dependent manner and is induced in response to nutrient starvation in PC-3 prostate carcinoma cells. Loss of function studies suggest that TRB3 is not required for PC-3 cell growth under 3D culture conditions while sufficient nutrients are present. Conversely, under conditions where glucose is limiting growth in 3D cultures can be significantly enhanced by overexpression of TRB3.

2. Experimental procedures

2.1. Cell culture

Human prostate carcinoma PC-3 and HeLa cells (American Type Culture Collection) were cultured as described [18]. COS-7 cells were cultured according to ATCC recommendations. GeneBloc transfections were carried out in 10-cm plates (30–50% confluency) as described previously [18]. Briefly, GeneBlocs (GB) were transfected by adding a preformed 5× concentrated complex of GB and lipid in serum-free medium to cells in complete medium. The total transfection volume was 10 ml. The final lipid concentration was 1.0 μg/ml; the final GB concentration was 60 nM unless otherwise stated. Plasmids were transfected using Effectene™ transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Hypoxia was induced by adding $CoCl_2$ (100 μM) directly to the tissue culture medium. Amino acid/glucose-free F-12K medium for PC-3 culture was purchased from Invitrogen-Gibco. Glucose-free EMEM medium for HeLa cell culture was prepared using MEM-amino acids, MEM-essential-amino acids and MEM-vitamins obtained from Cambrex (Vervier, Belgium). Dialyzed FBS was obtained from Invitrogen-Gibco (USA). Cells were exposed to nutrient free culture conditions by aspirating the medium, washing twice with DPBS and addition of medium deprived of the specific nutrient. LY294002 (LY) and Rapamycin (both Calbiochem) were dissolved in dimethylsufoxide (DMSO) and used at 5 μM or 20 ng/ml, respectively.

To assay cell growth on Matrigel matrix, PC-3 cells were transfected with GBs for 72 h. After trypsinization, the cells were seeded in duplicates on 24-well plates (150,000 cells per well) pre-coated with 250 μl Matrigel basement membrane matrix (Becton Dickinson, Mountain View, CA). HeLa cell were transfected with plasmids, medium was aspirated and glucosefree medium added after 24 h. After additional 24 h cells were trypsinized and seeded on Matrigel (100,000 cells per well on 24-well plates). Forty-eight hours later photographs were taken at ×1.25 or ×2.5 magnification with an Axiocam camera attached to an Axiovert S100 microscope (Zeiss, Oberkochem, Germany).

2.2. Northern blotting and Taqman analysis

RNA from cells was isolated and purified using the Invisorb spin cell RNA mini kit (InVitek GmbH, Berlin). Relative mRNA levels were detected by quantitative real-time RT-PCR (Taqman) analysis as described previously [19] using the following primers and probes: TRB3-fwd 5′ -AGT ATG GAC CTG GGA TTG TGG A-3′ ; TRB3-rev 5′ -ATT AGG CAG GGT CTG TCC TGT G-3′ ; TRB3-prb 5′ -Fam-CCA AGT GTC CCC AGA AGA GTC CCA CCT-Tamra-3′ ; p110β-fwd 5′ -TTG TGG GAT TGT CTT GGA TGG-3′ ; p110β-rev 5′ -CTA AGT TTT CAG GGA TGG ATG GTT-3′ ; p110β-prb 5′ -Fam-TGG CTA AAA CAA ACA TAT CCA CCA GAG CAT G-Tamra-3′ ; β-actin-fwd 5′ -GCA TGG GTC AGA AGG ATT CCT AT-3′ ; β-actin-rev 5′ -TGT AGA AGG TGT GGT GCC AGA TT-3′ ; β-actin-prb 5′ -Vic-TCG AGC ACG GCA TCG TCA CCA A-Tamra-3′ . For Northern blotting RNA was isolated using the Invisorb RNA kit (Invitek, Berlin). Twenty microgram of RNA were denatured by DMSO/glyoxal treatment for 1 h at 50 °C and subsequently separated in a sodium phosphate-buffered 1% agarose gel. During electrophoresis the buffer was recirculated. The RNA was transferred to nylon membranes (Nytran Supercharge; Schleicher and Schuell, Germany) and UV cross-linked. The blot was hybridized with a radiolabeled full-length TRB3 probe (RediprimeII, Amersham) according to standard protocols and analyzed using a PhosphorImager (Amersham Biosciences).

2.3. Plasmids and antisense oligonucleotides (GeneBlocs)

Full length and truncated versions of the TRB3 cDNA were amplified by PCR using the following primers: TRB3 forward: 5′ -CT TCT AGA ATG GCT CAT ATG CGA GCC ACC CCT CTA GCT GCT-3′ and TRB3 reverse: 5′ -AT GGA TCC TCA GGC GCC CCC GCC ATA CAG AAC CAC TTC TCT GTC-3′ ; TRB3-Δ1 forward: 5′ -CT TCT AGA ATG GCT CAT ATG CGA GCC ACC CCT CTA GCT GCT-3′ , TRB3-Δ1reverse 5′ -AT GGA TCC TCA GGC GCC CCC GCC CGA GTA TGA GGC CCG TGA-3′ , TRB3-Δ2 forward 5′ -CT TCT AGA ATG GCT CAT ATG CGA GCC ACC CCT CTA GCT GCT-3′ ; TRB3-Δ2 reverse 5′ -AT GGA TCC TCA GGC GCC CCC CAT GCT TGT GCG GGG GCA GCC-3′ ; TRB3-Δ3 forward 5′ -CT TCT AGA ATG GCT CAT AAG GCA GCC GAT GTC TGG AGC-3′ ; TRB3-Δ3 reverse 5′ -AT GGA TCC TCA GGC GCC CCC GCC ATA CAG AAC CAC TTC TCT GTC-3′ . The amplified product was inserted into the pCR4-TOPO cloning vector (Invitrogen, Carlsbad, CA) and confirmed by DNA sequence analysis. N-terminally HA-tagged TRB3 expression constructs were generated by subcloning the TRB3-cDNA into the mammalian expression vector pCG-HA via *Xba*I/*BamH*I ends [20].

GeneBlocs (GBs) represent third generation of gapmer antisense oligonucleotides; their composition and synthesis