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**The Par6 α /aPKC complex regulates Akt1 activity by
phosphorylating Thr34 in the PH-domain**

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Key Words

Par6, Atypical Protein Kinase C, Akt1, Glycogen Synthesis, Insulin Signalling, Insulin Resistance

Summary

A single nucleotide polymorphism in the partitioning defective protein-6 α (Par6 α) promoter is coupled with lower Par6 α expression and better insulin sensitivity, whereas overexpression of Par6 α in C2C12 myoblasts inhibits insulin-induced protein kinase B/Akt1 activation and glycogen synthesis. Here we show that a direct interaction of Par6 α with atypical protein kinase C (aPKC) is crucial for this inhibition. A Δ PB1-Par6 α 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 Par6 α 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 Par6 α /aPKC-mediated inhibition was also reflected by reconstitution of insulin-induced glycogen synthesis. In summary, Par6 α mediated inhibition of insulin-dependent glycogen synthesis in C2C12 cells depends on the direct interaction of Par6 α with aPKC and on aPKC-mediated T34 phosphorylation of Akt1.

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: PKC ζ and PKC λ/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 PKC ζ -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 PKC ζ with protein kinase B α /Akt1, which negatively regulates Akt1 catalytic activity (Doornbos et al., 1999). Saturated non-esterified fatty acids (NEFAs) or ceramide enhance the formation of the PKC ζ -Akt1 complex (Bourbon et al., 2002; Powell et al., 2004), and PKC ζ 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 et al., 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 6 α (Par6 α), a scaffold protein that interacts with the regulatory domain of aPKC. We

previously identified a single nucleotide polymorphism in the human Par6 α promoter that was coupled with lower Par6 α expression and better insulin sensitivity (Weyrich et al., 2005). Overexpression of human Par6 α 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 Par6 α effect depends on its interaction with aPKC and that phosphorylation of Akt1 at T34 by the Par6 α /aPKC complex impairs insulin action.

Materials and Methods

Chemicals and Antibodies – Antibodies against Akt1 and PKC ι 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-PKC ζ/λ were from Cell Signalling (Cell Signalling Technology, Danvers, MA, USA). The Par6 α 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-humanPKC ζ comprising amino acids (aa) 148-584 or GST-humanPar6 α (aa247-345) fusion proteins.

Plasmid Construction – The cDNAs of human Akt1, PKC ζ/ι and Par6 α (Genbank Acc. N° *NM_016948*) were cloned into the pRK expression vector containing the cytomegalovirus immediate-early promoter, and the cDNA of Par6 α also into the retroviral expression vector pLXSN. The deletion mutant Δ PB1-Par6 α lacking amino acids 15-95 (see Fig. 2a) was obtained by overlap extension PCR: 0.5 μ M of the primers pRK_fw TTGCCTTTCTCTCCACAGGTGT (vector specific) and Δ PB1-Par6 α _rev GGAGTCAGCTTCGCTATCGGGACTGCGCGCCGG, Δ PB1-Par6 α _fw

GCGCGCAGTCCCGATAGCGAAGCTGACTCCAGCGGCCTG 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 CTCCTTGTAGCCAATGAAGGCGCCATCATTCTTGAGGAG (25 cycles at 1 min 94°C, 1 min 50°C, 1 min 68°C), Akt1T34A_fw CTCCTCAAGAATGATGGCGCCTTCATTGGCTACAAGGAG 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 3 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.

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 (1mg/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 μ g/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 1×10^5 /ml 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 Par6 α . For cell culture experiments, clones overexpressing comparable amounts of transfected proteins were selected, seeded at a density of 5000 cells/cm² and grown until confluency. Before insulin stimulation (15 min), C2C12 cells were serum-starved (0.5% FCS) for 24 h.

RT-PCR for Par6 α – 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 Par6 α 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 (TGCCAGCACCAGGAAGTAG) 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).

Immunoprecipitation – HEK293 cells were lysed in 50 mM HEPES (pH 7.5), 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 150 mM NaCl, 1 mM EGTA, 100 mM NaF,

10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 $\mu\text{g}/\text{ml}$ aprotinin. After adjustment for equal protein concentration (800 $\mu\text{g}/\text{ml}$) 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 3 times with HNTG and Laemmli buffer added. Proteins were size separated by sodiumdodecyl (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blotting.

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 $\text{Na}_4\text{P}_2\text{O}_7$, 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 3 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 3 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 3 independent experiments for each condition was additionally corrected for Akt1

expression, determined by densitometric scanning of Western blots of the corresponding cell lysates.

Measurement of Glycogen Synthesis – Cells were washed (3x) with HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO₄, 1 mM CaCl₂, 0.1% bovine serum albumin, pH 7.4) and stimulated with insulin (3 nM) for 60 min at 37°C. A mixture of D-glucose/D-[¹⁴C]-glucose (5 mM final concentration, 0.3 μCi/well, Du Pont-NEN, Boston, MA, USA) was added for further 60 min (37°C). Cells then were washed again (3x) with ice-cold PBS and lysed in 30% (w/v) KOH (30 min, RT). Lysates were heated for 30 min at 96°C and cooled on ice. The glycogen was precipitated in 900 μl ice-cold ethanol by centrifugation (4.5 min, 9000 x g), washed with ethanol and resuspended in 500 μl H₂O. Radioactivity was determined by liquid scintillation counting.

Murine Tissue Lysates – For detection of endogenous Par6α in different murine organs, C57Bl/6 wild type mice (Charles River Laboratories Inc., MA, USA) were studied. Mice were maintained under normal light/dark cycle and kept on a regular chow. Animals at the age of 8 weeks and 6 months were sacrificed after ketamine anaesthesia, tissue samples were dounce homogenized on ice and extracted in hyperosmolaric tissue lysis buffer (55 mM HEPES, 30 mM NaF, 2.5 mM Na₄P₂O₇, 360 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 10% glycerol, 1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.5; 30 min at 4°C). Lysates were clarified by centrifugation at 12.000 g for 20 min (4°C). Protein content of supernatants was determined by the method of Bradford, and equal amounts of protein were loaded on SDS-polyacrylamide gels for Western blot analysis. All procedures were conducted according to the guidelines of laboratory animal care and were approved by the local governmental commission for animal research.

Statistical Analyses and Densitometry – The EasyWin32 Herolab© Software was used for scanning densitometry of phosphosignals with subsequent normalisation to the corresponding protein expression levels. Results are expressed as mean ± standard error of

the mean. For statistical analysis, a (two-tailed) Student's t test was used. $P < 0.05$ was considered to be statistically significant.

Results

Par6 α is expressed in insulin-sensitive tissues of juvenile and adult C57/Bl6 mice

Expression of Par6 α has been reported in four insulin sensitive tissues (muscle, pancreas, liver, brain) by cloning or Northern blotting (Joberty et al., 2000; Johansson et al., 2000). To underscore the relevance of Par6 α for insulin signalling, we investigated its expression in typical insulin sensitive cell lines like C2C12, L6 or 3T3L1. With the antibodies available, we were not able to detect Par6 α on a protein level, independent of the cell differentiation status (data not shown). However, we could confirm the presence of Par6 α mRNA in C2C12 myoblasts by RT-PCR (Fig. 1A). To verify the presence of Par6 α protein in insulin sensitive tissues, we analysed lysates derived from male juvenile (8 weeks) and adult (6 months) mice by Western blot (Fig. 1B). Par6 α was strongly expressed in all insulin-sensitive tissues (liver, muscle, fat, pancreas and brain). With the exception of lung, where Par6 α was weakly expressed in juvenile but not in adult animals, the age of the mice did not influence Par6 α expression. Par6 α expression patterns were identical in female mice (data not shown). Thus, Par6 α is expressed in all insulin responsive tissues and may be relevant for in-vivo insulin signalling.

Direct interaction of Par6 α with aPKC is required for inhibition of insulin-induced Akt1 activation in C2C12 cells

Par6 α comprises an amino-terminal Phox and Bem1p (PB1) domain, a Cdc42/Rac1-interactive-binding (CRIB)-like motif and a PSD-95/Discs large/Zo-1 (PDZ) domain (Fig. 2A). To prove that the effects of Par6 α on insulin signalling are specifically mediated by

the aPKC kinases and not other associating proteins, we constructed a Δ PB1-Par6 α mutant by deletion of the PB1-domain (aa15-95) that is responsible for the interaction with aPKC (Noda et al., 2003). The inability of our Δ PB1-Par6 α mutant to interact with aPKCs was verified by overexpressing wild-type Par6 α or Δ PB1-Par6 α together with either PKC ι or PKC ζ in HEK293 cells. As a control, aPKCs were overexpressed alone. After cell lysis, proteins were immunoprecipitated with an antibody directed against the carboxyl-terminus of Par6 α . As shown in the top panel of Fig. 2B, co-immunoprecipitation of aPKCs and Par6 α depended on the expression of Par6 α and was aborted when its PB-1 domain was deleted. The two lower panels in Fig. 2B confirm the presence of the overexpressed proteins in the cell lysates. We next generated C2C12 cells stably expressing the Δ PB1-Par6 α mutant (C2C12 ^{Δ PB1-Par6 α}) to investigate the functional consequences of a Par6 α that cannot interact with aPKC on Akt1 activation. Since the T308 phosphosite in the catalytic domain of Akt1 is targeted by phosphoinositide-dependent kinase 1 at the plasma membrane and is generally accepted to reflect Akt1 kinase activity (Brazil et al., 2004), we determined insulin-dependent Akt1-T308 phosphorylation (pT308-Akt1). C2C12 cells were stimulated with different insulin concentrations, lysed and proteins analysed by Western blotting with antibodies specific for phosphorylated T308 of Akt1. In C2C12 ^{Δ PB1-Par6 α} , insulin-dependent pT308-Akt1 was similar as in parental cells, whereas wild-type Par6 α overexpression led to a significant reduction of pT308-Akt1 under these conditions (Fig. 2C).

As the increase of aPKC activity accounts for the observed Par6 α -mediated inhibitory effects on Akt1 (Weyrich et al., 2004), we also investigated how deletion of the Par6 α -PB1 domain affects T410/403 phosphorylation of PKC ζ/λ (pT410/403-aPKC). This aPKC phosphosite is also targeted by phosphoinositide-dependent kinase 1 after insulin stimulation and reflects aPKC kinase activity (Standaert et al., 2001). As shown in Fig.

2C, pT410/403-aPKC remained unchanged in C2C12^{ΔPB1-Par6α} cells compared to untransfected C2C12 cells, whereas it was markedly increased in C2C12^{Par6α} cells. These results support our hypothesis that inhibition of insulin-dependent Akt1 activity in C2C12^{Par6α} is a direct consequence of the Par6α/aPKC complex formation and does not involve interaction of Par6α with other proteins.

C2C12 cells overexpressing Akt1^{T34A} are resistant to Par6α/aPKC-mediated inhibition of Akt1 activation and inhibition of glycogen synthesis

The T34-phosphosite in the PH-domain of Akt1 is targeted by ceramide-activated aPKC and responsible for inhibition of insulin-induced Akt1 activation (Powell et al., 2003). As ceramide is discussed as a mediator of insulin resistance in skeletal muscle (Adams et al., 2004), we wanted to know whether Par6α activated aPKC use the same mechanism to regulate Akt1. Thus, we investigated whether the T34 phosphosite is also responsible for the Par6α/aPKC-mediated downregulation of Akt1 activity in C2C12 myoblasts. Unfortunately, commercially available antibodies were not functional and neither detected a difference in T34-Akt1 phosphorylation nor recognized an overexpressed Akt1-T34D mutant, a mimetic of phosphorylated T34, in Western blot analysis. Therefore, we were not able to determine the extent of T34 phosphorylation in our cell lines. Instead, we generated C2C12 cell lines that overexpressed Akt1 or Akt1^{T34A}. These cells were then superinfected with Par6α encoding retrovirus and clones selected that expressed similar levels of Akt1 and Par6α proteins, as shown in Fig. 3A.

Cells were stimulated with different concentrations of insulin and phosphorylation of T308-Akt1 determined by Western blot analysis of cell lysates (Fig. 3B), and data were quantified by densitometric scanning (Fig. 3C). Like endogenous Akt1 in insulin-stimulated untransfected C2C12 cells, pT308-Akt1 of overexpressed Akt1 was significantly reduced when Par6α was co-overexpressed (-43.9%, p=0.012 for 10 nM

insulin). By contrast, pT308-Akt1 of the Akt1^{T34A} mutant was not significantly altered (+8.1%, $p=0.40$ for 10 nM insulin) upon Par6 α co-overexpression. Of note, the T34A mutation itself did not enhance T308-phosphorylation of Akt1 under unstimulated, basal conditions.

To verify that T308-phosphorylation actually reflects catalytic activity of Akt1 or the Akt1^{T34A} mutant in our cellular system, we next performed an immunokinase assay, using lysates from untreated or insulin stimulated cells. Akt1 was immunoprecipitated and in-vitro phosphorylation of a substrate peptide determined. Comparing Akt1 from unstimulated native C2C12 with Akt1 from C2C12^{Par6 α +} cells, there was no difference in basal kinase activity. However, after insulin stimulation (10 nM, 15 min), Akt1 kinase activity was significantly reduced when Par6 α was co-overexpressed (-59.3%; $p<0.05$; Fig. 4A) compared to untransfected C2C12. By contrast, neither basal nor insulin stimulated Akt1^{T34A} kinase activity was significantly altered in the presence of Par6 α (+15.9%, $p=0.31$ and +2.9%, $p=0.41$, respectively; Fig. 4B). Thus, Akt1^{T34A} kinase activity is resistant to Par6 α /aPKC regulation.

Finally, we tested whether the resistance of Akt1^{T34A} kinase activity to Par6 α -inhibition is of metabolic relevance in C2C12 cells. Glycogen synthesis was measured after stimulation with a physiological concentration of insulin (3 nM). While glycogen synthesis was increased by $37\pm 2\%$ in C2C12^{Akt1}, it was enhanced by only $23\pm 1\%$ in cells co-overexpressing Par6 α . This difference in increase was statistically not significant ($p=0.33$ at $n=3$). A likely explanation for the lack of statistical significance is the basal glycogen synthesis rate that was high in Akt1 overexpressing cells. In C2C12^{Akt1-T34A}, the increase in insulin-induced glycogen synthesis did not differ, whether Par6 α was co-overexpressed or not (30 ± 3 vs. $28\pm 3\%$ respectively, Fig. 4C). In summary, the ability of Par6 α to inhibit

insulin-stimulated glycogen synthesis is dependent on the complex formation with aPKC and subsequent aPKC-mediated Akt1 inhibition via phosphorylation of T34-Akt1.

Discussion

Serine/threonine phosphorylation of specific regulatory sites is a common mechanism for regulation of the insulin signalling cascade, and IRS proteins seem to be a major target of this regulation. Interestingly, some of the serine/threonine kinases that negatively regulate IRS proteins are also insulin-sensitive, like e. g. IKK β (I-kappa B kinase β), JNK (c-Jun N-terminal kinase) and PKC ζ , thereby providing negative feedback loops in insulin action (Zick, 2004). The role of Akt1-T34 as a target of aPKC has been identified in L6 myotubes stimulated with ceramide (Powell et al., 2003), a sphingolipid enriched in skeletal muscle of insulin-resistant subjects (Adams et al., 2004). In C2C12 cells, ceramide also caused Akt1 down-regulation, but this effect was independent of an increased aPKC activity and relied on a protein phosphatase 2A-like activity dephosphorylating Akt1 (Cazzolli et al., 2001). By contrast, our results indicate that the Par6 α /aPKC complex employs Akt1-T34 phosphorylation in C2C12 cells. Interestingly, serine/threonine phosphorylation of the PH domain with subsequent disruption of phosphoinositide binding was also reported for IRS1 (Nawaratne et al., 2006) and diacylglycerol kinase δ 1 (Imai et al., 2004), which indicates that this phosphorylation may represent a general mechanism for inhibition of translocation to the membrane.

Although we were not able to detect Par6 α in C2C12 cells by Western blot analysis, Par6 α was detected by RT-PCR which indicates that the protein is only weakly expressed in this cell line. However, C57Bl/6 mice tissue lysates clearly showed that Par6 α is expressed in all insulin-sensitive tissues, including muscle, which supports a role of Par6 α for in-vivo insulin signalling. Par6 activation of aPKC depends on activated (GTP-bound)

Rho-GTPases Cdc42 or TC10 (Kanzaki et al., 2004; Yamanaka et al., 2001). Of note, the Par6-aPKC-cdc42/TC10 complex enhanced glycogen-synthase-kinase 3 β (GSK3 β) phosphorylation in primary rat astrocytes and 3T3L1 adipocytes (Etienne-Manneville et al., 2003; Kanzaki et al., 2004), which could imply a positive role of this heteromeric Par6-aPKC-cdc42/TC10 complex for insulin signalling. In C2C12 cells, however, Par6 α overexpression inhibited insulin-dependent phosphorylation of both GSK3 α and GSK3 β , and a decreased GSK3 α / β phosphorylation leads to a stronger phosphorylation/inhibition of glycogen synthase and glycogen synthesis (Weyrich et al., 2004). One explanation for this seemingly contradictory finding may be that Par6 does not only interact with aPKC but is a scaffold for Par3, another member of the Par proteins, and cdc42 as well. Complexes of different Par6 isoforms may also involve Lgl and Pals1 and thus activate different signalling pathways (Hurd et al., 2003; Plant et al., 2003). It is currently unclear which of these pathways contributes to GSK3 β phosphorylation, whether they differ between distinct cellular models and how the expression levels of the signalling components affect the outcome. In addition, recent data indicate that at least in rat muscle there may be different pools of GSK3 β (Jensen et al., 2007). Another explanation for the difference in GSK3 β phosphorylation between Par6 overexpressing C2C12 myoblasts and 3T3L1 adipocytes may be that in the adipocyte model Par6 β was investigated, which acts in a different way than the α -isoform of Par6 (Gao et al., 2004). In C2C12 cells, the use of the Δ PB1-Par6 α mutant clearly indicates that the Par6 α /aPKC complex acts negatively on Akt1 and insulin-induced glycogen synthesis, and that the major contribution to this inhibition comes directly from aPKC.

In sum, this study indicates that Par6 α is expressed in various insulin-sensitive tissues and that the Par6 α /aPKC complex uses T34 phosphorylation of Akt1 to promote an aPKC-dependent negative feedback loop in insulin signalling. Since a single nucleotide

polymorphism in the human Par6 α promoter leads to reduced Par6 α expression and is associated with increased insulin sensitivity in humans (Weyrich et al., 2005), this mechanism could contribute to the development of insulin resistance in the in-vivo situation for individuals with a higher expression of Par6 α .

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Legends for figures

Figure 1

Detection of endogenous Par6 α expression in C2C12 cells and tissue lysates of C57Bl/6 mice. A) Expression of Par6 α was verified by PCR analysis on cDNA derived from C2C12 cells. The original RNA preparation was used as a template to exclude contamination by genomic DNA. B) Detection of endogenous Par6 α by Western blot analysis in tissue lysates (60 μ g per lane) of juvenile (=j; 8 weeks) and adult (=a; 6 months) C57Bl/6 mice. For both Western blots the amino-terminal anti-Par6 α antibody was used.

Figure 2

The Par6 α -mediated inhibitory effect on Akt1 relies on the Par6 α /aPKC interaction and on increased aPKC phosphorylation. (A) Schematic presentation of human Par6 α with its interaction domains PB1 (Phox and Bem1p), CRIB (Cdc42/Rac1-interactive binding) and PDZ (PSD-95/Discs large/Zo-1). hPar6 α , human Par6 α ; aa, amino acids. (B) HEK293 cells were transfected with the two human aPKC isoforms ι and ζ , Par6 α and the Δ PB1-Par6 mutant as indicated. After 24 h of serum starvation, cell lysates were subjected to immunoprecipitation (IP) with a Par6 α antibody (directed against the carboxyl-terminus) with identical binding affinity to wild-type and Δ PB1-Par6 α . Western blot analysis of protein expression is shown in the lower panels. (C) Parental C2C12 cells (upper panel) and C2C12 cells stably overexpressing wild-type Par6 α (middle panel) or Δ PB1-Par6 α (lower panel) were stimulated with the indicated concentrations of insulin, lysed and analysed by Western blotting. Filters were first probed with a phosphospecific antibody directed against pT308 of Akt1 and then reprobated with a T410/403-specific

phosphoantibody for PKC ζ / ι . When loading lysates, the amount corresponded to 40 μ g of protein.

Figure 3

The T34 phosphorylation site is crucial for Par6 α -dependent Akt1 down-regulation. (A) Native C2C12 and C2C12 cells overexpressing wild-type Akt1 or the Akt1^{T34A} mutant, and in addition Par6 α as indicated, were lysed and lysate aliquots (40 μ g of protein) analysed by Western blotting using antibodies specific for Akt1 and Par6 α . (B) C2C12 cells overexpressing either Akt1 (upper panel) or Akt1^{T34A} (lower panel), and in addition Par6 α as indicated, were stimulated with different concentrations of insulin, lysed and lysate aliquots analysed by Western blotting using antibodies against phosphorylated T308 of Akt1. To confirm a similar loading of protein (40 μ g of protein per each lane), Akt1 protein was detected by stripping and reblotting the filter. (C) Corresponding densitometric data of insulin dependent Akt1-T308 phosphorylation (n=4; means + SEM). Significantly differing column pairs are indicated by an asterisk.

Figure 4

Akt1^{T34A} is resistant against Par6 α -mediated inhibition of Akt1 kinase activity and inhibition of insulin-induced glycogen synthesis. C2C12 cells overexpressing wild type Akt1 (A) or Akt1^{T34A} (B) and in addition Par6 α as indicated were left untreated or stimulated with 10 nM (15 min) insulin. After cell lysis, an immunokinase assay was performed. The phosphorylation of a substrate peptide was measured by scintillation counting and data are shown as means + SEM (n=4). Significantly differing kinase activities are indicated by an asterisk (p<0.05). (C) The cell lines used in A and B were left untreated or stimulated with 3 nM (60 min) of insulin and glycogen synthesis

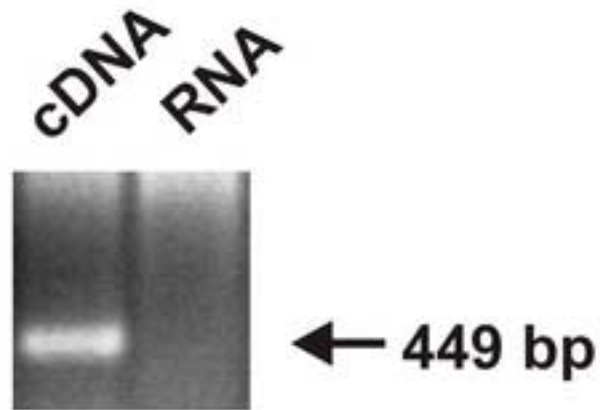
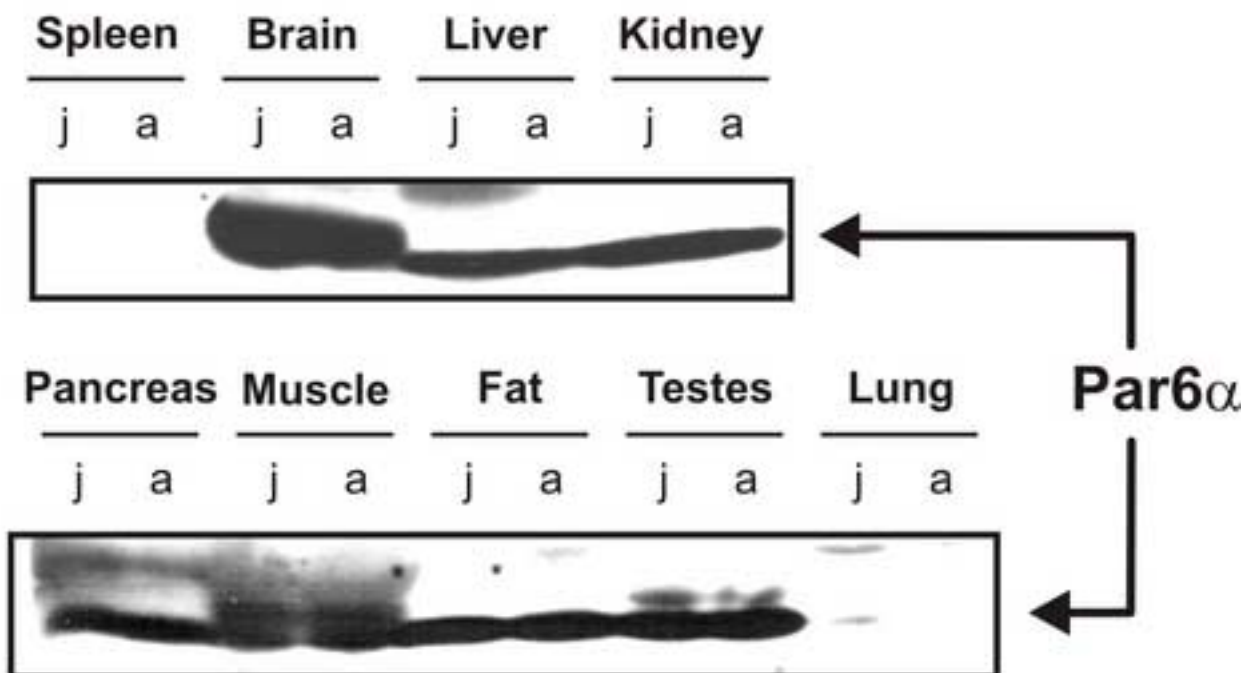
Fig.1**A****B**

Fig. 2

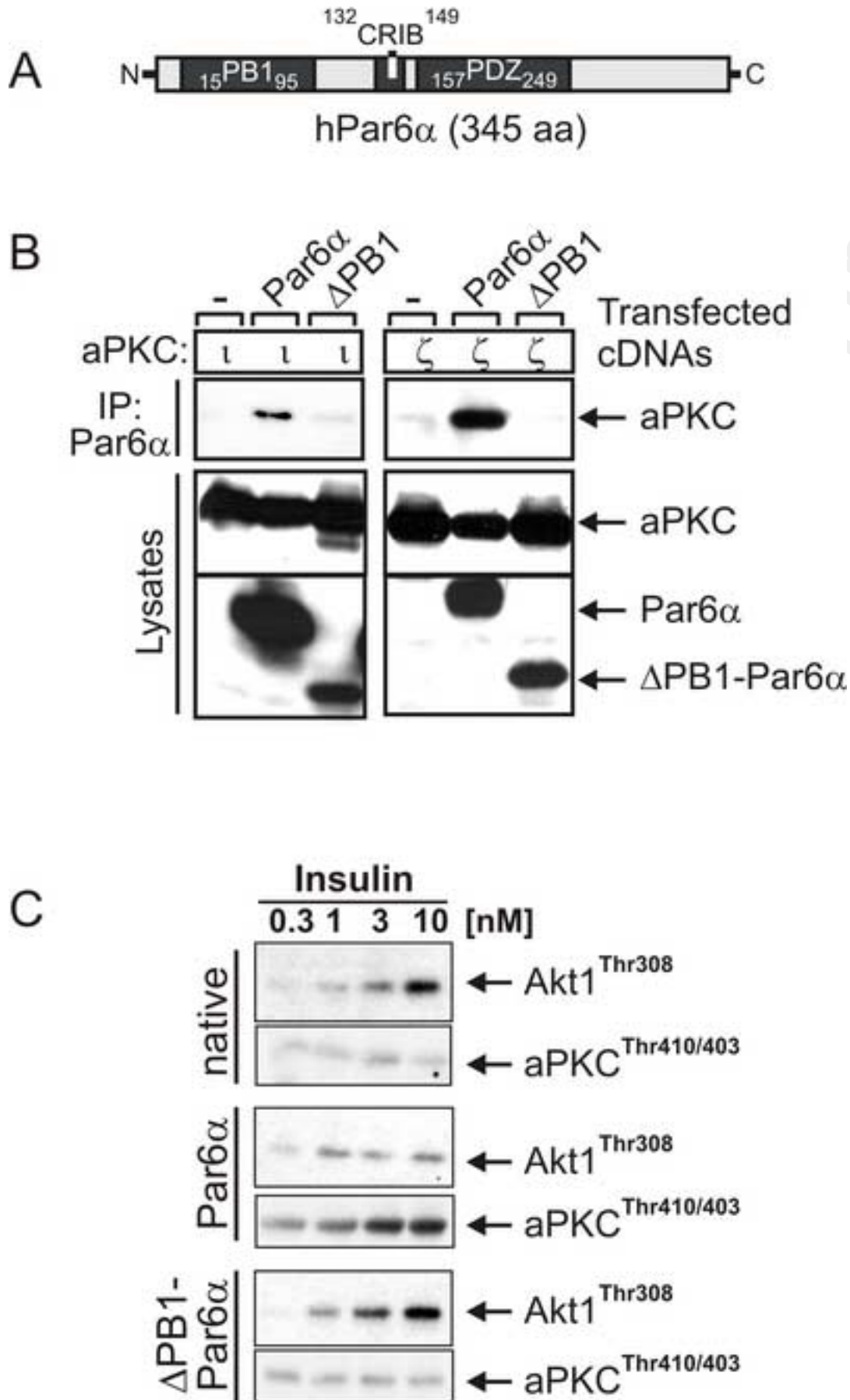


Fig.3

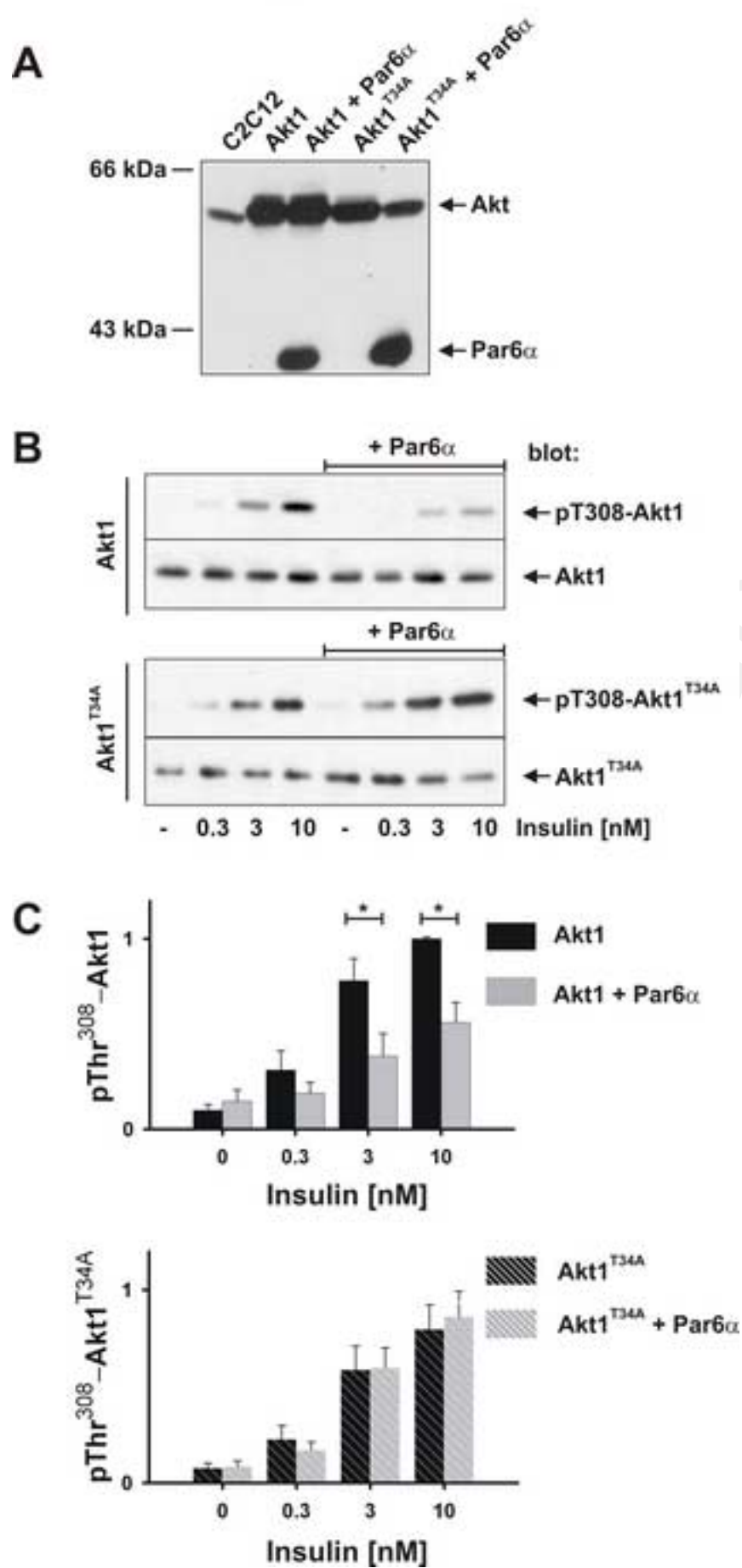


Fig.4

