Phosphorylation of Serine 1137/1138 of Mouse Insulin Receptor Substrate (IRS) 2 Regulates cAMP-dependent Binding to 14-3-3 Proteins and IRS2 Protein Degradation*

Received for publication, April 8, 2013, and in revised form, April 23, 2013 Published, JBC Papers in Press, April 24, 2013, DOI 10.1074/jbc.M113.474593

Sabine S. Neukamm†§, Jennifer Ott†, Sascha Dammeier†, Rainer Lehmann†§, Hans-Ulrich Häring†§**, Erwin Schleicher†§, and Cora Weigert†§

From the †Division of Clinical Chemistry and Pathobiology of the University of Tuebingen (Paul Langerhans Institute Tuebingen), Tuebingen, Germany and the ‡Division of Endocrinology, Diabetology, Vascular Medicine, Nephrology and Clinical Chemistry, Department of Internal Medicine IV, the §German Center for Diabetes Research (DZD), and the ¶Medical Proteome Center, Institute for Ophtalmic Research, University Hospital Tuebingen, Tuebingen 72076, Germany and the **Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at the University of Tuebingen (Paul Langerhans Institute Tuebingen), Tuebingen, Germany

Background: Regulation of insulin receptor substrate (IRS) 2 protein levels is crucial for glucose homeostasis.

Results: Elevated cAMP levels increase phosphorylation of serine 1137/1138 residues on IRS2 protein, which mediates its binding to 14-3-3 proteins and enhances its stability.

Conclusion: Serine 1137/1138 are novel cAMP-dependent phosphorylation sites on IRS2 that regulate its protein degradation via interaction with 14-3-3 proteins.

Significance: Novel cAMP-dependent mechanism to control IRS2 protein levels.

Insulin receptor substrate (IRS) 2 as intermediate docking platform transduces the insulin/IGF-1 (insulin like growth factor 1) signal to intracellular effector molecules that regulate glucose homeostasis, β-cell growth, and survival. Previously, IRS2 has been identified as a 14-3-3 interaction protein. 14-3-3 proteins can bind their target proteins via phosphorylated serine/threonine residues located within distinct motifs. In this study the binding of 14-3-3 to IRS2 upon stimulation with forskolin or the cAMP analog 8-(4-chlorophenylthio)-cAMP was demonstrated in HEK293 cells. Binding was reduced with PKA inhibitors H89 or R8-Br-cAMPS. Phosphorylation of IRS2 on PKA consensus motifs was induced by forskolin and the PKA activator N6-Phe-cAMP and prevented by both PKA inhibitors. The amino acid region after position 952 on IRS2 was identified as the 14-3-3 binding region by GST-14-3-3 pulldown assays. Mass spectrometric analysis revealed serine 1137 and serine 1138 as 14-3-3 binding region by GST-14-3-3 pulldown assays. Mass spectrometric analysis revealed serine 1137 and serine 1138 as novel cAMP-dependent phosphorylation sites on IRS2 and show their importance in 14-3-3 binding and IRS2 protein stability.

Insulin receptor substrate (IRS) 2 belongs to the family of insulin receptor substrates and serves as an intermediate docking platform to transmit the insulin and IGF-1 signal upon ligand binding to intracellular effector molecules for the regulation of glucose and lipid metabolism. Knock-out models have shown the physiological importance of IRS2 for glucose homeostasis, β-cell growth, and survival (1–3). The dynamic regulation of IRS2 protein levels in liver is important for the adaptation of the glucose metabolism to fasting and refeeding, whereas in pancreatic β-cells sufficient IRS2 protein levels are crucial for growth and survival (4). β-Cell-specific expression of IRS2 in IRS2 knock-out, obese, and streptozotocin-treated mice prevented diabetes in these mice (5). Ligand-stimulated autophosphorylation of tyrosine residues on insulin/IGF-1 receptors leads to their interaction with IRS proteins and other Src homology domain 2 containing proteins such as the p85 regulatory subunit of PI 3-kinase or Grb2. Serine and threonine residues on IRS2 can be phosphorylated by several kinases including ERK, JNK, or glycogen synthase kinase 3 (6–9) and has been associated with a negative feedback control of IRS2 in IRS2 knock-out, obese, and streptozotocin-treated mice prevented diabetes in these mice (5). Ligand-stimulated autophosphorylation of tyrosine residues on insulin/IGF-1 receptors leads to their interaction with IRS proteins and other Src homology domain 2 containing proteins such as the p85 regulatory subunit of PI 3-kinase or Grb2. Serine and threonine residues on IRS2 can be phosphorylated by several kinases including ERK, JNK, or glycogen synthase kinase 3 (6–9) and has been associated with a negative feedback control of IRS2 in IRS2 knock-out, obese, and streptozotocin-treated mice prevented diabetes in these mice (5). Ligand-stimulated autophosphorylation of tyrosine residues on insulin/IGF-1 receptors leads to their interaction with IRS proteins and other Src homology domain 2 containing proteins such as the p85 regulatory subunit of PI 3-kinase or Grb2.
insulin signaling (10, 11). The serine/threonine phosphorylation patterns of IRS proteins may also have stimulatory consequences for insulin/IGF-1 signal transduction (12–14). An important mechanism to control insulin signaling at the level of IRS2 is the regulation of IRS2 mRNA and total protein amounts. They are tightly regulated by fasting and refeeding (2, 7, 15). Elevated cAMP levels are a major stimulus for increased expression of IRS2 in the liver and pancreatic β-cell. IRS2 gene activation is initiated by activation of CREB (cAMP response element-binding protein) (16) through phosphorylation by PKA (protein kinase A) with TORC2 (transducer of regulated CREB activity 2) as an important co-activator for CREB-dependent hepatic IRS2 expression (15). Insulin suppresses IRS2 protein expression by reducing the rate of transcription of the IRS2 gene (17) and induces post-translational modifications on the IRS2 molecule itself, thus leading to proteasomal degradation (18).

14-3-3 proteins are versatile regulators of a variety of intracellular processes and participate in neuronal development and control of cell cycle, cell growth, gene transcription, and apoptosis. In mammals, 7 genes encode for the 7 14-3-3 isoforms that are entitled with the Greek letters β, γ, ε, η, σ, τ, and χ (19). 14-3-3 proteins can also be found in varying numbers in yeast, plants, and other eukaryotes. A special feature is the high sequence homology of all 14-3-3 proteins and the finding that 14-3-3 proteins we set out to characterize that can be exerted by 14-3-3 proteins we set out to characterize this interaction and its consequences further.

**EXPERIMENTAL PROCEDURES**

**Materials**—PKA substrate antibody (catalog number 9621), β-actin antibody (4970), p-Thr-308 Akt/PKB antibody (9275), p-Thr-202/p-Tyr-204 ERK antibody (9101), ERK1/2 protein antibody (9102), and PD98059 (9900) were from Cell Signaling Technology (Frankfurt, Germany) and p-Ser-157 VASP antibody (ab58555) was from Abcam (Cambridge, UK). GFP (green fluorescent protein) antibody (sc-8334) was from Santa Cruz (Santa Cruz, CA) and Protein A-Sepharose (17-5280-04), Protein G-Sepharose (17-0618-01), gluthione-Sepharose 4B (17-0756-01), and GST (glutathione S-transferase) antibody (27-4577-01) from GE Healthcare Europe (Munich, Germany). Recombinant human 14-3-3ζ-HRP (HRP-2669) was from R&D Systems (Minneapolis, MN) and IRS2 antibody (06-506 and MAB515) from Millipore (Schwalbach, Germany). InSolu™ Akt Inhibitor VIII Akt-1/2 (124017), 14-3-3 antagonist I (100081), and H89 (371963) were from Calbiochem (Schwalbach, Germany) and forskolin (A2165) from Appli-chem (Darmstadt, Germany). IGF-1 (I3769), EGF (E9644), 8-(4-chlorophenylthio) (CPT)-cAMP (C3912), and phospha-tase inhibitors (sodium fluoride, sodium pyrophosphate, sodium orthovanadate, and β-glycerophosphate) were from Sigma. Polyethyleneimine (24765) was from Polysciences (Eppelheim, Germany). Akt/PKB protein antibody (610861) was from BD Transduction Laboratories (Erenbodegem, Belgium). N2-Phe-cAMP (P006), 8-pCPT-2′-O-Me-cAMP (C041), and Rg-8-Br-cAMPS (B001) were from BioLog (Bremen, Germany). Flip-In HEK293 cells and 4–12% BisTris mini gels (NP0321) were obtained from Invitrogen (Karlsruhe, Germany) and GFP-Trap (gta-20) was from Chromotek (Martinsried, Germany). HEK293 cells were from The European Collection of Cell Cultures (Salisbury, UK) and Myc antibody and Myc-14-3-3-ζ expression vector was provided by Reiner Lammers (University Hospital Tuebingen, Tuebingen, Germany).

**Plasmid Constructs**—The coding region for mouse IRS2 (NM_001081212.1) was amplified from a pRK5 IRS2 vector (kindly provided by M. F. White, Boston, MA) using primers 5′-gagatctatcagtcgcgccccctgctg-3′ and 5′-ctgccgccgctcgactacactctctctctctc-3′, cloned into vector pSC-B (Stratagene) and sequenced. IRS2 was subcloned from this plasmid into pcDNA5/FRT/TO-GFP as a BamHI/NotI insert to give vector pcDNA5/FRT/TO-GFP-IRS2 in which IRS2 is tagged with GFP at the N terminus. For truncated IRS2 versions containing the IRS2 sequence from amino acids 1–300 and 1–600 a stop codon was created after position 300 (sense, 5′-gagttccggcctcgactacactctctctctctctctc-3′; antisense, 5′-ctgcccctgctgactacactctctctctctctctctc-3′) or 600 (sense, 5′-ctcatgccgcccctgctgactacactctctctctctctctc-3′; antisense, 5′-gagttccggcctcgactacactctctctctctctctctc-3′), respectively. For the constructs 301–1321 and 601–1321, a BamHI restriction site was created prior to positions 301 and 601 and the obtained inserts were ligated into pcDNA5/FRT/TO-GFP. The constructs mentioned so far were generated at the University of Dundee (Dundee, UK). Construct GFP-IRS2-601-952 was generated by creating a stop codon at position 952 and pcDNA5/
FRT/TO-GFP-IRS2-601-1321 served as template. The following constructs were generated with the Stratagene QuikChange XL Site-directed Mutagenesis method (200522-5, Stratagene, La Jolla, CA): GFP-IRS2-601-952, S1137A, S1138A, S1163A, and S1137/ S1138A, and verified by sequencing.

**Cell Culture and Transfections**—All cells were kept at 37 °C with 5% CO2 and 95% humidity. Fp-In HEK293 and HEK293 cells were cultivated in DMEM containing 4.5 g/liter of glucose (BE12–741F, Lonza, Cologne, Germany) supplemented with 10% FBS (SV30160.03, HyClone, Thermo Fisher Scientific, Schwerte, Germany), 1% glutamine, 100 units/ml of penicillin, and 100 units/ml of streptomycin (DE17–602E, Lonza, Cologne, Germany). Fp-In HEK293 cells were stably transfected by the calcium phosphate method (35) and positive clones were selected with hygromycin B, whereas HEK293 cells were transiently transfected using polyethylenimine in 25 mM HEPES, pH 7.5, containing phosphate inhibitors (1 mM sodium fluoride, 0.5 mM sodium pyrophosphate). Cells were seeded at a density of 7 × 106 cells onto 15-cm diameter cell culture dishes and transfected 24 h later with 10 μg of pDNA/plate using 80 μg of polyethylenimine/plate. 45 h later, cells were serum starved and incubated for 30 min with 20 μM forskolin alone or after preincubation with 30 μM H89 for 30 min. Cells were lysed with 900 μl of lysis buffer, after which GFP-IRS2 was purified from 5 mg of precleared total protein using GFP-Trap. Prior to LC-MS/MS analysis, samples were separated on 4–12% BisTris gels and after Coomassie staining bands corresponding to IRS2 were cut and subjected to tryptic proteolyis as described by Glocenkner et al. (37). Resulting peptides were separated using the UltiMate 3000 nano-HPLC system. They were eluted from the trap column onto the analytical column (Acclaim PepMap RSLC 75 μm × 25-cm C18 2-μm 100 Å) by applying an acetonitrile gradient of 2 to 35% of eluent (80% acetonitrile, 0.08% formic acid) for 33 min. The eluted peptides were analyzed by mass spectrometry (LTQ Orbitrap Velos, Thermo Fisher Scientific). Mass spectra were extracted by Mascot Daemon without charge state deconvolution and de-isotoping and the results were analyzed using Mascot (Matrix Science version 2.3.0, Boston, MA). Because mouse IRS2 was transfected, Mascot was set up to search the *Mus musculus* subset of the SwissProt database (release 2011_12, 533657 entries) assuming trypsin as the digestion enzyme. Mascot was searched with a fragment ion mass tolerance of 1 Da and a parent ion tolerance of 10 ppm. Oxidation of methionine, deamidation of asparagines and glutamine, and phosphorylation of serine, threonine, and tyrosine were specified in Mascot as variable modifications. Carbamidomethylation of cysteine was specified as a fixed modification. The Mascot result files were loaded into Scaffold software (Scaffold version 3.3.1, Proteome Software Inc., Portland, OR) to validate MS/MS-based peptide identifications. Peptide identifications were accepted if they could be established at a probability greater than 80%, as specified by the Mascot Daemon. Further analysis of the...
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MS/MS data with regard to post-translational modifications was performed using the Proteome Discoverer software (version 1.3, Thermo Fisher Scientific) and Skyline (version 1.2) (38).

Statistical Analysis—Statistical analysis was performed with JMP 10.0.0 (SAS Institute Inc., NC) and data are presented as mean ± S.E. t test was employed to test for significant differences between groups and a result was considered significant if $p < 0.05$.

RESULTS

Elevated cAMP Levels Enable 14-3-3 Binding to IRS2 in a PKA-dependent Manner—Dubois et al. (34) reported an interaction of IRS2 with 14-3-3 proteins upon activation of the PI 3-kinase pathway by IGF-1 stimulation in HEK293 cells. We tested if other intracellular pathways can mediate 14-3-3 binding to IRS2 in HEK293 cells expressing GFP-IRS2. The adenylyl cyclase activator forskolin increased binding of 14-3-3 to IRS2 as shown by an overlay assay (Fig. 1A). Dependence of the IRS2/14-3-3 interaction on elevated cAMP levels was also shown with the cell-permeable cAMP analog CPT-cAMP (Fig. 1B). The interaction was completely abrogated with the PKA inhibitor H89 (Fig. 1, A and B) or reduced to basal levels with the specific PKA inhibitor $R_{p}$-8-Br-cAMPS (Fig. 1C). These data suggest the involvement of a PKA-mediated phosphorylation of IRS2 for its interaction with 14-3-3 proteins. PKA-dependent phosphorylation of IRS2 was studied with a PKA substrate antibody in HEK293 cells expressing GFP-IRS2 after GFP pulldown. Forskolin induced the phosphorylation of IRS2 (Fig. 1, D–F). Successful stimulation with forskolin was shown by phosphorylation of Ser-157 of vasodilator-stimulated protein (VASP) (Fig. 1, D and E). Elevated cAMP levels cannot only enhance phosphorylation of IRS2 via PKA, but can also involve exchange proteins directly activated by cAMP (EPAC) and downstream kinases ERK, Akt/PKB, and p90 ribosomal S6 kinase (RSK). Moreover, the PKA substrate antibody used recognizes a consensus site that partially overlaps with other arginine-directed kinases such as Akt/PKB and RSK. Therefore, the putative involvement of these kinases was studied. Stimulation with EGF (epidermal growth factor) as an activator of the ERK pathway did not lead to phosphorylation of any of the sites recognized by the PKA substrate antibody (Fig. 1D). Successful EGF stimulation was confirmed by checking the phosphorylation status of ERK. Pretreatment with the MEK1 inhibitor PD98059 before forskolin treatment did not influence cAMP-dependent phosphorylation of PKA sites on IRS2 (Fig. 1D).

Next, cells were treated with IGF-1 and the Akt/PKB inhibitor Akti. Successful IGF-1 stimulation was confirmed by checking the phosphorylation of threonine 308 of Akt/PKB. However, the stimulation by IGF-1 did not lead to phosphorylation of any of the sites recognized by the PKA substrate antibody (Fig. 1E). Furthermore, we tested the direct PKA activator N'-Phe-cAMP. Stimulation of the cells with this compound increased the phosphorylation of IRS2, whereas the EPAC activator 8-pCPT-2′-O-Me-cAMP did not increase the phosphorylation to a similar extend (Fig. 1F). The use of the specific PKA inhibitor $R_{p}$-8-Br-cAMPS prevented the phosphorylation (Fig. 1, F and G). These results indicate that elevated cAMP levels via activation of PKA lead to phosphorylation of IRS2 and interaction between IRS2 and 14-3-3. Furthermore, phosphorylation of IRS2 on PKA sites was detected in primary hepatocytes after forskolin stimulation, which was prevented by H89 pretreatment (Fig. 1H).

Identification of the cAMP-dependent 14-3-3 Binding Region on IRS2—Fragments spanning different regions of the IRS2 protein were generated. The two fragments GFP-IRS2-(1–300) and GFP-IRS2-(1–600) encompass the front part of the IRS2 molecule including the pleckstrin homology and the phosphotyrosine binding domains. The two fragments GFP-IRS2-(301–1321) and GFP-IRS2-(601–1321) comprise the rear part of IRS2 with the kinase regulatory loop binding domain (Fig. 2A). HEK293 cells were transiently transfected with wild type IRS2 and IRS2 fragments and treated with forskolin and H89. Phosphorylation of PKA sites was tested after GFP pulldown (Fig. 2B). The antibody detected PKA phosphorylation sites on IRS2 wild type and fragments GFP-IRS2-(301–1321) and GFP-IRS2-(601–1321) after forskolin stimulation that were not detectable when cells were pretreated with H89. No signal was detected on fragments GFP-IRS2-(1–300) and GFP-IRS2-(1–600). Fragment GFP-IRS2-(601–952) was generated to further narrow down the area for forskolin-dependent phosphorylation of IRS2. Because the PKA substrate antibody did not detect any signals from this fragment we assumed that the PKA phosphorylation sites on IRS2 were located after amino acid position 952 (Fig. 2C). To test if the fragments containing PKA phosphorylation sites were also able to interact with 14-3-3 proteins, pulldown experiments with a GST-14-3-3 fusion protein were performed (Fig. 2D). IRS2 wild type protein interacted only in the forskolin-stimulated sample with GST-14-3-3β. GFP-IRS2-(301–1321) and GFP-IRS2-(601–1321) also showed strong interaction with GST-14-3-3β in the forskolin-stimulated condition, and only minimal interaction in the unstimulated or H89 pretreated condition. No interaction between fragment GFP-IRS2-(601–952) and GST-14-3-3β could be detected. Incubation with the PKA substrate antibody confirmed the presence of PKA phosphorylation sites on wild type IRS2 and IRS2 fragments 301–1321 and 601–1321. Therefore we concluded that PKA phosphorylation sites on IRS2 that mediate interaction with 14-3-3 were located after amino acid position 952.

Mass Spectrometric Identification of Potential PKA Phosphorylation and 14-3-3 Binding Sites—We applied mass spectrometry to identify possible candidates for PKA phosphorylation of IRS2. HEK293 cells were transiently transfected with GFP-IRS2 and treated with forskolin and H89. GFP-IRS2 was captured from cell lysates and after Coomassie staining, bands corresponding to GFP-IRS2 were cut and prepared for mass spectrometry analysis. This experiment was performed three times and only sites that matched the following criteria were considered for further testing: (i) present in all three replicates in the forskolin-stimulated sample and not present in the H89 pretreated sample, (ii) matched the consensus sequence for PKA (RXXpS), and (iii) matched a 14-3-3 binding motif (mode I, RXpS/pTXp; mode II, RX(F/Y)XS/pTXp). From that evaluation three candidates emerged: serine 1137, serine 1138, and serine 1163, depicted with their surrounding amino acids in Fig. 3A.
A. Of note, phosphorylated serine 1137 and serine 1138 could be found in tryptic peptides as single phosphorylated residues but also doubly phosphorylated peptides were detectable as shown in Fig. 3B. Serine 1137 (GRRRH51137SETFSS) and serine 1138 (GRRRHSS1138ETFSS) could probably compensate for each other, because in the case of substitution of either residue with an alanine there would still be the same PKA recognition motif present. Therefore, not only were the single residues mutated to alanine but an additional double mutant S1137A/S1138A was also generated. Transient expression of GFP, GFP-IRS2, GFP-IRS2-S1137A, GFP-IRS2-S1138A, GFP-IRS2-S1163A, or GFP-IRS2-S1137A/S1138A in HEK293 cells was

FIGURE 1. 14-3-3 interacts with IRS2 upon elevated cAMP levels and PKA phosphorylates IRS2. A, HEK293 cells were transiently transfected with GFP-IRS2. Cells were incubated for 30 min with 20 μM forskolin alone or after preincubation with 30 μM H89 for 30 min. 100 μg of protein was used for GFP pulldown. 14-3-3 binding was visualized by performing an overlay assay and the membrane was stripped and reprobed for GFP-IRS2 as expression and loading control. Densitometric analysis of 14-3-3 binding is shown as band intensities normalized for GFP-IRS2. GFP-IRS2 treated with forskolin was set as 1 (mean ± S.E., n = 3, *, p < 0.05, forskolin versus unstimulated; #, <0.05, forskolin versus H89). B, cells were stimulated with 100 μM CPT-cAMP for 30 min or after preincubation with 30 μM H89 for 30 min. 200 μg of protein was used for GFP pulldown. GFP-IRS2 treated with CPT-cAMP was set as 1 (mean ± S.E., n = 3, *, p < 0.05, CPT-cAMP versus unstimulated; #, <0.05, CPT-cAMP versus H89). C, cells were incubated for 30 min with 20 μM forskolin alone or after preincubation with 100 μM Rp-8-Br-cAMPS for 30 min. 400 μg of protein was used for GFP pulldown. GFP-IRS2 treated with forskolin was set as 1 (mean ± S.E., n = 3, *, p < 0.05, forskolin versus unstimulated; #, <0.05, forskolin versus Rp-8-Br-cAMPS). D and E, GFP-IRS2 was transiently expressed in HEK293 cells and cells were incubated for 30 min with 1 μM Akt inhibitor VIII Akti 1/2 (Akti), 20 μM forskolin, 50 ng/ml of IGF-1, 50 μM PD98059, and 100 ng/ml of EGF as indicated. 200 μg of total protein was used for GFP pulldown. Membrane was incubated with PKA substrate antibody. Phosphorylation of serine 157 of VASP was assessed as forskolin stimulation control, phosphorylation of threonine 308 of Akt/PKB as IGF-1 stimulation control, whereas phosphorylation of threonine 202/tyrosine 204 of ERK was checked as control for successful EGF stimulation. Corresponding IRS2, Akt/PKB, and ERK reblots are also shown (n = 3). F and G, GFP-IRS2 was expressed transiently in HEK293 cells. Cells were incubated with the following reagents for 30 min: 100 μM N6-Phe-cAMP, 100 μM 8-pCPT-2′,O-Me-cAMP, 100 μM Rp-8-Br-cAMPS or 20 μM forskolin. 400 μg of protein was used for GFP pulldown. Membrane was incubated with PKA substrate antibody, after which it was stripped and reprobed with IRS2 antibody (n = 3). H, primary hepatocytes were incubated with 20 μM forskolin alone or after preincubation with 30 μM H89 for 30 min, respectively. 100 μg of protein was separated on SDS gel and membranes were incubated with PKA substrate antibody, phosphoserine 157 VASP antibody, or β-actin antibody. PKA substrate antibody membrane was stripped and reprobed with IRS2 antibody (n = 3).
followed by stimulation with forskolin and H89 and subsequent GFP pulldown. The PKA substrate antibody only detected signals in the forskolin-treated samples of GFP-IRS2 and GFP-IRS2-S1163A (Fig. 3C). This result argued against serine 1163 as a PKA phosphorylation site and argued for serine 1137 and serine 1138 as PKA phosphorylation sites on IRS2. Due to the detection of peptides phosphorylated on both serine residues 1137 and 1138 and the PKA consensus site still present in the single mutants, the double mutant GFP-IRS2-S1137A/S1138A was used for further studies. Sequence alignments of IRS2 protein sequences from human, mouse, rat, and frog revealed sequence conservation of these potential PKA phosphorylation sites (Fig. 3D).

**Mutation of Serine 1137/1138 on IRS2 Reduces Interaction with 14-3-3**—If serine 1137 and serine 1138 are important for cAMP-mediated interaction with 14-3-3 proteins, mutation of these sites should reduce this interaction. This hypothesis was tested by stimulating cells that transiently expressed GFP-IRS2 and GFP-IRS2-S1163A with forskolin. GST pulldown experiments revealed that wild type IRS2 interacted slightly with GST-14-3-3β for 2 h and samples were separated by SDS-PAGE. Transfer onto nitrocellulose membranes followed by incubation with GFP antibody to visualize the amount of protein that interacted with GST-14-3-3 and with GST to ensure equal pulldown and loading. Incubation with PKA substrate antibody was carried out to visualize phosphorylation of PKA motifs (n = 3).
action with GST-14-3-3ε in the basal condition and a minor interaction with GST-14-3-3β. There was also an interaction detectable in the forskolin-treated samples, but the extent was significantly less in comparison to wild type IRS2 (Fig. 4B). To further confirm these results we performed co-immunoprecipitation assays, where HEK293 cells were transiently transfected with GFP, GFP-IRS2, GFP-IRS2-S1137A, GFP-IRS2-S1138A, GFP-IRS2-S1163A, or GFP-IRS2-S1137A/S1138A. Cells were stimulated with 20 μM forskolin for 30 min alone or after pretreatment with 30 μM H89 for 30 min. GFP pulldown assay was performed with 200 μg of total protein and after SDS-PAGE and Western blotting the membrane was incubated with PKA substrate antibody. The corresponding blot reprobed with IRS2 antibody is also shown (n = 3). D, Amino acid areas surrounding serine 1137 and serine 1138 from IRS2 in different species are shown: Homo sapiens (NP_003740.2), Mus musculus (NP_001074681.1), Rattus norvegicus (NP_001162104.1), and Xenopus laevis (AAH72768.1).

FIGURE 3. Mass spectrometry identifies three candidate residues in IRS2 for phosphorylation by PKA. A, sequences surrounding the potential PKA phosphorylation sites serine 1137, serine 1138, and serine 1163 are shown according to IRS2 mouse numbering. B, MS/MS analysis of a tryptic peptide with the parent mass (M+3H) = 951.4145 revealed a mass spectrum consisting of single- and double-charged fragment masses (preferably ions most relevant to phosphorylation sites are annotated) matching the doubly phosphorylated IRS2-peptide sequence RHspSSETFSSTTVPSFAHNSK. C, HEK293 cells were transiently transfected with GFP, GFP-IRS2, GFP-IRS2-S1137A, GFP-IRS2-S1138A, GFP-IRS2-S1163A, or GFP-IRS2-S1137A/S1138A. Cells were stimulated with 20 μM forskolin for 30 min alone or after pretreatment with 30 μM H89 for 30 min. GFP pulldown assay was performed with 200 μg of total protein and after SDS-PAGE and Western blotting the membrane was incubated with PKA substrate antibody. The corresponding blot reprobed with IRS2 antibody is also shown (n = 3).

Stimulation with Forskolin Prevents Protein Degradation of IRS2—A strong effect of elevated cAMP levels on the IRS2 protein amount via transcriptional activation has already been established (16). Based on our results we hypothesized additional effects of cAMP, namely on IRS2 protein stability. HEK293 cells stably expressing IRS2 were generated, because stable transfection provides the advantage of a continuous expression of the desired protein at considerably lower levels than in transiently transfected cells, thus preventing an obscuring of IRS2 protein half-life due to permanently high expression levels. To visualize IRS2 protein degradation cells were incu-
bated with cycloheximide to inhibit protein translation. After 3 and 6 h of incubation, IRS2 protein content was reduced by 80%. Concomitant incubation with forskolin reduced the IRS2 protein content only by about 25% (Fig. 5, A and B). Comparable results were also obtained in primary hepatocytes (Fig. 5C).

14-3-3 and Serine 1137/1138 Are Important for IRS2 Protein Stability—For clarification of whether 14-3-3 binding to IRS2 is important for its stability, a dipeptidyl-phosphoserine compound was used (39). It binds to endogenous 14-3-3 proteins, thereby disrupting its binding to target proteins. Treatment of cells with cycloheximide, forskolin, and the 14-3-3 antagonist in parallel abolished the increased IRS2 protein stability that was observed after incubation with cycloheximide and forskolin alone (Fig. 6, A and B). The involvement of the identified PKA phosphorylation sites of serine 1137/1138 in IRS2 protein stability was also tested. GFP-IRS2 stably expressed in Flp-In HEK293 cells showed increased protein stability upon stimulation with forskolin compared with the S1137A/S1138A mutant (Fig. 6, C and D). Thus, the presence of phosphorylated serine 1137/1138 and the interaction with cAMP-dependent IRS2 protein stability.

**DISCUSSION**

An increasing number of studies have shown the importance of 14-3-3 proteins in controlling cellular processes like cell cycle, cell growth, gene transcription, and apoptosis as reviewed in Refs. 40–43. IRS proteins and IRS1 and IRS2 in particular serve as intracellular docking and adapter molecules that integrate stimuli from different cellular pathways. Our results provide clear evidence that IRS2 and 14-3-3 are binding partners under conditions of increased cAMP levels. The binding is at least partially mediated via PKA-dependent phosphorylation of IRS2 on serine 1137/1138 and enhances IRS2 protein stability. The binding of 14-3-3 proteins to IRS2 has been shown by Ogihara et al. (33) in Sf9 cells and can also be regulated by an IGF-1/insulin-dependent pathway (34, 44). Now we
could demonstrate a second, IGF-1/insulin-independent regulatory mechanism induced by elevated cAMP levels.

Our results indicate that the cAMP-activated phosphorylation of IRS2 involves PKA. Elevated cAMP levels can also activate EPAC (45, 46) and as downstream kinases ERK, RSK, and Akt/PKB (47). Application of different activators and inhibitors of the above mentioned targets revealed that PKA is the major kinase responsible for the phosphorylation of IRS2 upon cAMP stimulation in our system. The activators of EPAC, ERK, and Akt/PKB did not lead to phosphorylation of IRS2 on PKA sites, whereas the PKA activator did. Inhibitors of Akt/PKB or ERK had no effect on forskolin-stimulated IRS2 phosphorylation, but H89 and the PKA inhibitor R8-Br-cAMPS prevented phosphorylation and the increased binding of IRS2 to 14-3-3 proteins. Taken together, these results provide clear indications of involvement of the cAMP-PKA pathway as mediator of IRS2 phosphorylation and binding to 14-3-3 proteins. The inhibitor H89 is very effective in reducing the interaction of IRS2 and the single mutation of either serine 1137 (RRHA1137ETF) and the double mutation of either residues 1137 and 1138 at the same time were detected phosphorylation sites. Because peptides phosphorylated on H89 can modulate the IRS2/14-3-3 interaction.

We could show in Flp-In HEK293 cells stably expressing either IRS2 or GFP-IRS2 and also in primary hepatocytes that the half-life of the IRS2 protein was increased when cells were stimulated with forskolin. Serine 1137/1138 proved to be crucial for this increased stability, because a double GFP-IRS2-S1137A/S1138A mutant did not show increased protein stability during forskolin stimulation. Based on the present data we hypothesize that phosphorylation on serine 1137/1138 is necessary for both, the cAMP-enhanced binding to 14-3-3 proteins and the stabilization of the protein. The use of a 14-3-3 antagonist supports the proposed molecular mechanism of cAMP/PKA-dependent IRS2 protein stabilization. Cells stably expressing GFP-IRS2 showed no increased IRS2 protein stabil-

FIGURE 5. Forskolin prevents protein degradation of IRS2. A, Flp-In HEK293 cells stably expressing IRS2 were incubated with 25 μg/ml of cycloheximide (CHX) alone or in combination with 20 μM forskolin (FSK) for 1, 3, and 6 h. 10 μg of protein was separated and membranes were incubated with antibodies against IRS2, phosphorylated serine 157 of VASP as stimulation control and β-actin as loading control. B, densitometric analysis of IRS2 protein bands. IRS2 content was normalized for β-actin and IRS2 from untreated cells was set as 100% (mean ± S.E., n = 8, * p < 0.05 cycloheximide (open squares) versus cycloheximide/forskolin-treated (filled squares)). C, hepatocytes were isolated from male C57BL/6 mice, plated onto 6-well plates, treated as described in A and 100 μg of protein was analyzed (n = 3).
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A. ChX CHX/FSK 14-3-3 antagonist

- 1 3 6 1 3 6

14-3-3 antagonist

h

IPRS2

β-actin

p-Ser-157 VASP

B. CHX CHX/FSK

- 1 3 6 1 3 6

IRS2

β-actin

p-Ser-157 VASP

C. CHX CHX/FSK

- 1 3 6 1 3 6

GFP-IRS2

GFP-IRS2-S1137A/S1138A

D. CHX CHX/FSK

- 1 3 6 1 3 6

GFP-IRS2

GFP-IRS2-S1137A/S1138A

FIGURE 6. 14-3-3 proteins and serine 1137/1138 are important for IRS2 protein stability. A, Flp-In HEK293 cells stably expressing GFP-IRS2 were treated for the indicated time points with 25 μg/ml of cycloheximide (ChX) alone or in combination with 20 μM forskolin (FSK). Another set of cells was treated with 20 μM 14-3-3 antagonist overnight and during the incubation times. 25 μg of protein was separated by SDS-PAGE and membranes were incubated with following antibodies: IRS2, phosphoserine 157 of VASP and β-actin. B, denitometric analysis of IRS2 protein degradation. IRS2 content was normalized for β-actin and IRS2 from untreated cells was set as 100% (mean ± S.E., n = 4, *, p < 0.05 cycloheximide/forskolin (open squares) versus cycloheximide/forskolin/14-3-3 antagonist-treated (filled squares) at 3 h). C, Flp-In HEK293 cells stably expressing GFP-IRS2 or GFP-IRS2-S1137A/S1138A were incubated with 25 μg/ml of CHX and 20 μM FSK for 1, 3, and 6 h. 25 μg of protein was separated by SDS-PAGE and membranes were incubated with IRS2 antibody, phosphoserine 157 of VASP and β-actin antibody. D, the extent of IRS2 protein degradation was quantified by scanning immunobots and normalization of IRS2 signal intensities for β-actin. Untreated cells expressing GFP-IRS2 or GFP-IRS2-S1137A/S1138A were set to 100% (mean ± S.E., n = 4, *, p < 0.05, GFP-IRS2 (open squares) versus GFP-IRS2-S1137A/S1138A (filled squares) at 3 and 6 h).

Our data indicate an additional mechanism for the up-regulation of IRS2 protein levels in physiological conditions with elevated cAMP concentrations. During fasting, blood glucagon levels are increased and are known to induce IRS2 mRNA expression through glucagon-dependent G protein-coupled increases in cAMP levels, activation of PKA, and subsequent activation of the transcription factor CREB and its coactivator TORC2 (15, 16). Of note, IRS2 expression in the fasting state is
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important to negatively regulate glucose output from the liver to avoid hyperglycemia (15). Increased IRS2 protein levels in hepatocytes during the fasting state ensure fast and effective signaling upon insulin stimulation in the transition from the fasted to the fed state. Notably, we were able to show phosphorylation of IRS2 on PKA sites in primary mouse hepatocytes after forskolin stimulation as well as increased IRS2 protein stability. The pronounced up-regulation of IRS2 protein levels observed already after 30 min of forskolin stimulation suggests that the primary cell culture model in particular, is responsive to the effects of cAMP on the regulation of IRS2 protein amount.

In pancreatic β-cells IRS2 expression is crucial for growth and survival (5). Increased blood glucose levels in the refed state are followed by insulin secretion of the β-cell. Glucose itself can induce IRS2 mRNA and protein expression in β-cells by a calcium-dependent mechanism (59). In addition, glucagon-like peptide 1 is released into the systemic circulation from enteroendocrine cells during feeding and potentiates insulin secretion. Glucagon-like peptide 1 increases cAMP levels in enteroendocrine cells during feeding and potentiates insulin secretion. Glucagon-like peptide 1 increases cAMP levels in β-cells, thus promoting β-cell viability via increased IRS2 expression (16). Our finding of increased IRS2 protein stability due to elevated cAMP levels could provide an additional mechanism for the β-cell to protect itself from reduced IRS2 protein levels. Van de Velde et al. (60) observed rapid gene expression of CREB target genes by forskolin stimulation that decreased to baseline levels after 4 h, whereas elevated IRS2 protein expression was measurable by Western blot up to 16 h in INS-1 cells. This discrepancy in decreased in IRS2 gene activation but elevated IRS2 protein levels could be explained with our finding of increased IRS2 protein stability due to cAMP-induced 14-3-3 binding to IRS2. Of course, our considerations are based on the mechanistic investigation of cAMP-dependent interaction between IRS2 and 14-3-3 in our study and have to be confirmed in β-cells and subsequently in vivo.

To conclude, we identified serine 1137/1138 as PKA phosphorylation sites on IRS2 that mediate 14-3-3 binding and regulate IRS2 protein stability. The increased IRS2 protein stability upon elevated cAMP levels provides an additional mechanism to cAMP-induced IRS2 mRNA and subsequent protein expression to ensure sufficient amounts of IRS2 protein.

Acknowledgments—We are grateful to Prof. Carol MacKintosh and Dr. Rachel Toth from the University of Dundee for their help in generation of some of the expression vectors used in this work and the initial experiments that led to this study. Furthermore, we thank Prof. Reiner Lammers for providing the Myc-14-3-3y construct and the Myc antibody, Angelika Hauser for GST-14-3-3 expression vectors, and Morris White for the pRKS IRS2 expression vector. In addition we thank Heike Runge and Ann Kathrin Pohl for their outstanding technical assistance and Madhura Parne for proofreading the manuscript.

REFERENCES
1. Kubota, N., Tobe, K., Terauchi, Y., Eto, K., Yamauchi, T., Suzuki, R., Tsumamoto, Y., Komeda, K., Nakano, R., Miki, H., Satoh, S., Sekihara, H., Sciacchitano, S., Lesniak, M., Aizawa, S., Nagai, R., Kimura, S., Akunama, Y., Taylor, S. I., and Kadowaki, T. (2000) Disruption of insulin receptor sub-
2. Kubota, N., Kubota, T., Itoh, S., Kumagai, H., Kozono, H., Takamoto, I., Mineyama, T., Ogata, H., Tokuyama, K., Ohsumi, M., Sasaki, T., Moroi, M., Sugii, K., Kakuta, S., Iwakura, Y., Noda, T., Ohnishi, S., Nagai, R., Tobe, K., Terauchi, Y., Ueki, K., and Kadowaki, T. (2008) Dynamic functional relay between insulin receptor substate 1 and 2 in hepatic insulin signaling during fasting and feeding. Cell Metab. 8, 49–64
3. Withers, D. J., Gutierrez, J. S., Towery, H., Burks, D. J., Ren, J. M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G. I., Bonner-Weir, S., and White, M. F. (1998) Disruption of IRS-2 causes type 2 diabetes in mice. Nature 391, 900–904
4. Schuppin, G. T., Pons, S., Hügıl, S., Aiello, L. P., King, G. L., White, M., and Rhodes, C. J. (1998) A specific increased expression of insulin receptor substate 2 in pancreatic beta-cell lines is involved in mediating serum-stimulated beta-cell growth. Diabetes 47, 1074–1085
5. Hennige, A. M., Burks, D. J., Ozcan, U., Kulkarni, R. N., Ye, J., Park, S., Schubert, M., Fisher, T. L., Dow, M. A., Leshan, R., Zakaria, M., Massa-Basha, M., and White, M. F. (2003) Up-regulation of insulin receptor substate-2 in pancreatic beta cells prevents diabetes. J. Clin. Invest. 112, 1521–1532
6. Sharfi, H., and Eldar-Finkelman, H. (2008) Sequential phosphorylation of insulin receptor substate-2 by glycol synthase kinase-3 and c-Jun NH2-terminal kinase plays a role in hepatic insulin signaling. Am. J. Physiol. Endocrinol. Metab. 294, E307–E315
7. Fritsche, L., Neukamm, S. S., Lehmann, R., Kremmer, E., Hennige, A. M., Hunder-Gugel, A., Schenk, M., Häring, H. U., Schleicher, E. D., and Weigert, C. (2011) Insulin-induced serine phosphorylation of IRS-2 via ERK1/2 and mTOR. Studies on the function of Ser-675 and Ser-907. Am. J. Physiol. Endocrinol. Metab. 300, E824–E836
8. Solinas, G., Naugler, W., Galimi, F., Lee, M. S., and Karin, M. (2006) Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. Proc. Natl. Acad. Sci. U.S.A. 103, 16454–16459
9. Oriente, F., Formisano, P., Miele, C., Fiory, F., Maitan, M. A., Vigliotta, G., Tiencia, A., Santopietro, S., Caruso, M., Van Obberghen, E., and Beguinot, F. (2001) Insulin receptor substate-2 phosphorylation is necessary for protein kinase Cz activation by insulin in L6Hir cells. J. Biol. Chem. 276, 37109–37119
10. Greene, M. W., and Garofalo, R. S. (2002) Positive and negative regulatory role of insulin receptor substate 1 and 2 (IRS-1 and IRS-2) serine/threonine phosphorylation. Biochemistry 41, 7082–7091
11. Gurevitch, D., Boura-Halton, S., Isaac, R., Shahaf, G., Alberstein, M., Ronen, D., Lewis, E. C., and Zick, Y. (2010) Elimination of negative feedback control mechanisms along the insulin signaling pathway improves beta-cell function under stress. Diabetes 59, 2188–2197
12. Weigert, C., Kron, M., Kalbacher, H., Pohl, A. K., Runge, H., Häring, H. U., Schleicher, E., and Lehmann, R. (2008) Interplay and effects of temporal changes in the phosphorylation state of serine-302, -307, and -318 of insulin receptor substate-1 on insulin action in skeletal muscle cells. Mol. Endocrinol. 22, 2729–2740
13. Danielsson, A., Ost, A., Nystrom, F. H., and Strålfors, P. (2005) Attenuation of insulin-stimulated insulin receptor substate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes. J. Biol. Chem. 280, 34389–34392
14. Coppers, K. D., Hancer, N. J., Opare-Ado, L., Qiu, W., Walsh, C., and White, M. F. (2010) Irs1 serine 307 promotes insulin sensitivity in mice. Cell Metab. 11, 84–92
15. Canettieri, G., Koo, S. H., Berdeaux, R., Heredia, J., Hedrick, S., Zhang, X., and Montmigny, M. (2005) Dual role of the coactivator TORC2 in modulating hepatic glucose output and insulin signaling. Cell Metab. 2, 331–338
16. Ishii, U. S., Canettieri, G., Scriver, R. A., Kulkarni, R. N., Krajeski, S., Reed, J., Walker, J., Lin, X., White, M., and Montmigny, M. (2003) cAmp promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. Genes Dev. 17, 1575–1580
17. Hirashima, Y., Tsuruzoe, K., Kodama, S., Igata, M., Toyonaga, T., Ueki, K., Kahn, C., and Araki, E. (2003) Insulin down-regulates insulin receptor substate-2 expression through the phosphatidylinositol 3-kinase/Akt
IRS2 Protein Stabilization Upon Phosphorylation of Serine 1137/1138

pathway. J. Endocrinol. 179, 253–266

18. Rui, L., Fisher, T. L., Thomas, J., and White, M.F. (2001) Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. J. Biol. Chem. 276, 40362–40367

19. Kleppe, R., Martinez, A., Deskeland, S. O., and Haavik, J. (2011) The 14-3-3 proteins in regulation of cellular metabolism. Semin. Cell Dev. Biol. 22, 713–719

20. Wilker, E. W., Grant, R. A., Artim, S. C., and Yaffe, M. B. (2005) A structural basis for 14-3-3σ functional specificity. J. Biol. Chem. 280, 18891–18898

21. Liu, D., Bienkowska, J., Petosa, C., Collier, R. J., Fu, H., and Liddington, R. (1995) Crystal structure of the ζ isoform of the 14-3-3 protein. Nature 376, 191–194

22. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phospho-serine. Cell 84, 889–897

23. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Atken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Canley, L. C. (1997) The structural basis for 14-3-3 phosphopeptide binding specificity. Cell 91, 961–971

24. Rittinger, K., Budman, J., Xu, J., Volinia, S., Cantley, L. C., Smerdon, S. J., Gamblin, S. J., and Yaffe, M. B. (1999) Structural analysis of 14-3-3 phosphopeptide complexes identifies a dual role for the nuclear export signal of 14-3-3 in ligand binding. Mol. Cell. 4, 153–166

25. Masters, S. C., Pederson, K. J., Zhang, L., Barbieri, J. T., and Fu, H. (1999) Interaction of 14-3-3 with a nonphosphorylated protein ligand, exoenzyme 5 of Pseudomonas aeruginosa. Biochemistry 38, 5216–5221

26. Sumiooka, A., Nagaishi, S., Yoshida, T., Lin, A., Miura, M., and Suzuki, T. (2005) Role of 14-3-3σ in F665-dependent gene transactivation mediated by the amyloid β-protein precursor cytoplasmic fragment. J. Biol. Chem. 280, 42364–42374

27. Seimiya, H., Sawada, H., Muramatsu, Y., Shimizu, M., Ohko, K., Yamane, K., and Tsuruo, T. (2000) Involvement of 14-3-3 proteins in nuclear localization of telomerase. EMBO J. 19, 2652–2661

28. O’Kelly, J., Butler, M. H., Zilberberg, N., and Goldstein, S. A. (2002) For-}

mer, T., and Paudel, H. K. (2003) 14-3-3 connects glycogen synthase kinase-3 (GSK-3) to Tau within a brain microtubule-associated tau phosphorylation complex. J. Biol. Chem. 278, 23489–23498

29. Osielska, V., Silhan, J., Boura, E., Teisinger, J., and Obsil, T. (2008) 14-3-3 proteins. A family of versatile molecular regulators. Physiol. Res. 57, S11–S21

30. Mackintosh, C. (2004) Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. Biochem. J. 381, 329–342

31. Obsilová, V., Silhan, J., Boura, E., Teisinger, J., and Obsil, T. (2008) 14-3-3 proteins. A family of versatile molecular regulators. Physiol. Res. 57, S11–S21

32. Oki, H., Subramanian, R., and Masters, S. C. (2000) 14-3-3 proteins. Structure, function, and regulation. Annu. Rev. Pharmacol. Toxicol. 40, 617–647

33. Neukamm, S. S., Toth, R., Morrice, N., Campbell, D. G., Mackintosh, C., Lehmann, R., Haering, H. U., Schleicher, E. D., and Weigert, C. (2012) Identification of the amino acids 300–600 of IRS-2 as 14-3-3 binding region with the importance of IGF-1/insulin-regulated phosphorylation of Ser-573. PLoS One 7, e43296

34. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) A family of cAMP-binding proteins that directly activate Rap1. Science 282, 2275–2279

35. de Rooij, J., Zwartkruiss, F. J., Verheijen, M. H., Cool, R. H., Nijman, M., Wittinghofer, A., and Bos, J. L. (1998) Epac is a Rap1 guanine-nucleotide exchange factor directly activated by cyclic AMP. Nature 396, 474–477

36. Roscioni, S. S., Elzinga, C. R., and Schmidt, M. (2008) Epac Effectors and biological functions. Naunyn Schmiedebergs Arch. Pharmacol. 377, 345–357

37. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. 351, 95–105

38. Yi, Z., Luo, M., Carroll, C. A., Weintraub, S. T., and Mandarino, L. J. (2005) Identification of phosphorylation sites in insulin receptor substrate-1 by hypothesis-driven high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. Anal. Chem. 77, 5693–5699

39. Short, J. D., Dere, R., Houston, K. D., Cai, S. L., Kim, J., Bergeron, J. M., Shen, J., Liang, J., Bedford, M. T., Mills, G. B., and Walker, C. L. (2010) AMPK-mediated phosphorylation of murine p27 at T197 promotes binding of 14-3-3 proteins and increases p27 stability. Mol. Carcinog. 49, 429–439

40. Li, X., Song, S., Liu, Y., Ko, S. H., and Kao, H.Y. (2004) Phosphorylation of the histone deacetylase 7 modulates its stability and association with 14-3-3 proteins. J. Biol. Chem. 279, 34201–34208

41. Yang, H. Y., Wen, Y. Y., Chen, C. H., Lozano, G., and Lee, M. H. (2003) 14-3-3σ positively regulates p35 and suppresses tumor growth. Mol. Cell. Biol. 23, 7096–7107

42. Rui, L., Yuan, M., Frantz, D., Shoelson, S., and White, M. F. (2002) SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J. Biol. Chem. 277, 42394–42398

43. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system. Annu. Rev. Biochem. 67, 425–479

44. Iakoucheva, L. M., Brown, C. J., Lawson, J. D., Obradovic, Z., and Dunker, A. K. (2002) Intrinsic disorder in cell-signaling and cancer-associated proteins. J. Mol. Biol. 323, 573–584

45. Yaffe, M. B. (2002) How do 14-3-3 proteins work? Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Lett. 513, 53–57
57. Hausser, A., Link, G., Hoene, M., Russo, C., Selchow, O., and Pfizenmaier, K. (2006) Phospho-specific binding of 14–3–3 proteins to phosphatidylinositol 4-kinase IIIβ protects from dephosphorylation and stabilizes lipid kinase activity. J. Cell Sci. 119, 3613–3621

58. Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., and Greenberg, M. E. (2000) 14–3–3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol. Cell 6, 41–51

59. Lingohr, M. K., Briaud, L., Dickson, L. M., McCuaig, J. F., Alárcon, C., Wicksteed, B. L., and Rhodes, C. J. (2006) Specific regulation of IRS-2 expression by glucose in rat primary pancreatic islet beta-cells. J. Biol. Chem. 281, 15884–15892

60. Van de Velde, S., Hogan, M. F., and Montminy, M. (2011) mTOR links incretin signaling to HIF induction in pancreatic beta cells. Proc. Natl. Acad. Sci. U.S.A. 108, 16876–16882