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Protein Kinase C-ζ-induced Phosphorylation of Ser^{318} in Insulin Receptor Substrate-1 (IRS-1) Attenuates the Interaction with the Insulin Receptor and the Tyrosine Phosphorylation of IRS-1*

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Klaus Moeschel‡, Alexander Beck‡, Cora Weigert‡, Reiner Lammers‡, Hubert Kalbacher§, Wolfgang Voelter§, Erwin D. Schleicher‡, Hans-Ulrich Häring‡, and Rainer Lehmann‡¶

From the ‡Department of Internal Medicine IV, Endocrinology, Metabolism, Pathobiochemistry and Clinical Chemistry, University Hospital Tübingen, and §Department of Physical Biochemistry, Institute for Physiological Chemistry, University of Tübingen, D-72076 Tübingen, Germany

Insulin receptor substrate-1 (IRS-1) was recently identified as a novel upstream substrate for the insulin-activated protein kinase C (PKC)-ζ. This interaction down-regulates insulin signal transduction under hyper-insulinemic conditions. To clarify the molecular mechanism of this feedback loop, we sought to identify the PKC-ζ phosphorylation sites of IRS-1 and to investigate their biological significance. Upon incubation of recombinant IRS-1 fragments with PKC-ζ, we identified Ser^{318} of rat IRS-1 (Ser^{323} in human IRS-1) as the major *in vitro* phosphorylation site (confirmed by mutation of Ser^{318} to alanine). To monitor phosphorylation of Ser^{318} in cellular extracts, we prepared a polyclonal phospho-site-specific antibody. The biological significance was studied in baby hamster kidney cells stably expressing the insulin receptor (BHK^{IR}). Using the phospho-Ser^{318}-specific antibody we observed that insulin stimulates phosphorylation of Ser^{318} in IRS-1, which is mediated, at least partially, by PKC-ζ. Moreover, we found that the previously described insulin-stimulated, PKC-ζ-mediated inhibition of the interaction of IRS-1 with the insulin receptor and the reduced tyrosine phosphorylation of IRS-1 was abrogated by mutation of IRS-1 Ser^{318} to alanine. These results, generated in BHK^{IR} cells, suggest that phosphorylation of Ser^{318} by PKC-ζ might contribute to the inhibitory effect of prolonged hyperinsulinemia on IRS-1 function.

Insulin resistance is associated with a variety of physiological and patho-physiological states, including type 2 diabetes, hypertension, glucose intolerance, and obesity; however, the molecular mechanisms that modulate insulin signaling under these conditions are difficult to resolve. One important early site of divergence in insulin signaling, which seems to be a relevant target for modulation of the signal, is insulin receptor substrate 1 (IRS-1)[1] (1). IRS-1 is a hydrophilic protein with an apparent molecular mass of 180 kDa containing a conserved pleckstrin homology domain located at the amino terminus, adjacent to a phosphotyrosine binding (PTB) domain (2). After insulin-stimulation, this domain interacts with the activated insulin receptor (IR), IRS-1 is subsequently tyrosine-phosphorylated, and the signal is transmitted further (1, 2).

In addition to tyrosine phosphorylation, IRS-1 undergoes Ser/Thr phosphorylation at multiple sites (3, 4). Many *in vitro* and *in vivo* studies (5–13) have shown that increased Ser/Thr phosphorylation of IRS-1, *e.g.* after treatment of cells with either activators of protein kinase C (PKC), Ser/Thr phosphatase inhibitors, high insulin concentrations, or activation of cellular stress pathways by tumor necrosis factor α and other cytokines, inhibits the IR-mediated tyrosine phosphorylation of IRS-1, thereby affecting insulin signal transduction.

The atypical PKC-ζ, which is activated by insulin, has been identified to transduce insulin signaling downstream from IRS-1 and phosphatidylinositol 3-kinase in mediating glucose uptake. However, as reported recently, prolonged insulin stimulation of PKC-ζ can also participate in the negative regulation of insulin signaling by phosphorylating IRS-1 at serine residues, as shown in Fao cells (6) and in NIH3T3 cells stably expressing the insulin receptor (8). The PKC-ζ-mediated IRS-1 phosphorylation inhibits both IRS-1 interaction with the IR and the ability of insulin to phosphorylate IRS-1 on tyrosine residues.

We aimed to identify and characterize the functionally relevant Ser/Thr sites of IRS-1 responsible for the PKC-ζ-mediated attenuation of insulin signaling. Based on our novel previously described mass spectrometric procedure for phosphopeptide screening (14), we identified Ser^{318} as the major site phosphorylated by PKC-ζ. Using polyclonal phospho-site-specific antibodies, we demonstrated that this site is also phosphorylated by PKC-ζ in cellular extracts. The functional relevance of the phosphorylation site was studied by mutation of Ser^{318} → Ala (S318A). Our results indicate that PKC-ζ promotes, at least partially, the phosphorylation of Ser^{318} and suggest that this phosphorylation of Ser^{318} might mediate the inhibitory effects of PKC-ζ on the first steps of insulin signal transduction, *i.e.* the complex formation between IR and IRS-1 and the tyrosine phosphorylation of IRS-1 under prolonged insulin stimulation.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media, supplements, fetal calf serum were purchased from Life Technologies, Inc. (Eggenstein, Germany), prote-

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¶ To whom correspondence should be addressed: Innere Medizin IV, Otfried-Müller-Strasse 10, D-72076 Tübingen, Germany. Tel.: 49-70-71-29-83193; Fax: 49-70-71-29-5348; E-mail: rainer.lehmann@med.uni-tuebingen.de.

[1] The abbreviations used are: IRS-1, insulin receptor substrate 1; PTB, phosphotyrosine binding; IR, insulin receptor; PKC, protein kinase C; HPLC, high-performance liquid chromatography; BHK^{IR}, baby hamster kidney cells stably expressing the human insulin receptor; GST, glutathione *S*-transferase; pSer, phospho-serine.

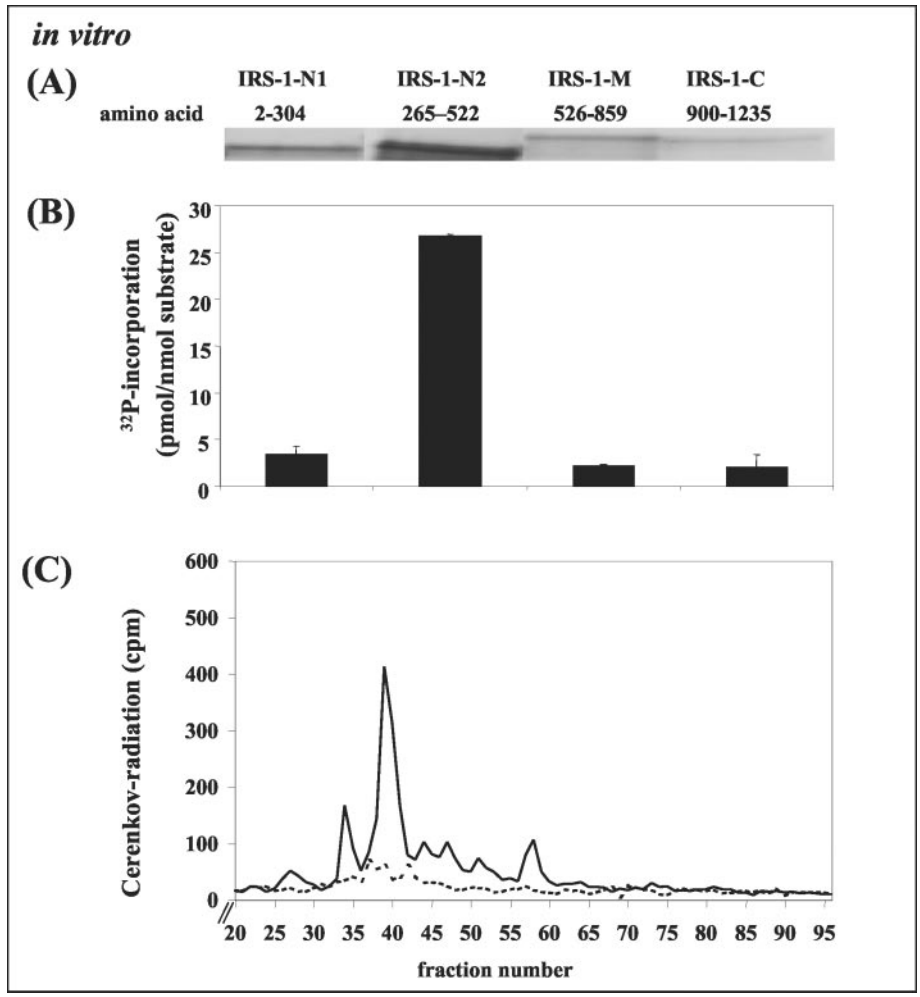

FIG. 1. **Ser^{318} in GST-IRS-1^{N2} is the major *in vitro* PKC-ζ phosphorylation site in IRS-1.** *A*, 25 pmol of each GST-IRS-1 fusion protein was phosphorylated by 10 milliunits of recombinant human PKC-ζ in an *in vitro* kinase assay in the presence of [γ-^{32}P]ATP. The total assay sample amount was separated by SDS-7.5% PAGE and visualized by autoradiography. *B*, densitometric evaluation of the autoradiograms (n = 3). *C*, ^{32}P-Cerenkov elution pattern generated by HPLC separation of tryptic peptides from wild-type GST-IRS-1^{N2} phosphorylated by PKC-ζ (*solid line*) in comparison to the S318A-GST-IRS-1^{N2} point mutant after a PKC-ζ *in vitro* assay (*dashed line*).

ase inhibitor mixture was purchased from Roche (Mannheim, Germany), phosphatase inhibitors were obtained from Sigma (Munich, Germany), protein G-Sepharose CL-4B and ^{32}P-ATP were purchased from Amersham Biosciences (Freiburg, Germany), antibodies against the β-subunit of the IR (sc711) and IRS-1 (sc560) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), the antibodies against phospho-Tyr (4G10) and IRS-2 (06–506) were purchased from Upstate Biotechnology (Lake Placid, NY), and the monoclonal anti-IRS-1 antibody (12H8) was made by NanoTools (Teningen, Germany). The pSer^{307}-antibody was a kind gift from M. F. White (Harvard Univ., Boston, MA), and baby hamster kidney cells stably expressing the human insulin receptor (BHK^{IR}) were kindly provided by N. P. Møller (Novo Nordisk, Bagsvaerd, Denmark). Polyclonal anti-pSer^{318} antiserum was raised against a synthetic peptide (SMVGGKPG*pS*-FRVRASSD) flanking Ser^{318} in IRS-1 (which is conserved among mouse, rat, and human). Polyclonal anti-IRS-2 antiserum for IRS-2 immunoprecipitation was raised in rabbits against a synthetic peptide of the C-terminal part of mouse IRS-2. The recombinant human PKC-ζ was purchased from Calbiochem (San Diego, CA). The cytomegalovirus promoter-based expression vectors for the rat IRS-1 and the human PKC-ζ are described in Ref. 5. All high-performance liquid chromatography (HPLC)-grade solvents were obtained from Merck (Darmstadt, Germany).

Purification and in Vitro Phosphorylation of GST-IRS-1 with PKC-ζ—The N-terminal, middle, and C-terminal portions of rat IRS-1 protein were generated as described (7). In brief, the fragments were ligated to glutathione *S*-transferase and cloned into the *pGEX-2T* vector (Amersham Biosciences, Freiburg, Germany). The DNAs encoding these regions of IRS-1 were synthesized by PCR using rat *IRS-1* cDNA as template and pairs of oligonucleotide primers that contained appropriate restriction sites bordering these fragments (15). Because of a low expression level, the N-terminal fragment was subcloned into two smaller fragments. Altogether, the following four constructs were generated: IRS-1^{N1}, amino acid residues 2–304, size 60.6 kDa; IRS-1^{N2}, amino acid residues 265–522, size 53.8 kDa; IRS-1^{M}, amino acid residues 526–859, size 62.6 kDa; IRS-1^{C}, amino acid residues 900–1235, size 61.8 kDa (IRS-1^{M} and IRS-1^{C} were kindly provided by X. J. Sun, Burlington, VT). The PCR products were isolated, digested with appropriate restriction enzymes, and subcloned into *pGEX-2T*, which were used to transform *E. coli BL* 21. The fusion proteins were expressed and purified by affinity chromatography. The assays were performed at 30 °C for 90 min with 25 pmol of the isolated GST-IRS-1 protein fragments, which was phosphorylated *in vitro* by 10 milliunits of PKC-ζ in 55 mM HEPES-NaOH, pH 7.5, 1.25 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM ATP, and 1 μCi of γ-^{32}P-ATP. The samples were analyzed by SDS-7.5% PAGE and visualized by autoradiography.

In-gel Digestion—The GST-IRS-1^{N2}-band at 54 kDa was excised and in-gel-digested as described elsewhere (14). The resulting peptides were separated using a Shimadzu HPLC-system with a C2/C18, 100 × 21-mm column (Amersham Biosciences); Detection was as follows: UV at λ = 214 nm; flow rate = 0.1 ml/min; buffer A, 0.05% trifluoroacetic acid in water (v/v); buffer B, 80% acetonitrile/0.05% trifluoroacetic acid (v/v); gradient, 0% buffer B (10 min), 10% buffer B (20 min), 40% buffer B (60 min), 100% buffer B (90 min), and return to 0% buffer B in 6 min. The collected fractions were analyzed in a Trilux 1450 MicroBeta Plus β-counter (PerkinElmer/Wallac, Turku, Finland).

Site-directed Mutagenesis—Mutation of S318A was made by oligonucleotide-mediated mutagenesis. The mutagenic upstream primer used was GGTGGGAAACCAGGTG*C*CTTCAGGGTGCGTGCC, with the wild-type IRS-1 expression vector serving as template. Positive clones were verified by sequencing.

Transfection, Cell Lysis, and Immunoprecipitation—BHK^{IR} cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. A minimum of 4.0 × 10^5 cells/well of a six-well plate were transfected using the $Ca_3(PO_4)_2$-DNA coprecipitation method (16). After incubation overnight at 37 °C in 5% CO_2, the cells were starved in Dulbecco's modified Eagle's medium (5.5 mM glucose) without fetal calf serum for 24 h and then stimulated with

insulin (100 nM) as indicated. Cells were lysed with 200 μl of lysis buffer/well (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, containing protease inhibitors and phosphatase inhibitors). Total protein (400 μg) was used for immunoprecipitation. Immunoprecipitated proteins or 20 μg of protein of the total extracts were separated by SDS-7.5% PAGE, and Western blot analysis was performed as described (5).

Statistical Analysis—All data are expressed as means ± S.E. Differences between the time curves were tested by analysis of variance using repeated measure design. $p < 0.05$ was considered to be statistically significant.

RESULTS

Identification of the Major in Vitro Phosphorylation Site of PKC-ζ in IRS-1—By developing a novel mass spectrometric approach for phosphopeptide screening out of complex phosphoprotein digest mixtures, we recently identified Ser^{318} as a PKC-ζ *in vitro* phosphorylation site in the IRS-1^{N2} fragment (IRS-1 sequence from amino acid 265–522) (14). To search for further *in vitro* PKC-ζ phosphorylation sites in IRS-1 not covered by the N2-fragment, the PKC-ζ-catalyzed ^{32}P-incorporation in the NH_2 terminal 1 (IRS-1-N1), NH_2 terminal 2 (IRS-1-N2), the middle (IRS-1-M), and the C-terminal part (IRS-1-C) were compared (Fig. 1, *A* and *B*). Incubation of PKC-ζ with N1, M, or C resulted in a low, essentially comparable ^{32}P-incorporation (Fig. 1, *A* and *B*). In contrast, we observed a 7- to 10-fold higher ^{32}P-incorporation in IRS-1^{N2} (Fig. 1, *A* and *B*). The ^{32}P-incorporation of the GST part was negligible, because very little radioactivity remained in the GST part after thrombin cleavage of GST-IRS-1^{N2} and subsequent gel-separation (data not shown).

The specificity of the PKC-ζ-mediated phosphorylation of Ser^{318} of GST-IRS-1^{N2} was studied by replacing the serine residue with alanine using site-directed mutagenesis (GST-Ala^{318}-IRS-1^{N2}). The HPLC elution profile of the tryptic digest of the wild-type GST-IRS-1^{N2} contained a single major tryptic ^{32}P-labeled phosphopeptide, which was not present in the HPLC elution pattern of the GST-Ala^{318}-IRS-1^{N2} digest (Fig. 1*C*). According to our previously published procedure (14), mass spectrometric peptide sequencing revealed that more than 85% of total incorporated ^{32}P-radioactivity is based upon peaks of different variants of the monophosphorylated phospho-Ser^{318}. Consequently, these peaks were not ^{32}P-phosphorylated in the HPLC-separated, mutated GST-Ala^{318}-IRS-1^{N2} digest. It is noteworthy that the $pSer^{318}$ containing tryptic peptide also contained the previously described Ser^{307} phosphorylation site (10, 17–19), but this was shown not to be phosphorylated by PKC-ζ in either GST-Ser^{318}IRS-1^{N2} or GST-Ala^{318}-IRS-1^{N2} using mass spectrometric analysis. Together, our *in vitro* results show that the IRS-1^{N2} fragment is a preferred substrate for PKC-ζ, and that Ser^{318} is the preferential phosphorylation site in this IRS-1 fragment and possibly also in the whole IRS-1 molecule.

Phospho-site-specific Antibodies Demonstrate Insulin-stimulated and PKC-ζ-mediated Phosphorylation of Ser^{318} in IRS-1—To determine whether the Ser^{318} site is also phosphorylated by PKC-ζ in living cells, a polyclonal antibody against a synthetic $pSer^{318}$ peptide was prepared. At first, the specificity of this antibody to detect IRS-1 phosphorylation on Ser^{318} was evaluated by *in vitro* studies, which demonstrated that the PKC-ζ-phosphorylated wild-type GST-IRS-1^{N2} but not the S318A mutated fragment was recognized by the $pSer^{318}$ antibody (Fig. 2*A*).

In BHK^{IR} cells transiently transfected with wild-type IRS-1 stimulation with insulin for 60 min resulted in the observation of a specific band corresponding to immunoprecipitated IRS-1 after immunoblotting with $pSer^{318}$ antibody, whereas no signal was observed in the absence of insulin (Fig. 2*B*, *left*, *lane 1*). Because PKC-ζ-mediated phosphorylation of IRS-1 has been

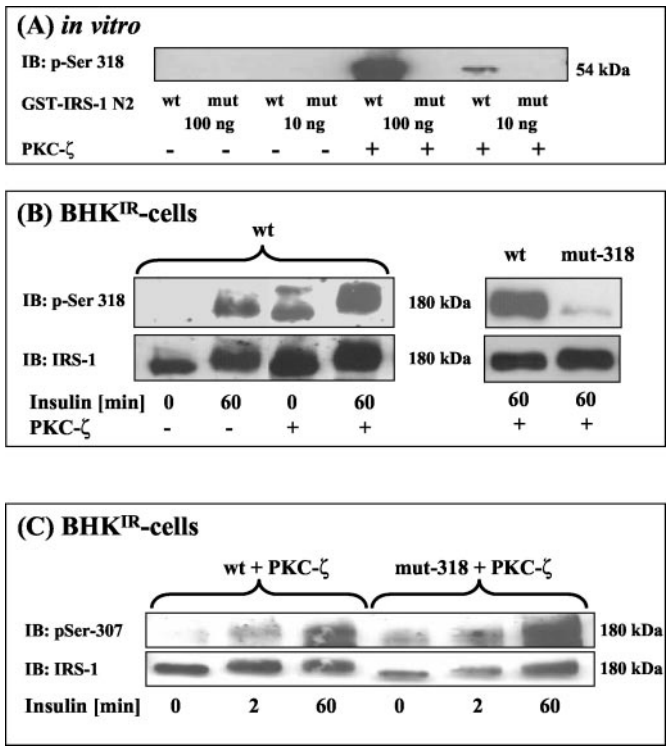

FIG. 2. **Insulin stimulates PKC-ζ-mediated phosphorylation of IRS-1 on Ser^{318} in BHK^{IR} cells.** *A*, immunoblot of an *in vitro* PKC-ζ phosphorylation assay using 10 ng or 100 ng of wild type (*wt*) or S318A mutated (*mut*) GST-IRS-1-N2-fusion protein ± PKC-ζ. *B*, *left panel*, baby hamster kidney cells stably expressing the human insulin receptor (*BHK^{IR}-cells*) transiently transfected with IRS-1 wild type alone or both IRS-1 and PKC-ζ were treated with 100 nM insulin for 60 min; *right panel*, immunoprecipitates of BHK^{IR} cells which were transiently co-transfected with either IRS-1 wild type (*wt*) or S318A-IRS-1 (*mut-318*) and PKC-ζ were stimulated with 100 nM insulin for 60 min. IRS-1 was immunoprecipitated with a monoclonal IRS-1 antibody (12H8) and immunoblotted with the phospho-site-specific Ser^{318} antibody. The same blot was stripped and reprobed with a polyclonal IRS-1 antibody (sc560). *C*, immunoprecipitates of BHK^{IR} cells which were transiently co-transfected with either IRS-1 wild type (*wt+PKC-ζ*) or S318A-IRS-1 (*mut-318+PKC-ζ*) and PKC-ζ were stimulated with 100 nM insulin for 2 and 60 min; immunoblot with the phospho-site-specific Ser^{307} antibody and reprobe of the same blot with the polyclonal IRS-1 antibody (sc560). Representative results are shown from three experiments.

shown to negatively regulate insulin signaling after 60 min of insulin-stimulation (6, 8), we used this condition for our experiments. Incubation of the cellular extracts with alkaline phosphatase or incubation at 37 °C for 30 min without phosphatase inhibitors led to a complete loss of immunoreactivity of the antibody (data not shown), indicating that dephosphorylated IRS-1 is not recognized. Moreover, in BHK^{IR} cells co-transfected with wild-type IRS-1 and PKC-ζ, and in the absence of insulin, a signal of the phosphorylated Ser^{318} was obtained, and this signal increased after 60 min of insulin stimulation (Fig. 2*B*, *left*, *lanes 3* and *4*). Similarly, we observed a strong increase in insulin-stimulated Ser^{318} immunoreactivity in HEK293 cells transiently co-transfected with wild-type IRS-1 and PKC-ζ (data not shown). These results indicate that Ser^{318} is strongly phosphorylated by PKC-ζ after 60 min of insulin stimulation. Furthermore, the data show that insulin stimulates a kinase, probably endogenous PKC-ζ, which phosphorylates IRS-1 at Ser^{318} (Fig. 2*B*, *left*, *lane 2*), and that transfected PKC-ζ alone is also capable of phosphorylating Ser^{318} in IRS-1 (Fig. 2*B*, *left*, *lane 3*).

To further verify that the Ser^{318} antibody specifically binds to $pSer^{318}$ in IRS-1, mutated S318A-IRS-1 or wild-type IRS-1

were transiently co-transfected with PKC-ζ in BHK^{IR} cells and stimulated with insulin for 60 min (Fig. 2*B*, *right*). The mutated S318A-IRS-1 was only marginally recognized by the $pSer^{318}$ antibody, which is likely to be the result of the presence of endogenous wild-type IRS-1, whereas in the cells transfected with wild-type IRS-1, a strong immunocomplex with Ser^{318} was seen. Both IRS-1 molecules were expressed to the same level, as shown by reblotting with a polyclonal IRS-1 antibody (Fig. 2*B*, *right*). The Ser^{318} antibody did not recognize the previously identified, insulin-dependent Ser^{307} phosphorylation site, because the Ser^{307} was phosphorylated in the mutated S318A-IRS-1, as demonstrated by the $pSer^{307}$-specific antibody (Fig. 2*C*). Moreover, Fig. 2*C* shows that insulin has a similar time-dependent effect upon Ser^{307} phosphorylation in cells with wild-type and S318A mutant forms of IRS-1, indicating that PKC-ζ-mediated Ser^{318} phosphorylation does not influence insulin-stimulated Ser^{307}. Furthermore, the $pSer^{307}$ antibody does not recognize $pSer^{318}$ in the GST-$IRS\text{-}1^{N2}$ fragment after *in vitro* PKC-ζ phosphorylation (data not shown), indicating that both antibodies react specifically for their target pSer epitopes.

Phosphorylation of Ser^{318} by PKC-ζ under Prolonged Insulin-stimulation Inhibits Complex Formation between IR and IRS-1 and Tyrosine Phosphorylation of IRS-1—To investigate whether PKC-ζ phosphorylation of Ser^{318} on IRS-1 inhibits the proximal insulin signaling, we studied the IRS-1 Ser^{318} phosphorylation in BHK^{IR} cells and compared it to the IRS-1 tyrosine phosphorylation stimulated at various time points with insulin (from 2 min to 60 min (prolonged)). Fig. 3, *A* and *B* shows a significant decrease in tyrosine phosphorylation after 60 min of insulin stimulation, whereas the Ser^{318} phosphorylation increased to a plateau.

To confirm that prolonged insulin stimulation inhibits tyrosine phosphorylation of IRS-1 by promoting phosphorylation of Ser^{318} by PKC-ζ, we prepared BHK^{IR} cells transiently expressing PKC-ζ and either wild-type IRS-1 or mutated IRS-1 containing the S318A substitution. The results show that the increase in tyrosine phosphorylation of IRS-1 after 2 min was not significantly different in wild type and mutated IRS-1 (Fig. 3*C*). However, after 60 min of insulin stimulation, cells transfected with mutated Ala^{318}-IRS-1 showed no decrease in tyrosine phosphorylation of IRS-1, whereas cells transfected with the wild-type IRS-1 showed a significant reduction in IRS-1 tyrosine phosphorylation. The difference in the time course of the tyrosine phosphorylation between mutated and wild-type IRS-1 was statistically significant ($p < 0.0005$). In BHK^{IR} cells incubated with 100 nM insulin and transfected either with wild-type IRS-1 or mutated Ala^{318}-IRS-1 but without PKC-ζ, no reduction of tyrosine phosphorylation of IRS-1 was observed after 60 min of insulin stimulation (data not shown), indicating that PKC-ζ is necessary to obtain the insulin-stimulated reduction in tyrosine phosphorylation. Next, we investigated whether the PKC-ζ-mediated phosphorylation of Ser^{318} of IRS-1 under prolonged insulin stimulation leads to a reduced complex formation between IR and IRS-1. The results show that in BHK^{IR} cells overexpressing wild-type IRS-1 and PKC-ζ, 60 min of insulin treatment resulted in an attenuated complex formation of IR/IRS-1 (Fig. 3*D*), which is comparable with the effects recently shown in Fao cells by Liu *et al.* (6). In contrast, complex formation was not altered under the same conditions when cells were co-transfected with the mutated Ala^{318}-IRS-1 and PKC-ζ ($p < 0.005$) (Fig. 3*D*). Furthermore, in cells transfected with wild type or mutated IRS-1 but without PKC-ζ, no attenuation of the complex formation was observed after 60 min of insulin treatment (data not shown), indicating that this effect is also PKC-ζ-dependent. These results are consistent with our hypothesis that the PKC-ζ-mediated phosphorylation of Ser^{318}, which is closely located to the PTB domain of IRS-1, inhibits the interaction between the IR and IRS-1. Together, these results suggest that both the inhibition of the insulin-stimulated complex formation between IR and IRS-1 and the inhibition of the insulin-stimulated tyrosine phosphorylation of IRS-1 are mediated, at least partially, by PKC-ζ-dependent phosphorylation of the Ser^{318} of IRS-1.

Phosphorylation of Ser^{318} by PKC-ζ under Prolonged Insulin-stimulation Did Not Attenuate the Tyrosine Phosphorylation of IRS-2—IRS-2 is structurally and functionally similar to IRS-1, and stimulation with insulin leads to the rapid tyrosine phosphorylation of both IRS-1 and IRS-2 (20, 21). Therefore, we examined the correlation of PKC-ζ-induced IRS-1 Ser^{318} phosphorylation with the insulin-stimulated tyrosine phosphorylation of IRS-2. As shown in Fig. 4*A*, the tyrosine phosphorylation of IRS-2 is not reduced by prolonged insulin treatment (60 min) in BHK^{IR} cells overexpressing IRS-1, IRS-2, and PKC-ζ, although the phosphorylation of the Ser^{318} of IRS-1 is increased at all time points (Fig. 4*B*). Moreover, in these cells expressing IRS-2, the phosphorylation of Ser^{318} is also increased in the unstimulated state (Fig. 4*B*). This apparently unexpected result may be explained by the recent report (22) that overexpression of IRS-2 leads to a constitutive increase in PKC-ζ activity levels in L6hIR muscle cells. Thus, tyrosine phosphorylation of IRS-2 is not negatively regulated by Ser^{318} phosphorylation of IRS-1 under prolonged insulin stimulation, in contrast to the inverse correlation of increased IRS-1 Ser^{318} phosphorylation and decreased IRS-1 tyrosine phosphorylation after 60 min of insulin stimulation (shown in Fig. 3, *A* and *B*).

DISCUSSION

In the present study, we (*i*) identified Ser^{318} as the major *in vitro* IRS-1 phosphorylation site of PKC-ζ, (*ii*) generated and characterized a phospho-Ser^{318}-specific antibody, and (*iii*) investigated the biological function of this phosphorylation site in BHK^{IR} cells. The advantage of the *in vitro* approach is that the phosphorylation sites present in the amino acid sequence of the investigated protein are much easier to analyze because the protein is essentially pure and much more protein is available. Analysis of the phosphorylation of GST-tagged protein fragments of IRS-1 (molecular mass ≃ 60 kDa) represents a good model for examination of PKC substrate specificity, assuming that these fragments are correctly folded. By using our *in vitro* experimental set-up, we detected only one major PKC-ζ phosphorylation site in the $IRS\text{-}1^{N2}$ fragment, although six consensus sites for PKC phosphorylation were predicted using the general consensus motif *X*-S/T-*X*-R/K (23). Moreover, our results further suggest that the other 10 potential phosphorylation consensus sites for the PKCs that are located in the other three IRS-1 fragments represent only minor phosphorylation sites. The situation, however, may be different *in vivo* because (*i*) the whole IRS-1 molecule may be differently folded, (*ii*) the intracellular environment is different, thus possibly affecting PKC-ζ substrate specificity, and (*iii*) other kinases, in particular other PKC isoforms, may phosphorylate Ser^{318}.

To verify that Ser^{318} is also phosphorylated in cells, a polyclonal antiserum against the peptide sequence surrounding phosphorylated Ser^{318} in IRS-1 was prepared. Our experimental data demonstrate that it specifically recognizes the phosphorylated Ser^{318} residue: it strongly immunoblots the *in vitro* PKC-ζ-phosphorylated wild-type $IRS\text{-}1^{N2}$ fragment but not the S318A mutated IRS-1 fragment. Again, in lysates from intact cells, the antibody strongly immunoblots IRS-1 immunoprecipitated from cells co-transfected with IRS-1 and PKC-ζ after

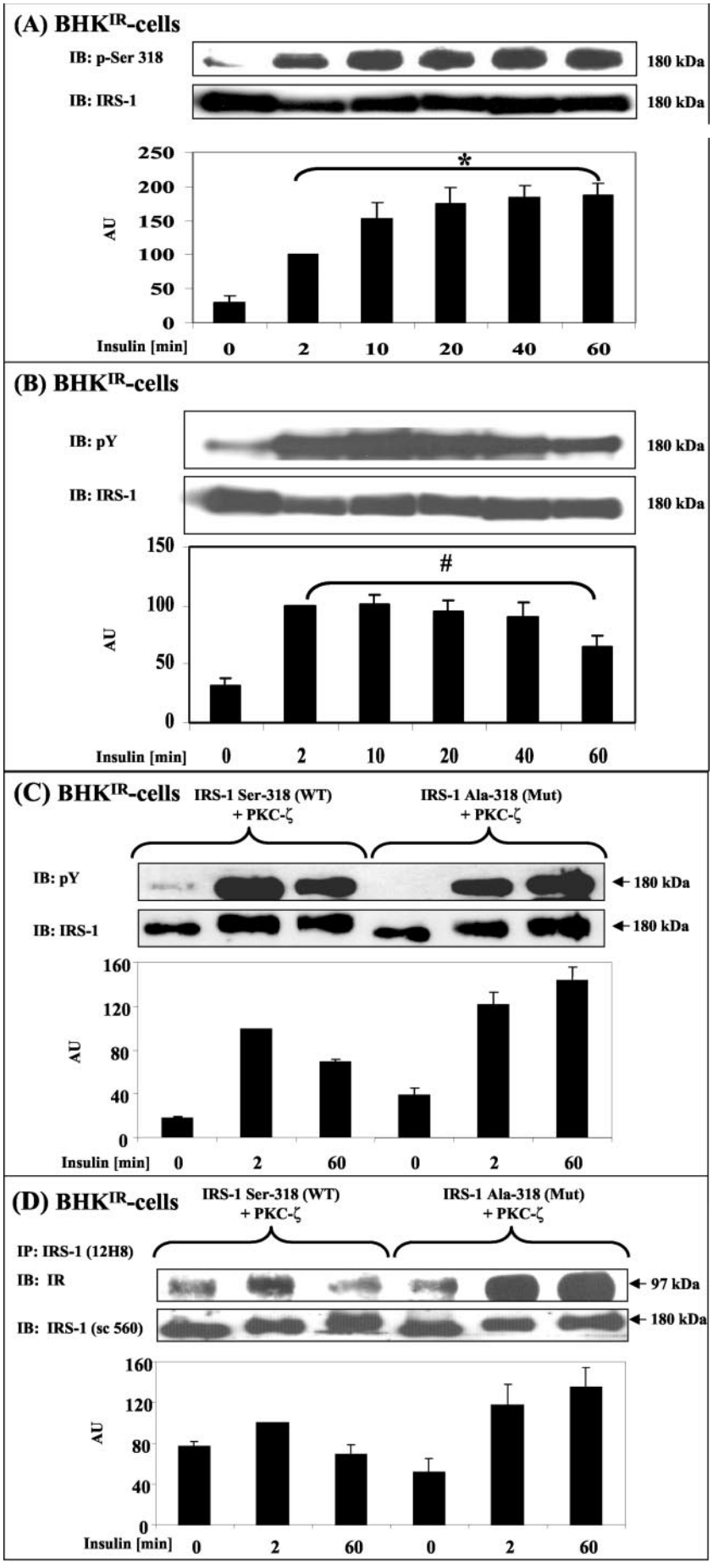

FIG. 3. **The PKC-ζ-mediated phosphorylation of Ser^{318} correlates inversely with the tyrosine phosphorylation of IRS-1 and the Ser^{318} → Ala point mutation prevents the impairment of the insulin-stimulated interaction of insulin receptor with IRS-1 and the decreased tyrosine phosphorylation of IRS-1.** BHK^{IR} cells were transiently transfected with wild-type IRS-1 and PKC-ζ, treated with 100 nM insulin as indicated, lysed, and analyzed by SDS-7.5% PAGE. The mean of values after 2 min of insulin stimulation was set as 100%. Quantification of the blot intensity based upon scanning densitometry of immunoblots normalized for IRS-1 protein is shown. *A*, time course of the phosphorylation of Ser^{318} stimulated with 100 nM insulin for the indicated time points. A representative immunoblot with the phospho-site-specific Ser^{318} antibody and the same blot reprobed with a polyclonal IRS-1 antibody (sc560) is shown (*, $p < 0.009$; 60 min of insulin *versus* 2 min; $n = 5$). *B*, time course of the IRS-1 tyrosine phosphorylation. The cells were stimulated with 100 nM insulin for the indicated time points, lysed, and 400 μg of total protein were immunoprecipitated with a monoclonal IRS-1 antibody (12H8). A representative immunoblot with the phospho-tyrosine antibody and the same blot reprobed with a polyclonal IRS-1 antibody (sc560) are shown (#, $p < 0.006$; 60 min of insulin *versus* 2 min; $n = 4$). *C*, for the analysis of the tyrosine phosphorylation of IRS-1, 20 μg of cell lysates were resolved, immunoblotted with an anti-phosphotyrosine antibody, and the membrane was reblotted with an anti-IRS-1 antibody. The time course of the tyrosine phosphorylation between wild-type IRS-1 and Ala^{318}-IRS-1 was significantly different ($p < 0.0005$, by analysis of variance; $n = 5$). *D*, for the analysis of IR/IRS-1 interaction, BHK^{IR} cells were transiently transfected with PKC-ζ and either wild-type IRS-1 or mutated Ala^{318}-IRS-1. An immunoblot with IR- or IRS-1-antibody is shown as indicated. The time course of the IR/IRS-1 interaction between wild-type IRS-1 and Ala^{318}-IRS-1 was significantly different ($p < 0.005$, by analysis of variance; $n = 7$).

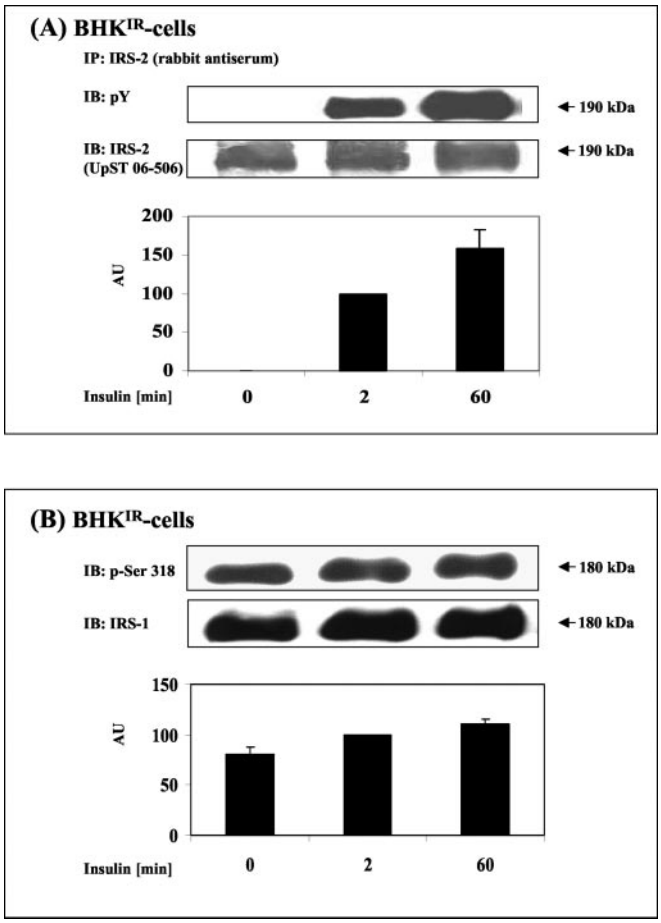

FIG. 4. **The insulin-stimulated tyrosine phosphorylation of IRS-2 is not impaired by PKC-ζ-mediated phosphorylation of Ser^{318} in BHK^{IR} cells.** BHK^{IR} cells were transiently transfected with IRS-1, IRS-2, and PKC-ζ, treated with 100 nM insulin for 2 or 60 min, lysed, and analyzed by SDS-7.5% PAGE. *A*, for analysis of the tyrosine phosphorylation of IRS-2, 400 μg of total protein were immunoprecipitated with anti-mouse IRS-2 rabbit antiserum and immunoblotted with the phospho-tyrosine-antibody ($n = 4$). The membrane was reblotted with a commercial anti-IRS-2 (sc 06–506) antibody. After 2 min of insulin-stimulation, the value of the mean was set as 100%. *B*, immunoblot with the phospho-site-specific Ser^{318} antibody and reprobe of the same blot with the polyclonal IRS-1 antibody (sc560) is shown. Phospho-tyrosine and Ser^{318} phosphorylation intensity were quantified based upon scanning densitometry of immunoblots normalized for IRS-1 and IRS-2 protein, respectively ($n = 4$).

insulin stimulation but reacts only very weakly if the mutated S318A-IRS-1 is transfected (likely because of endogenous wild-type IRS-1). Together, these results demonstrate that the pSer^{318}-antibody reacts specifically with phosphorylated Ser^{318} in IRS-1, and that PKC-ζ mediates Ser^{318} phosphorylation during insulin-stimulation. It is noteworthy that our results also show that insulin stimulated Ser^{318} phosphorylation in the non-PKC-ζ-transfected cells, possibly by means of endogenous PKC-ζ. Moreover, transfection of PKC-ζ stimulated Ser^{318} phosphorylation without insulin addition, indicating that PKC-ζ mediates the phosphorylation at this site. Our results do not exclude the possibility that other insulin-stimulated kinases, particularly other PKC-isoforms, phosphorylate the Ser^{318} of IRS-1.

To investigate the biological relevance of this phosphorylation site, we first compared the Ser^{318} phosphorylation of IRS-1 from acute (2 min) to prolonged (60 min) insulin stimulation with the tyrosine phosphorylation of IRS-1 and found an inverse correlation, indicating that Ser^{318} phosphorylation precedes the down-regulation of tyrosine phosphorylation, *i.e.* inactivation of IRS-1. This effect seems to be specific for IRS-1, because IRS-2 tyrosine phosphorylation is not concomitantly decreased. However, our results also show that basal Ser^{318} phosphorylation is influenced by IRS-2 expression, probably by means of the previously shown IRS-2-mediated PKC-ζ activation (22).

To further characterize the functional role of this site, Ser^{318} was mutated to Ala. Although we observed no change after 2 min of insulin stimulation, the recently described reduction of IR/IRS-1 binding and IRS-1 tyrosine phosphorylation (3, 6, 8) was not observed after 60 min of insulin treatment in BHK^{IR} cells transfected with the S318A-IRS-1 and PKC-ζ. Because, after 2 min of insulin-stimulation, the alanine point mutation shows a normal complex formation between IR and IRS-1 and a normal tyrosine phosphorylation of IRS-1, site-directed mutagenesis does not obviously alter the structural integrity of IRS-1. Together, the results indicate that the presence of Ser^{318} is necessary to obtain the reduced, PKC-ζ-mediated IR/IRS-1 complex formation and IRS-1 tyrosine phosphorylation after prolonged insulin-stimulation, effects which have been demonstrated previously in Fao cells (6) and in NIH3T3^{IR} fibroblasts (8). Furthermore, it was shown that a kinase-inactive form of PKC-ζ fails to mimic the inhibitory effects of its wild-type counterpart when transiently overexpressed in Fao or H-35 cells (6) and NIH3T3^{IR} cells (8). In addition, it was shown that endogenous IRS-1 coprecipitates with endogenous PKC-ζ, and this association is increased 2-fold upon insulin-stimulation, which further supports the finding that IRS-1 is a substrate for PKC-ζ (6, 8). Thus, PKC-ζ might promote feedback inhibition of IR/IRS-1 complex formation and IRS-1 tyrosine phosphorylation through phosphorylation of Ser^{318} without a negative regulatory effect on IRS-2 tyrosine phosphorylation. Based upon our findings in cells overexpressing IR, IRS-1, and/or IRS-2 and/or PKC-ζ, future experiments in non-transfected, tissue-specific cells should be performed to confirm the importance and potential physiological relevance of our findings.

Extensive studies have provided evidence that phosphorylation of Ser^{307} in IRS-1 inhibits IR/IRS-1 complex formation and IRS-1 tyrosine phosphorylation after prolonged insulin-stimulation similar to our results (10, 17–19). However, this signaling pathway involves the activation of N-terminal Jun kinase. Furthermore, phosphorylation of Ser^{307} may also be stimulated by tumor necrosis factor-α by means of a mitogen-activated protein-1-dependent pathway. These results, together with our findings, imply that insulin-stimulated activation of different kinases via differing pathways may lead to phosphorylation of either Ser^{307} or Ser^{318} in IRS-1 and that phosphorylation of either serine residue leads to attenuation of the proximal insulin signaling. Thus, the two distinct pathways might converge at this IRS-1 region (Ser^{307}/Ser^{318}) and cause the inhibition of IRS-1 signaling.

The mechanism by which the phosphorylation of Ser^{307} or Ser^{318} inhibits IRS-1 tyrosine phosphorylation is not known. Prolonged insulin-stimulation inhibits IRS-1 binding to the phosphorylated NPEY motif in the juxta-membrane region of the IR β-subunit (9). Because Ser^{307} or Ser^{318} is adjacent to the PTB domain, its phosphorylation might disrupt the interaction between the phosphorylated NPEY motif of the IR and IRS-1. Although the PTB domain is not absolutely required for IRS-1 tyrosine phosphorylation, its presence strongly facilitates the process, and partial disruption of PTB domain function is expected to reduce coupling of IR to IRS-1 at endogenous levels of these proteins (24, 25). Alternatively, phosphorylation of Ser^{307} or Ser^{318} might recruit signaling molecules, which sterically inhibit interactions between the PTB domain and the IR β-subunit. The dissociated IRS protein fails to undergo further tyro-

sine phosphorylation by the insulin receptor kinase, while being subjected to the action of protein tyrosine phosphatases that reduce its phosphotyrosine content.

In summary, phosphorylation of Ser^{318} is critical for the inhibitory effect of prolonged insulin-stimulation by PKC-ζ in transfected cell lines, which might be mediated by the association of activated PKC-ζ with IRS-1. Our results are consistent with the assumption that phospho-Ser^{318} blocks the interaction between the IRS-1 PTB domain and the IR, which might significantly reduce the coupling between the activated IR and IRS-1. Based upon our findings in cells overexpressing insulin receptor, IRS-1, and/or PKC-ζ, we hypothesize that the phosphorylation state of Ser^{318} and other sites, *e.g.* Ser^{307}, might predict, in certain cells or tissues, the ability of IRS-1 to mediate the insulin response.

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