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Molecular and Cellular Endocrinology 268 (2007) 30–36

MCE
Molecular and Cellular Endocrinology

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The Par6α/aPKC complex regulates Akt1 activity by phosphorylating Thr34 in the PH-domain

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Received 13 September 2006; received in revised form 16 January 2007; accepted 18 January 2007

Abstract

A single nucleotide polymorphism in the partitioning defective protein-6α (Par6α) promoter is coupled with lower Par6α expression and better insulin sensitivity, whereas overexpression of Par6α in C2C12 myoblasts inhibits insulin-induced protein kinase B/Akt1 activation and glycogen synthesis. Here we show that a direct interaction of Par6α with atypical protein kinase C (aPKC) is crucial for this inhibition. A ΔPB1-Par6α deletion mutant that does not interact with aPKC neither increased aPKC activity nor interfered with insulin-induced Akt1 activation in C2C12 cells. Further, T34 phosphorylation of Akt1 through aPKC is important for inhibition of Akt1. When Par6α was overexpressed, activation of wild-type Akt1 (−59.3%; $p = 0.049$), but not T34A-Akt1 (+2.9%, $p = 0.41$) was reduced after insulin stimulation. The resistance of T34A-Akt1 to Par6α/aPKC-mediated inhibition was also reflected by reconstitution of insulin-induced glycogen synthesis. In summary, Par6α-mediated inhibition of insulin-dependent glycogen synthesis in C2C12 cells depends on the direct interaction of Par6α with aPKC and on aPKC-mediated T34 phosphorylation of Akt1.
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Keywords: Par6; Atypical protein kinase C; Akt1; Glycogen synthesis; Insulin signalling; Insulin resistance

1. Introduction

Type 2 diabetes mellitus has become one of the main causes of morbidity throughout many countries. As insulin resistance is a key feature for type 2 diabetes development, it is important to understand the molecular basis that underlies this phenomenon. Atypical protein kinases C (aPKC: PKCζ and PKCλ/ι) are interesting candidates that may be involved in insulin resistance. These serine-threonine kinases exert both positive and negative effects on insulin signalling. Many studies have shown that aPKC activation is important for insulin-stimulated glucose transport in muscle cells and adipocytes (Farese et al., 2005). On the other hand, negative effects of aPKC on insulin signal transduction were also reported. These negative feedback loops are accomplished by PKCζ-mediated serine phosphorylation of insulin receptor substrate 1 (IRS1) with subsequent loss of phosphatidylinositol-3-kinase (PI3K) activity (Liu et al., 2001; Moeschel et al., 2004; Ravichandran et al., 2001) or by direct interaction of PKCζ with protein kinase Bα/Akt1, which negatively regulates Akt1 catalytic activity (Doornbos et al., 1999). Saturated non-esterified fatty acids (NEFAs) or ceramide enhance the formation of the PKCζ-Akt1 complex (Bourbon et al., 2002; Powell et al., 2004), and PKCζ then phosphorylates the T34 residue in the amino-terminal pleckstrin homology (PH) domain of Akt1. This in turn reduces phosphatidylinositol-3,4,5-triphosphate binding of Akt1 and down-regulates Akt1 activation (Powell et al., 2003). As both, elevated plasma NEFAs and increased skeletal muscle ceramide content reflect typical features of type 2 diabetes (Summers and Nelson, 2005), aPKC-dependent Akt1-T34 phosphorylation may be relevant for development of insulin resistance.

A potential regulator of aPKC in insulin signalling is partitioning-defective protein 6α (Par6α), a scaffold protein that interacts with the regulatory domain of aPKC. We previously identified a single nucleotide polymorphism in the human Par6α promoter that was coupled with lower Par6α expression and better insulin sensitivity (Weyrich et al., 2005). Overexpression of human Par6α in C2C12 murine myoblasts increased aPKC activity and resulted in repression of insulin-induced glycogen synthesis by enhancing the negative aPKC–IRS1–PI3K feedback loop (Weyrich et al., 2004). In the present study we show that the Par6α effect depends on its interaction with aPKC and

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doi:10.1016/j.mce.2007.01.011

that phosphorylation of Akt1 at T34 by the Par6α/aPKC complex impairs insulin action.

2. Materials and methods

2.1. Chemicals and antibodies

Antibodies against Akt1 and PKCι were purchased from BD (for Western blotting; BD Transduction Laboratories, Franklin Lakes, NJ, USA) and Upstate (for Akt immunokinase assay; Upstate Biotechnology Inc., Lake Placid, NY, USA). The phosphospecific antibodies directed against pT308-Akt1 and pT410/403-PKCζ/λ were from Cell Signalling (Cell Signalling Technology, Danvers, MA, USA). The Par6α specific antibody detected the amino-terminus of the protein and was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). We also used antibodies obtained from rabbit immunisation with GST-human PKCζ comprising amino acids (aa) 148–584 or GST-human Par6α (aa247-345) fusion proteins.

2.2. Plasmid construction

The cDNAs of human Akt1, PKCζ/ι and Par6α (Genbank Acc. No. NM_016948) were cloned into the pRK expression vector containing the cytomegalovirus immediate-early promoter, and the cDNA of Par6α also into the retroviral expression vector pLXSN. The deletion mutant ΔPB1-Par6α lacking amino acids 15–95 (see Fig. 2A) was obtained by overlap extension PCR: 0.5 μM of the primers pRK_fw TTGCCTTTCTCTCCACAGGTGT (vector specific) and ΔPB1-Par6α_rev GGAGTCAGCTTCGCTATCGGGACT-GCGCGCCGG, ΔPB1-Par6α_fw GCGCGCAGTCCCGATAGCGAAGCT-GACTCCAGCGGCCTG and pRK_rev CCATTATAAGCTGCAATAAAC (vector specific) in the first round (25 cycles; 1 min at 94 °C melting, 1 min at 50 °C annealing, 1 min at 68 °C extension for both PCRs with Pfx-DNA-polymerase, Invitrogen, Carlsbad, CA, USA); and 0.5 μM of the primers pRK_fw and pRK_rev for the overlap extension (35 cycles with 1 min 94 °C, 1 min 40 °C and 1.5 min 68 °C with TripleMaster Polymerase Mix, Eppendorf AG, Hamburg, Germany). The $Akt1^{T34A}$ construct was obtained using 0.5 μM of the primers pRK_fw and Akt1T34A_rev CTCCTTGTAGCCAAT-GAAGGCGCCATCATTCTTGAGGAG (25 cycles at 1 min 94 °C, 1 min 50 °C, 1 min 68 °C), Akt1T34A_fw CTCCTCAAGAATGATGGCGCCTTCATTGGC-TACAAGGAG and pRK_rev (25 cycles at 1 min 94 °C, 1 min 50 °C, 2 min 68 °C) and 25 cycles with pRK_fw and pRK_rev (1 min 94 °C, 1 min 50 °C and 2.5 min 68 °C) for the overlap extension (all three reactions with Pfx-Polymerase). The polymerase buffer systems provided by the manufacturers were used, and 0.2 mM dNTP mixture was present in all PCR reactions. Mutagenesis was verified by sequencing.

2.3. Cell culture, transfection and treatment

HEK293 and C2C12 cells were cultured in DMEM/F12 or DMEM, respectively, and supplemented with 10% FCS and 2 mM glutamine. Transfection of HEK293, BOSC23 and C2C12 cells was performed by calcium-phosphate/DNA co-precipitation (Chen and Okayama, 1987). For stable transfection of C2C12 with wild type Akt1 and the T34A-Akt1 mutant, the donor DNA was transfected together with $pSV2_{neo}$ as a neomycin resistance providing selection plasmid in a 9:1 ratio. Stably transfected C2C12 clones were selected by supplementation of culture media with G418 racemate (1 mg/ml) for 14 days. To additionally overexpress Par6α in these clones, the retroviral vector pLXSN (Clontech, Palo Alto, CA, USA) encoding Par6α was used and virus produced from transfected BOSC23 cells (Pear et al., 1993). To this end, the cells were transfected with the DNA, washed with DMEM/F12 + 0.5% FCS + 2 mM glutamine 15 h after transfection and subsequently cultivated in full (10% FCS, 2 mM glutamine) medium. Virus-containing supernatant was harvested 24 h later and in the presence of polybrene (4 μg/ml) added to GP + E − 86 packaging cells that were subconfluent and had been seeded 18 h earlier. Infected cells were selected with G418 as above, pooled and virus containing supernatant collected from confluent cells after overnight incubation. The virus titer of these cells was about $1 \times 10^5\ ml^{-1}$ and used to infect C2C12 cells stably overexpressing Akt1 or $Akt1^{T34A}$. Infection was done in the presence of polybrene as indicated above, but fresh virus-containing supernatant was added after 5 h and 10 h to achieve optimal infection. Infected cells were then diluted to grow individual colonies which were picked, expanded and evaluated for double expression of Akt1 or its T34A mutant and Par6α. For cell culture experiments, clones overexpressing comparable amounts of transfected proteins were selected, seeded at a density of 5000 cells/cm^2 and grown until confluency. Before insulin stimulation (15 min), C2C12 cells were serum-starved (0.5% FCS) for 24 h.

2.4. RT-PCR for Par6α

RNA from confluent C2C12 myoblasts was obtained by use of the TRIZOL® Reagent from Invitrogen and purified by the RNAase-free DNAase from the PAXGene™ Blood RNA Kit (PreAnalytiX, Qiagen, Hombrechtikon, CH). Synthesis of cDNA was done with the 1st Strand cDNA Synthesis KIT for RT-PCR AMV (Roche Diagnostics, Basel, CH). PCR for Par6α on C2C12 cDNA was done with the primers 166_fw (TTCCGACGCTTTGCACTAC) and 1119_rev (TCCGCTAACATCACCTCG), followed by a nested PCR with 593_fw (CCCTGGGCTTCTACATTCG) and 1042_rev (TGCCAGCACCAGGAAG-TAG) to ensure specificity, 35 cycles each with Taq-Polymerase from Eppendorf AG (5 min 94 °C for initial melting, then 1 min 94 °C, 1 min 50 °C and 1 min 72 °C, 0.2 mM dNTP, Taq-buffer from Eppendorf).

2.5. Immunoprecipitation

HEK293 cells were lysed in 50 mM HEPES (pH 7.5), 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml aprotinin. After adjustment for equal protein concentration (800 μg/ml) with HNTG (20 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, pH 7.5), lysates were placed on a rotating wheel at 4 °C for 4 h in the presence of antibody (2 μg) and 20 μl of a 1:1 slurry of Protein A-Sepharose. The Sepharose was washed three times with HNTG and Laemmli buffer added. Proteins were size separated by sodiumdodecyl (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blotting.

2.6. Immunokinase assay

Akt kinase activity was determined with an Akt specific substrate peptide (RPRAATF) surrounding the phosphorylation site of GSK-3 according to the manufacturer's protocol (Upstate). In brief, cells were lysed with 50 mM TRIS (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 5 mM $Na_4P_2O_7$, 10 mM β-glycerolphosphate, 1 mM Na_3VO_4 and 0.1% β-mercaptoethanol. After determination of protein concentration and adjustment of the lysates for equal protein content (800 μg for each reaction), Akt1 was immunoprecipitated with Protein-G-sepharose-bound sheep antibody (Upstate) directed against aa 466–480 of Akt1 (with identical affinity for both wild type Akt1 and the T34A-Akt1 mutant). Beads were washed three times with 50 mM TRIS (pH 7.5), 0.03% Brij-35, 0.1 mM EGTA and preincubated with assay buffer (10 mM MOPS, 12.5 mM β-glycerolphosphate, 2.5 mM EGTA, 0.5 mM Na_3VO_4, 1 mM DTT) on ice. After addition of substrate peptide (final concentration 100 μM) and [γ-^{32}P]-ATP (80 μM final concentration, 10 μCi per assay, Amersham Pharmacia, Buckinghamshire, UK), the reaction mixture was incubated for 10 min at 30 °C. Finally, ^{32}P-labelled Akt1 substrate was trapped on p81 phosphocellulose filters and radioactivity measured after three washes (5 min, RT) with 40 ml phosphoric acid (0.75%) and an additional washing step in 20 ml acetone (5 min, RT). Kinase activity (expressed in counts per minute) from at least three independent experiments for each condition was additionally corrected for Akt1 expression, determined by densitometric scanning of Western blots of the corresponding cell lysates.

2.7. Measurement of glycogen synthesis

Cells were washed (3×) with HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1% bovine

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