The Protein-Tyrosine-Phosphatase SHP2 Is Phosphorylated on Serine Residues 576 and 591 by Protein Kinase C Isoforms α, β1, β2, and η

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Received June 25, 2001; Revised Manuscript Received October 26, 2001

ABSTRACT: To study whether protein kinase C (PKC) isoforms can interact with protein-tyrosine-phosphatases (PTPs) which are connected to the insulin signaling pathway, we co-overexpressed PKC isoforms together with insulin receptor, docking proteins, and the PTPs SHP1 and SHP2 in human embryonic kidney (HEK) 293 cells. After phorbol ester induced activation of PKC isoforms α, β1, β2, and η, we could show a defined gel mobility shift of SHP2, indicating phosphorylation on serine/threonine residues. This phosphorylation was not dependent on insulin receptor or insulin receptor substrate-1 (IRS-1) overexpression and did not occur for the closely related phosphatase SHP1. Furthermore, PKC phosphorylation of SHP2 was completely blocked by the PKC inhibitor bisindolylmaleimide and was not detectable when SHP2 was co-overexpressed with kinase negative mutants of PKCβ1 and -β2. The phosphorylation also occurred on endogenous SHP2 in Chinese hamster ovary (CHO) cells stably overexpressing PKCβ2. Using point mutants of SHP2, we identified serine residues 576 and 591 as phosphorylation sites for PKC. However, no change of phosphatase activity by TPA treatment was detected in an in vitro assay. In summary, SHP2 is phosphorylated on serine residues 576 and 591 by PKC isoforms α, β1, β2, and η.

Insulin resistance of target tissues plays a major role in the pathogenesis of type 2 diabetes mellitus (*1*). However, the molecular mechanisms which lead to insulin resistance are still poorly understood. Among the most interesting candidates for a negative modulation of the insulin signal are serine kinases and protein-tyrosine-phosphatases (PTPs).[1]

The serine/threonine kinase family protein kinase C (PKC) is of particular interest as a potential inhibitor of the insulin signaling chain. Many studies have reported an involvement of PKC in the inhibition of the insulin receptor kinase activity [reviewed in (*2*)]. PKC isoforms represent structurally and functionally related proteins which differ in their regulatory domains, in their dependence on calcium, and in their tissue distribution (*3*, *4*). They are classified into three major groups, classical (cPKCα, -β1, -β2, and -γ), new (nPKCδ, -ε, -η, and -θ), and atypical (aPKCζ and -ι/λ) isoforms. In previous studies, we and others have shown that PKC stimulation by phorbol esters or hyperglycemic conditions reduced insulin receptor tyrosine autophosphorylation and insulin-dependent tyrosine phosphorylation of downstream elements of the insulin signaling chain (*5*−*8*). Activation of PKC isoforms β1 and β2 caused a reduced autophosphorylation of the human insulin receptor (HIR) in human embryonic kidney (HEK) 293 cells (*9*). When the docking protein insulin receptor substrate-1 (IRS-1) was coexpressed in the cells, the effect of PKCβ was increased, and an inhibitory action was also shown for PKC isoforms α, δ, and θ (*8*). This observation suggested a complex interaction of PKC isoforms with different elements of the insulin signaling chain.

Several protein phosphatases such as SHP1, SHP2, PTPα, PTP1B, and LAR (*10*−*14*) can regulate insulin signaling. Among these, the ubiquitously expressed, cytoplasmic protein-tyrosine-phosphatase SHP2 has been shown to interact through its two SH2 domains with members of the insulin signaling cascade like the HIR and IRS-1. Binding of SHP2 to the insulin receptor and phosphorylation on its tyrosine residues resulted in increased phosphatase activity. But different from IRS-1, the insulin receptor is not dephosphorylated by SHP2 (*11*, *15*). Another study has described a positive role of SHP2 in the ras/mitogen-activated protein (MAP) kinase pathways (*16*).

Zhao et al. first showed the ability of PKC and MAP kinase to phosphorylate SHP2 in vitro but did not observe any effect on its enzyme activity (*17*). Other studies demonstrated that MAP kinase phosphorylation of SHP2 in vitro resulted in an inhibition of the phosphatase activity (*18*). Thus, there is still a controversial discussion about the role of SHP2 in insulin signaling and its interaction with PKC. In particular, nothing is known about an interaction of SHP2

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[1] Abbreviations: HEK, human embryonic kidney; HIR, human insulin receptor; IP, immunoprecipitation; IRS, insulin receptor substrate; MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PKC, protein kinase C; PTP, protein-tyrosine-phosphatase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SH2, src homology domain 2; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Van, vanadate.

10.1021/bi011327v CCC: $22.00
Published on Web 12/01/2001

and PKC in vivo, a differential activity of PKC isoforms, or the identification of the phosphorylation sites.

In this report, we have described the interaction of PKC isoforms and the PTP SHP2 in intact cells and its impact on the early steps of the insulin signaling chain. We have identified serines 576 and 591 as phosphorylation sites of distinct PKC isoforms. Since phosphorylation on these sites had no effect on the SHP2 phosphatase activity toward the insulin receptor or IRS-1, the physiological significance of the interaction still has to be determined.

MATERIALS AND METHODS

Materials. Cell culture reagents and fetal calf serum were purchased from Gibco (Egenstein, Germany); culture dishes were from TPP (Frickenhausen, Germany). Recombinant human insulin was from Lilly (Indianapolis, IN). TPA, aprotinin, phenylmethylsulfonyl fluoride, Na_3VO_4, Triton X-100, and dithiothreitol were from Sigma (Munich, Germany). The reagents for SDS−PAGE and Western blotting were from Roth (Karlsruhe, Germany) and BioRad (Munich, Germany). Protein-A Sepharose CL 4B was from Pharmacia (Uppsala, Sweden). Nitrocellulose was from Sartorius (Göttingen, Germany). [γ-^{32}P]-ATP was obtained from NEN-DuPont (Bad Homburg, Germany). Recombinant insulin receptor substrate-1 (IRS-1) was from Upstate Biotechnology Inc. (Lake Placid, NY). All other reagents were from the best grade commercially available.

The numbering of the SHP2 amino acid sequence is according to Vogel et al. (*19*). The cDNAs for the PKC isoforms γ, δ, ϵ, ζ, η, θ, and ι were kindly provided by Dr. H. Mischak (National Institutes of Health, Bethesda, MD). The cDNA for IRS-1 and the polyclonal rabbit antibody against IRS-1 were gifts from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). Polyclonal isoform-specific PKC antibodies were purchased from Gibco Life Technologies (Karlsruhe, Germany). The monoclonal mouse antibody against phosphorylated tyrosine residues was from Leinco Technology Inc. (Ballwin, MO). A polyclonal antibody recognizing the insulin receptor β-subunit (αIR) as well as a polyclonal antibody recognizing SHP2 were raised against peptides representing the carboxy-terminal amino acids of these proteins.

Construction of Expression Plasmids. For transient expression in HEK293 cells, all cDNAs were cloned by standard procedures into the cytomegalovirus immediate early promotor-based expression plasmid pRK5 (*20*). For generation of serine/threonine to alanine SHP2 mutants, the phosphatase inactive SHP2* (C459A) mutants, and the kinase negative PKCβ1/2 (K371A) mutants, the respective codons were mutated using the method of Kunkel et al. (*21*). All mutagenesis oligos were purchased from Gibco Life Technologies (Karlsruhe, Germany). The sequences of the constructs were verified by fluorescence sequencing, and plasmid DNA was prepared using a Qiagen Plasmid Kit (Qiagen, Hilden, Germany).

Transient Expression in HEK293 Cells. Human embryonic kidney (HEK) 293 cells (ATCC CRL 1573) were grown in Dulbecco's MEM/Nutrient Mix F12 medium (Gibco Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal calf serum. Cells were transfected according to the protocol of Chen and Okayama (*22*). Briefly, cells were seeded in 6-well dishes at a density of 3×10^5 cells per well in 2 mL of medium. A total of 4 μg of supercoiled plasmid DNA was mixed with 0.25 M $CaCl_2$ in a final volume of 0.1 mL. To this an equal amount of 2× transfection buffer (50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4) was added, and after incubation for 10 min at room temperature, the mixture was given dropwise to the cells. After incubation for 16 h at 37 °C and 3% CO_2, the cells were serum-starved for 24 h in DMEM (1 g/L of glucose) containing 0.5% fetal calf serum.

Generation of Stable Transfected CHO Cells. PKCβ2 or -θ stably overexpressing CHO cells (CHO-PKCβ2/θ) were generated by retroviral infection of exponentially growing cells. The retroviruses were obtained by transfection of 293-BOSC cells (*23*) with the retroviral expression vector pLXSN-PKCβ2/θ (*24*). CHO cells were incubated with supernatants of 293-BOSC cells containing the recombinant retroviruses for 12 h at 37 °C in the presence of 8 μg/mL Polybrene. Virus-containing medium was then removed, and cells were grown in medium containing 750 μg/mL Geneticin (G418). After 2 weeks of selection, G418-resistant clones were picked and analyzed for the expression level of the transduced protein.

Stimulation, Cell Lysis, and Immunoprecipitation. Serum-starved cells were stimulated with 200 nM bisindolylmaleimide for 60 min, 100 nM TPA for 30 min, and 100 nM insulin for 5 min at 37 °C and lysed in 0.2 mL per well of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/mL aprotinin, 100 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4). Crude lysates were cleared by centrifugation (20 min/12000*g*) and analyzed on SDS−PAGE (40 μg of protein per lane), or used for immunoprecipitations. To this, 150 μL of cleared lysates was diluted with the same volume of IP buffer (50 mM HEPES, pH 7.5, 0.1% Triton X-100, 150 mM NaCl, 10% glycerol, 2 mM Na_3VO_4), combined with 20 μL of Protein-A Sepharose and antiserum, and rotated overnight at 4 °C. Immunoprecipitates were washed 3 times with 1 mL of IP buffer, boiled for 5 min in 1× Laemmli buffer, and separated by 7.5% SDS−PAGE.

Western Blotting. After SDS−PAGE, proteins were transferred to nitrocellulose membranes by semi-dry blotting (transfer buffer: 20 mM NaH_2PO_4, 20 mM Na_2HPO_4, pH 8.8), and filters were blocked with NET-G buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton X-100, and 0.25% gelatin, pH 7.4) for 1 h. Subsequently, filters were incubated with the first antibody overnight at 4 °C. The membranes were washed 3 times with NET-G buffer before incubation with peroxidase-conjugated anti-mouse or anti-rabbit IgG for 1 h at room temperature. Signals were detected by enhanced chemiluminescence (ECL).

Phosphatase Assay. PTP activity was measured toward recombinant IRS-1 that had been phosphorylated by activated HIR kinase in the presence of [γ-^{32}P]-ATP as described by Krützfeldt et al. with minor modifications (*25*). Phosphorylated IRS-1 was bound to immunoprecipitated SHP2 generated from transfected and stimulated HEK293 cells. Dephosphorylation reactions were performed at room temperature for 0−110 min, and phosphorylation remaining on IRS-1 was determined by liquid scintillation counting.

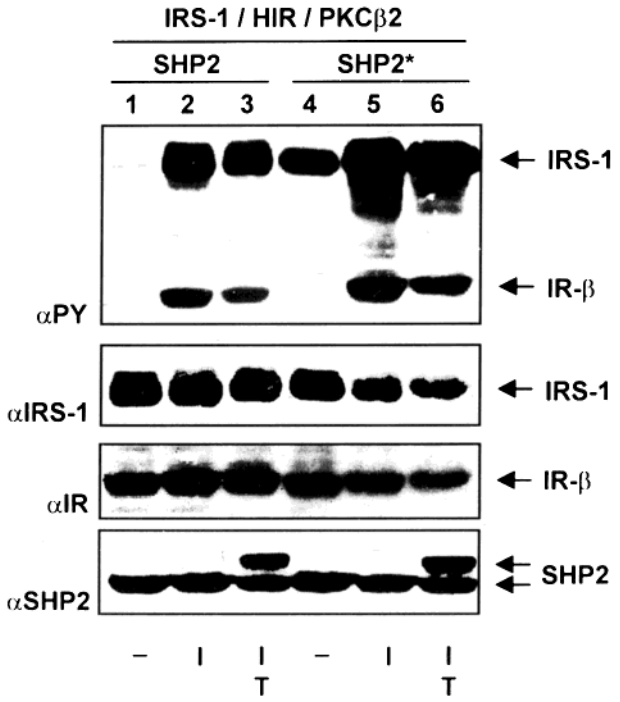

FIGURE 1: SHP2 has no effect on TPA-induced inhibition of insulin receptor tyrosine phosphorylation. HEK293 cells overexpressing IRS-1, HIR, PKCβ2, and SHP2 or phosphatase-inactive SHP2* were stimulated as indicated with 100 nM insulin (I, 5 min) and 100 nM TPA (T, 30 min) at 37 °C. Whole cell lysates were prepared; the proteins were separated by SDS−PAGE, transferred to nitrocellulose, and blotted with antibodies against phosphotyrosine (aPY). Similar levels of overexpression were confirmed by reprobing the membranes with antibodies against IRS-1, HIR, and SHP2. Shown are representative immunoblots of four independent experiments.

RESULTS

To study the potential involvement of PTPs for PKC-induced receptor inhibition, we coexpressed IRS-1, HIR, and PKCβ2 together with the PTP SHP2 or its catalytically inactive mutant SHP2* in HEK293 cells. After stimulation with insulin and TPA, whole cell lysates were prepared, and the proteins were separated by SDS−PAGE and transferred to nitrocellulose. Figure 1 shows in the upper panel an immunoblot against phosphorylated tyrosine residues. Overexpression of inactive SHP2* (lanes 4−6) led to an increased tyrosine phosphorylation of the receptor β-subunit and, even in the basal state, IRS-1 when compared to SHP2 wild type (lanes 1−3). However, the degree of phosphorylation varied between experiments. In reblotting experiments to verify the expression of proteins, we noted a double band for both SHP2 and its mutant after TPA treatment, suggesting a phosphorylation of SHP2 by PKC. Subsequent experiments confirmed this result, and control experiments revealed that the effect was independent of insulin receptor or IRS-1 coexpression (data not shown).

Next, we asked whether other PKC isoforms are able to induce the SHP2 phosphorylation. Figure 2 shows a representative immunoblot of our experiments in which we transfected HEK293 cells with cDNAs of SHP2 and nine different PKC isoforms. Cells were either left untreated or stimulated with TPA or insulin and the inhibitor bisindolylmaleimide. The lower panels of Figure 2 show that PKCs α, β1, β2, and, to a lesser extent, η were able to induce a mobility shift of SHP2 after TPA stimulation. This shift was prevented by preincubation with the PKC inhibitor bisindolylmaleimide but not with a MAP kinase inhibitor (not shown). To investigate whether the shift of SHP2 depends on the kinase activity of PKC, a similar experiment was performed using kinase negative mutants of PKCβ1 and -β2 (PKCβ1/β2-KA). Again, HEK293 cells were transfected and stimulated, and after separation on SDS−PAGE, the respective proteins were detected by immunoblotting (Figure 3). When PKCβ1-KA (lane 5) or PKCβ2-KA (lane 11) was coexpressed with SHP2, no shift was detectable after TPA stimulation (Figure 3, lower panels).

SHP1 is a homologous cytosolic phosphatase which had been shown previously to be a substrate of PKC (*26*, *27*). To test whether it could also be phosphorylated under these conditions, SHP1 was coexpressed together with different PKC isoforms and cells treated with TPA. None of these coexpression experiments resulted in a mobility shift of SHP1, as shown in a representative Western blot of PKCβ2 and PKCθ coexpression (Figure 4).

We were able to obtain similar effects for SHP2 shifts in transient expression experiments using other cell lines (CHO, BHK, NIH3T3; data not shown). In addition, we confirmed the SHP2 phosphorylation in CHO cell lines stably overexpressing different PKC isoforms. Figure 5 shows representative immunoblots of three different cell lines (parental, PKCβ2-, PKCθ-overexpressing) which were treated with TPA and the PKC inhibitor bisindolylmaleimide. After cell lysis, equal amounts of protein were separated by SDS−PAGE and transferred to nitrocellulose, and SHP2 and PKCβ2 were visualized by immunoblotting with the respective antibodies. As shown in the lower panel of Figure 5, only in PKCβ2 overexpressing CHO cells was a mobility shift of the endogenous SHP2 after TPA preincubation detected (lane 5) whereas untransfected or PKCθ overexpressing cells showed no effect.

To investigate possible physiological consequences, we examined whether the phosphorylation of SHP2 had an effect on the phosphatase activity toward its substrate IRS-1. To this end, we established a phosphatase assay in which recombinant IRS-1 had been phosphorylated by activated insulin receptor kinase in the presence of [γ-^{32}P]-ATP in vitro and was incubated with SHP2 immunoprecipitates from different transfected and stimulated HEK293 cells. As shown in Figure 6A, the molecular weight shift of SHP2 after TPA preincubation was also detected in immunoprecipitates (lane 2, lower panel). When immunoprecipitates of SHP2 from untreated cells were incubated with phosphorylated IRS-1 at room temperature, a 60% dephosphorylation of IRS-1 occurred within 110 min (Figure 6B). Similar dephosphorylation kinetics were detectable when SHP2 immunoprecipitates from TPA preincubated cells were used. In control reactions that included the phosphatase inhibitor vanadate (6 mM), only little, time-dependent dephosphorylation of IRS-1 was detected, similar to dephosphorylation in the presence of the phosphatase inactive SHP2*. We conclude that the PKC-induced serine phosphorylation of SHP2 had no significant effect on its phosphatase activity toward IRS-1 as a substrate in this experimental system.

To identify the PKC phosphorylation sites in SHP2, we examined the amino acid sequence of SHP2 and found 15 putative phosphorylation sites for PKC, based on the minimal

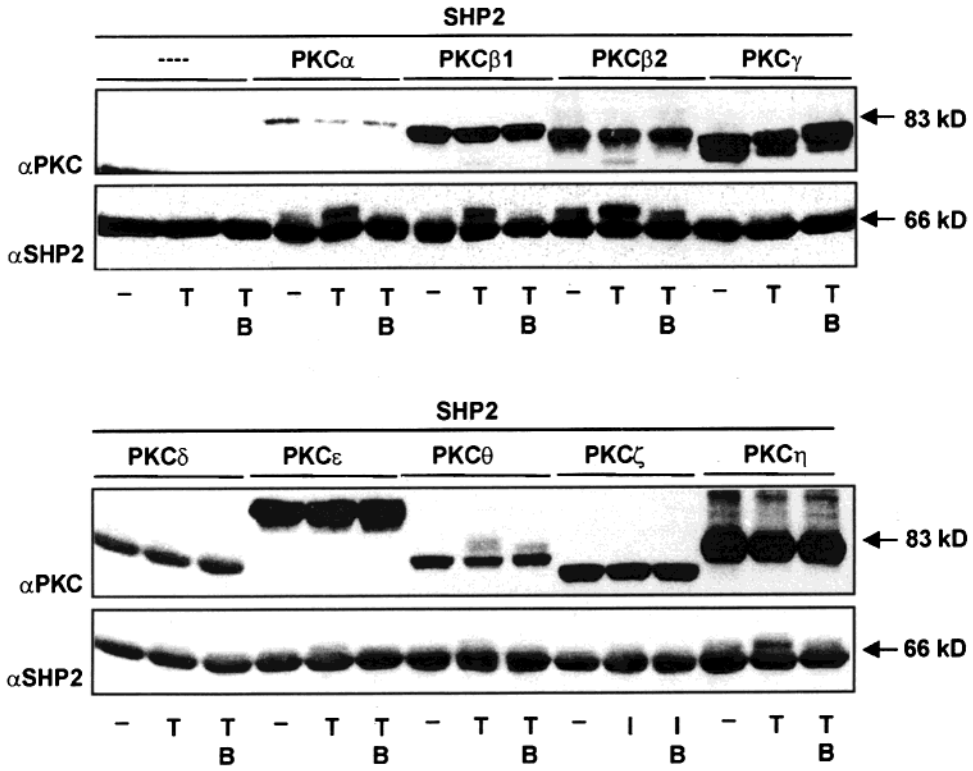

FIGURE 2: SHP2 is a substrate of several PKC isoforms. HEK293 cells overexpressing SHP2 and the indicated PKC isoforms were treated as indicated with 100 nM insulin (I, 5 min), 100 nM TPA (T, 30 min), and 200 nM bisindolylmaleimide (B, 60 min) at 37 °C. Whole cell lysates were prepared; the proteins were separated by SDS−PAGE, transferred to nitrocellulose, and blotted with antibodies against the different PKC isoforms (upper panel) and SHP2 (lower panel). Results shown are representative immunoblots of three separate experiments.

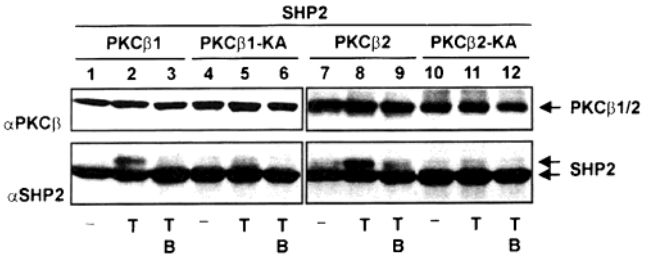

FIGURE 3: Kinase inactive mutants of PKC cannot induce a mobility shift of SHP2. HEK293 cells overexpressing SHP2 and the indicated PKC constructs were stimulated with or without 100 nM TPA (T, 30 min) and 200 nM bisindolylmaleimide (B, 60 min) at 37 °C. Whole cell lysates were prepared; the proteins were separated by SDS−PAGE, transferred to nitrocellulose, and blotted with antibodies against PKCβ1/2 (upper panel) and SHP2 (lower panel). Immunoblots representative of three separate experiments are shown (KA, lysine to alanine mutation in inactive PKC).

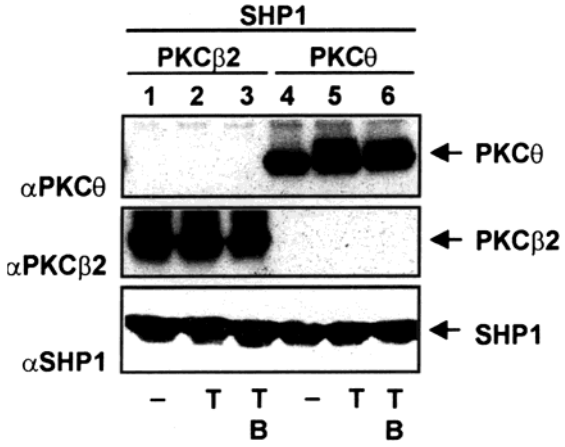

FIGURE 4: SHP1 is not a substrate of PKC. HEK293 cells overexpressing SHP1 and the indicated PKC isoforms were stimulated with or without 100 nM TPA (T, 30 min) and 200 nM bisindolylmaleimide (B, 60 min) at 37 °C. Whole cell lysates were prepared; the proteins were separated by SDS−PAGE, transferred to nitrocellulose, and blotted with antibodies against PKCθ (upper panel), PKCβ2 (middle panel), and SHP1 (lower panel). Representative immunoblots of three independent experiments are shown.

consensus motif S/T-X-R/K. We prepared SHP2 constructs with mutations of all of these sites (serine/threonine to alanine) by in vitro mutagenesis. If mutagenesis changes a serine that is substrate of the PKC, the mobility shift of SHP2 should be abolished. Analysis of these constructs by co-expression with PKCβ2 in HEK293 cells to our surprise identified two sites, serine residues 576 and 591, that were involved in phosphorylation. As shown in Figure 7, substitution of serine 576 clearly reduced the extent of the mobility shift after TPA incubation (lane 10), in contrast to SHP2-WT (lane 4) and a mutant without effect (S535A, lane 12). When serine 591 was substituted with alanine (lane 8), the shift was completely supressed, as was the case in the double mutant SHP2-S576/591A (lane 6). These results suggest that phosphorylation of SHP2 occurs at two sites, serine 591 and serine 576. While mutation of S576 still allows phosphorylation at S591, indicated by a partial shift, a mutation of S591 abolishes subsequent phosphorylation at S576, as indicated by the absence of any shift. Further experiments with PKCα, -β1, and -η revealed that serines 576 and 591 serve as phosphorylation sites for these PKC isoforms as well (data not shown).

Although the phosphorylated form of SHP2 had no different activity against IRS-1 in the in vitro assay, we tested whether an overexpression of the double mutant SHP2-S576/

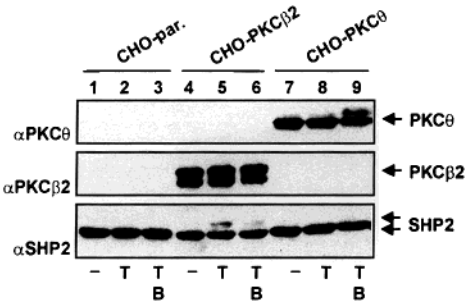

FIGURE 5: Endogenous SHP2 is phosphorylated in PKC overexpressing CHO cells. CHO cells were stimulated with or without 100 nM TPA (T, 30 min) and 200 nM bisindolylmaleimide (B, 60 min) at 37 °C. Whole cell lysates were prepared; the proteins were separated by SDS−PAGE, transferred to nitrocellulose, and blotted with antibodies against PKCθ (upper panel), PKCβ2 (middle panel), or SHP2 (lower panel). An immunoblot representative of four separate experiments is shown.

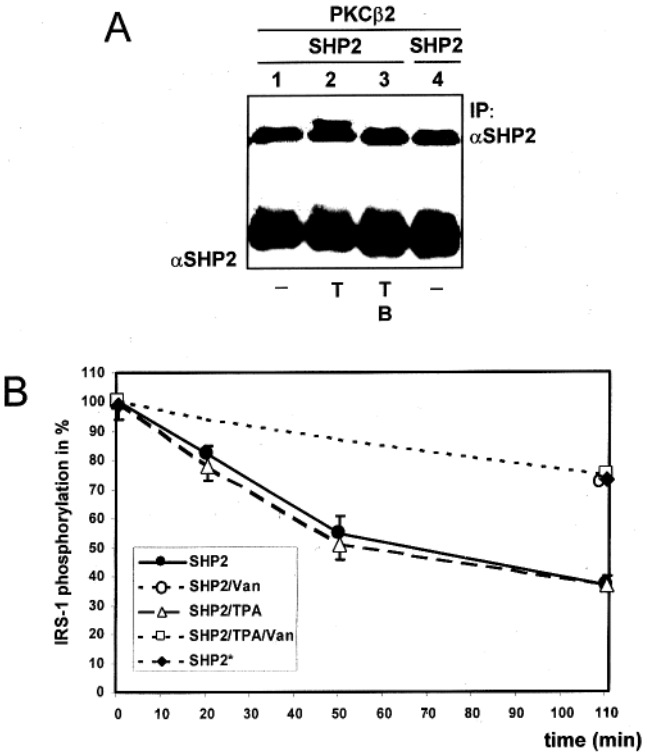

FIGURE 6: Analysis of phosphatase activity of SHP2 from untreated versus TPA-treated cells. (A) Expression and molecular weight shift of SHP2 after TPA preincubation in anti-SHP2 immunoprecipitates. (B) Phosphatase activity was measured toward recombinant IRS-1 that had been phosphorylated by activated insulin receptor kinase in the presence of [γ-^{32}P]-ATP. Phosphorylated IRS-1 was bound to SHP2 immunoprecipitates from untreated (●) or TPA-treated (△, 100 nM, 30 min) cells overexpressing PKCβ2 and SHP2 or catalytically inactive SHP2* (◆). Dephosphorylation reactions were performed at room temperature for 0−110 min. Control reactions were performed in the presence of the phosphatase inhibitor vanadate (○, □). After incubations were stopped on ice, phosphorylation remaining on IRS-1 was determined by β-counting. Shown are means of three independent experiments (IP, immunoprecipitation; Van, vanadate).

591A would have an impact on the PKC-induced inhibition of early signaling elements in intact cells. To this end, we repeated the experiment shown in Figure 1 but overexpressed the SHP2-S576/591A mutant together with IRS-1, HIR, and PKCβ2. As expected, the double mutant SHP2-S576/591A had no differential effect on insulin receptor and IRS-1

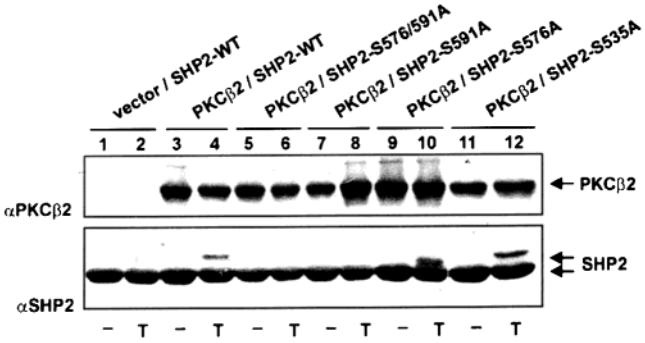

FIGURE 7: Serine residues 576 and 591 are phosphorylated by PKCβ2. HEK293 cells overexpressing PKCβ2 and SHP2 wild type (WT) or the indicated SHP2 mutants were stimulated with 100 nM TPA (T, 30 min) at 37 °C as indicated. Whole cell lysates were prepared, and the proteins were separated by SDS−PAGE. After transferring the proteins to nitrocellulose, the membranes were incubated with antibodies against PKCβ2 (upper panel) or SHP2 (lower panel). A representative immunoblot of five independent experiments is shown.

tyrosine phosphorylation compared to SHP2-WT overexpressing cells. Further, we looked for a differential association of phosphorylated or nonphosphorylated SHP2 with IRS-1. 293 cells overexpressing the IR, IRS-1, SHP2, and PKCβ were stimulated with insulin and TPA, lysed, and IRS-1 immunoprecipitated. Analysis with an antibody directed against SHP2 revealed the phosphorylated and the nonphosphorylated form (data not shown).

DISCUSSION

In this study, we have shown an interaction of SHP2 with PKC isoforms α, β1, β2, and η in intact cells that results in phosphorylation of serine residues 576 and 591. Expression of the single mutant SHP2-S576A together with PKCβ led to the expected reduced mobility shift in accordance with phosphorylation on only one site. Surprisingly, coexpression of PKCβ with the mutant SHP2-S591A completely prevented the shift, indicating the absence of serine phosphorylation. This observation suggests a model in which the activated PKC phosphorylates SHP2 first on serine 591 which in turn allows for the kinase to phosphorylate subsequently serine 576. Mutation of other potential PKC sites had no effect on the gel mobility of SHP2. In addition, we generated constructs in which serine 558 or threonines 546/566 were changed to alanine since work by Peraldi et al. had indicated that these amino acids serve as phosphorylation sites for MAP kinase (*18*). However, substitution of the phosphorylation sites for MAP kinase as well as pretreatment with a potent MAP kinase inhibitor (data not shown) had no effect on the shift of SHP2 in this experimental system. These results strongly suggest a direct phosphorylation of SHP2 by PKC. The phosphorylation process does not require a strong interaction of the proteins since coimmunoprecipitation was not possible.

In our earlier studies, we had seen that activation of PKC led to a reduced tyrosine phosphorylation of HIR and IRS-1 (*8, 9*). Since 293 cells express a significant level of endogenous SHP2, we wondered whether it could be part of an inhibitory complex. Although we detected an interaction of both proteins, we found no evidence for an enhanced activity of serine phosphorylated SHP2 toward IRS-1 in the in vitro assay. Furthermore, no significant impact on PKC-

induced receptor inhibition was observed after overexpression of phosphatase-inactive SHP2* which is also phosphorylated by PKCβ2. Control experiments with the double mutant and therefore not phosphorylated SHP2-S576/591A (data not shown) also showed no difference in the PKC-induced receptor inhibition. Therefore, we have currently no evidence that the observed phosphorylation of SHP2 has an impact on the early steps of the insulin signaling chain. In addition, we have tested the activity toward other substrates such as SHPS-1/SIRPα and Pyk2 which have been shown to be dephosphorylated by SHP2 (*33*, *34*) but did not find any differences.

Independent from the insulin signal, serine phosphorylation of SHP2 may be important for other pathways. For example, the affinity of Grb2 for wild type or mutated SHP2 could be of great interest. This adaptor protein binds with its SH2 domain to phosphorylated tyrosines 546 or 584 on SHP2 connecting the PDGF receptor to the MAP kinase pathway (*35*). Therefore, a modulation of the PDGF/Grb2-dependent MAP kinase activation has to be determined in further studies. There was, however, no effect of SHP2-S576/591A expression on insulin-dependent activation of the ERK, as determined by Western blotting with a phosphospecific antiserum against ERK (data not shown).

In conclusion, we have identified two serine residues of SHP2 that are phosphorylated by several members of the PKC family.

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BI011327V