Cellular Signalling 26 (2014) 233–239

Contents lists available at ScienceDirect

Cellular Signalling

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

Evidence for a regulatory role of Cullin-RING E3 ubiquitin ligase 7 in insulin signaling

Florian Scheufele [a,1], Benjamin Wolf [a,1], Michael Kruse [b,f], Thomas Hartmann [a], Justine Lempart [a], Susanne Muehlich [c], Andreas F.H. Pfeiffer [b,d], Loren J. Field [e], Maureen J. Charron [f,g,h], Zhen-Qiang Pan [i], Stefan Engelhardt [a,j], Antonio Sarikas [a,j,*]

[a] Institute of Pharmacology and Toxicology, Technische Universität München, Biedersteiner Straße 29, 80802 Munich, Germany
[b] German Institute of Human Nutrition Potsdam-Rehbrücke, Department of Clinical Nutrition, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany
[c] Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University, Goethestraße 33, 80336 Munich, Germany
[d] Department of Endocrinology, Diabetes and Nutrition, Charité University Medicine, 10117 Berlin, Germany
[e] Krannert Institute of Cardiology, University of Indiana, Indianapolis, IN 46202, USA
[f] Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
[g] Department of Medicine (Endocrinology), Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
[h] Department of Obstetrics & Gynecology and Women's Health, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
[i] Mount Sinai School of Medicine, Icahn Medical Institute, Department of Oncological Sciences, 1425 Madison Avenue, New York, NY 10029, USA
[j] DZHK (German Center for Cardiovascular Research), Partner Site Munich Heart Alliance, 80802 Munich, Germany

ARTICLE INFO

Article history:
Received 8 October 2013
Accepted 1 November 2013
Available online 9 November 2013

Keywords:
Ubiquitin
E3 ubiquitin ligase
Proteasome
Insulin
Cell signaling

ABSTRACT

Dysfunctional regulation of signaling pathways downstream of the insulin receptor plays a pivotal role in the pathogenesis of insulin resistance and type 2 diabetes. In this study we report both in vitro and in vivo experimental evidence for a role of Cullin-RING E3 ubiquitin ligase 7 (CRL7) in the regulation of insulin signaling and glucose homeostasis. We show that *Cul7*$^{-/-}$ mouse embryonic fibroblasts displayed enhanced AKT and Erk MAP kinase phosphorylation upon insulin stimulation. Depletion of CUL7 by RNA interference in C2C12 myotubes led to increased activation of insulin signaling pathways and cellular glucose uptake, as well as a reduced capacity of these cells to execute insulin-induced degradation of insulin receptor substrate 1 (IRS1). In vivo, heterozygosity of either *Cul7* or *Fbxw8*, both key components of CRL7, resulted in elevated PI3 kinase/AKT activation in skeletal muscle tissue upon insulin stimulation when compared to wild-type controls. Finally, *Cul7*$^{+/-}$ or *Fbxw8*$^{+/-}$ mice exhibited enhanced insulin sensitivity and plasma glucose clearance. Collectively, our findings point to a yet unrecognized role of CRL7 in insulin-mediated control of glucose homeostasis by restraining PI3 kinase/AKT activities in skeletal muscle cells.

1. Introduction

Diabetes mellitus is a major metabolic disorder affecting more than 285 million people worldwide [1]. 90% of the patients suffer from type 2 diabetes, which is characterized by glucose intolerance, insulin resistance and defects in pancreatic insulin secretion [2]. Insulin exerts its effects in target tissues by binding to insulin tyrosine kinase receptors, resulting in the recruitment and phosphorylation of insulin receptor substrate (IRS) proteins. In particular IRS1 and IRS2 were shown to play critical roles in insulin signaling by transmitting receptor stimulation to the activation of PI3 kinase/AKT and Erk MAPK pathways, which mediate the metabolic and mitogenic effects of insulin, respectively [3]. In addition, activation of PI3 kinase also triggers the translocation of vesicles containing glucose transporter 4 (GLUT4) from intracellular storage pools to the cell membrane, thereby enabling uptake of glucose into the cell [4].

A major cause of insulin resistance and type 2 diabetes is thought to be dysfunctional regulation of cellular signal transduction downstream

Abbreviations: ANOVA, analysis of variance; CUL7, Cullin7; CRL7, Cullin RING E3 ubiquitin ligase 7; FBXW8, F-box/WD repeat-containing protein 8; GLUT4, glucose transporter 4; HSP90, heat shock protein 90; IRS, insulin receptor substrate; IGF-1, insulin-like growth hormone 1; ITT, insulin tolerance test; MAPK, mitogen-activated pathway kinase; MEF, mouse embryonic fibroblast; PI3K, phosphoinositol-3 kinase; RING, really interesting new gene; mTOR, mammalian target of rapamycin; S6K, p70 S6 kinase; UPS, ubiquitin-proteasome-system; WT, wild type; 2-DOG, 2-deoxy-D-(^{3}H)-glucose.

* Corresponding author: Antonio Sarikas, Institute of Pharmacology and Toxicology, Technische Universität München, Biedersteiner Straße 29, 80802 Munich, Germany. Tel.: +49 89 4140 3298; fax: +49 89 4140 3261.

E-mail addresses: scheufele@ipt.med.tum.de (F. Scheufele), bennitto@gmx.de (B. Wolf), michael.kruse@dife.de (M. Kruse), Hartmann@ipt.med.tum.de (T. Hartmann), justinel@gmx.de (J. Lempart), susanne.muehlich@lrz.uni-muenchen.de (S. Muehlich), afhp@dife.de (A.F.H. Pfeiffer), ljfield@iupui.edu (L.J. Field), maureen.charron@einstein.yu.edu (M.J. Charron), zhen-qiang.pan@mssm.edu (Z.-Q. Pan), stefan.engelhardt@tum.de (S. Engelhardt), sarikas@ipt.med.tum.de (A. Sarikas).

[1] Contributed equally as first authors.

0898-6568/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cellsig.2013.11.005

of the insulin receptor [5,6]. Several studies provided evidence for dysregulation of key signaling molecules, such as IRS1, IRS2 and AKT1, in insulin resistant cells and tissues due to altered transcriptional or posttranslational (such as ubiquitin-mediated proteasomal degradation) activities [7]. Timely degradation of intracellular proteins by the ubiquitin-proteasome system (UPS) is a precisely controlled process that regulates a broad spectrum of fundamental cellular functions, ranging from cell cycle progression to signal transduction. Central to this process is the recognition of a substrate protein by an E3 ubiquitin ligase, a pivotal step for initiating the ubiquitination reaction that joins the target protein covalently with polyubiquitin chains, thereby leading to its degradation by the 26S proteasome [8].

We have previously identified IRS1 as a proteolytic target of Cullin-RING E3 ubiquitin ligase 7 (CRL7), a multimeric enzyme composed of the scaffold protein Cullin7 (CUL7), the RING finger protein ROC1 and the SKP1-FBXW8 substrate targeting subunit [9–11]. It was shown that CRL7 induced degradation of IRS1 is part of a negative feedback loop via mammalian target of rapamycin complex 1 (mTORC1) and p70 S6 kinase (S6K) activities to restrain PI3 kinase/AKT signaling upon activation of the insulin-like growth factor 1 (IGF-1) receptor [9,10]. Collectively, these studies provided experimental evidence for a novel role of CRL7 in IGF-1 signaling in vitro. Based on these observations, we sought to investigate whether CRL7 also contributes to the regulation of insulin signaling and evaluate its biological relevance in vivo.

2. Materials and methods

2.1. Animals

Cul7$^{+/-}$ mice were generated by replacing exons 2–4 of the *Cul7* gene with a PGK-neo cassette as described in detail previously [9]. *Fbxw8*$^{+/-}$ mice were generated by injecting embryonic stem cells harboring a gene trap insertion in intron 3 of the *Fbxw8* gene (Clone RRT057, BayGenomics gene-trap resource) into C57BL/6J blastocysts [12]. The injected blastocysts were then transplanted into pseudopregnant females using standard protocols [13]. A similar approach was used by DeCaprio and colleagues [14]. Both strains were maintained on a hybrid 129/SvJ X C57BL/6 background. All experiments were performed with female 8–12 week old mice that were kept on a 12 hour light/dark cycle with free access to food and water. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine (New York, USA) and the State Government of Bavaria (Germany).

2.2. Insulin tolerance tests

For insulin tolerance tests (ITT), 6 hour fasted mice were intraperitoneally injected with a bolus of human insulin at 0.75 units per kg of body weight (Novolin R; Novo Nordisk, Denmark) according to standard protocol [15]. Control animals received an intra-peritoneal bolus of saline (0.9% NaCl). Blood glucose levels were determined in tail vein blood at the indicated times (0 to 120 min) with a glucometer (Aventis Pharma, Frankfurt, Germany).

2.3. Insulin signaling studies in vivo

For assessment of insulin signaling pathways in vivo, mice were fasted for 6 h and intra-peritoneally injected with human insulin at 2 units per kg of body weight (Humalog, Lilly, Bad Homburg, Germany) or 0.9% NaCl (control group). After 5 min mice were sacrificed by cervical dislocation and tissue collected. Gastrocnemius muscles were removed and snap frozen in liquid nitrogen. Tissues were homogenized in ice-cold lysis buffer (50 mM HEPES, pH 8.0, 5 mM EDTA, 15 mM NaPP tetrabasic, 25 mM NaF, 100 mM BGP, 2 mM Na_3VO_4, 0.1% Triton X-100 and phosphatase and protease inhibitors) using a motor-driven ultra turrax (IKA, Staufen, Germany). Homogenates were centrifuged at 14,000 ×*g* for 10 min at 4 °C, and supernatants stored at −80 °C until analysis.

2.4. Insulin signaling studies in vitro

For insulin signaling studies in vitro, mouse embryonic fibroblasts (MEFs) of *Cul7*$^{-/-}$ and *Cul7*$^{+/-}$ embryos were isolated from pregnant mice at day 14.5 p.c. and grown in DMEM with 10% FCS as described previously [9]. For analysis of signaling pathways, MEFs were serum starved for 16 h and stimulated with 100 nM insulin for various time intervals. Cell extracts (50 μg of protein) were separated by 10% SDS-PAGE followed by immunoblot analysis. Murine C2C12 myoblasts were obtained from ATCC (Wesel, Germany) and cultured in DMEM (4500 mg/l glucose) containing 10% FCS. For differentiation to myotubes, medium was switched to DMEM with 2% horse serum for 7 to 10 days. For signaling experiments, C2C12 myotubes were transfected with CUL7 (On Target Plus SMART pool, Dharmacon, Colorado) or scramble siRNA for 48 h following the manufacturer's instructions. At 32 h post-transfection, C2C12 myotubes were serum starved for 16 h before exposure to insulin (100 nM) or DMEM (controls) for various time intervals (15, 30 and 45 min). For analysis of insulin triggered IRS1 degradation kinetics, C2C12 myotubes were serum deprived for 4 h, treated with emetine (10 μm) and stimulated with insulin (100 nM) for various time intervals (4, 8 and 16 h). Cells were then washed with ice-cold PBS and lysed in buffer containing 20 mM Tris–HCl (pH 8.0), 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 10% glycerol, 0.02 mM p-amidino phenylmethanesulfonyl fluoride, 1% Nonidet® P-40 substitute, 0.5 mM Na_3VO_4 and 20 mM $Na_4P_2O_7$.

2.5. Immunoblotting analysis

Lysate protein was resolved by SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Richmond). Phosphorylated and total proteins were identified by immunoblotting using the following primary antibodies: polyclonal anti-P-AKT (Ser473), anti-P-Erk (Thr202/Tyr204) and anti-AKT, monoclonal anti-Erk 1/2 (Cell Signaling, Danvers, MA); polyclonal anti-IRS1 (Millipore, Billerica, MA); monoclonal anti-CUL7 (Sigma-Aldrich, Steinheim, Germany) and anti-HSP90 α/β (F-8) (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary horseradish peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG antibodies were from Cell Signaling (Danvers, MA).

2.6. 2-[^{3}H] deoxy-D-glucose uptake assays

To quantify cellular glucose influx upon insulin stimulation, 2-[^{3}H] deoxy-D-glucose (2-DOG) uptake measurements were carried out in C2C12 myotubes according to standard protocol [16]. Briefly, cells were incubated for 10 min in Krebs-Ringer buffer containing 2-[^{3}H] deoxy-D-glucose (0.2 mCi/ml) in the presence or absence of 100 nM insulin. Cytochalasin B (20 μM) was used to block non-specific glucose uptake. Cells were lysed in PBS containing 0.2 M NaOH and radioactivity measured with a liquid scintillation counter (Packard, Canberra, Australia).

2.7. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and horse serum were from Pan-Biotech (Aidenbach, Germany). All other reagents used were of analytical grade from Sigma-Aldrich (Steinheim, Germany) or Applichem (Darmstadt, Germany).