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# Prolonged tyrosine kinase activation of insulin receptor by pY27-caveolin-2

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ABSTRACT

Caveolin-2 regulation of insulin receptor (IR) tyrosine kinase activity was investigated. An insulin time course revealed that rapidly induced tyrosine phosphorylation of IR was steadily maintained over a 180 min time period. In parallel, insulin-exerted IR interaction with caveolin-2 was detected as early as 5 min throughout until 180 min. Down-regulation of caveolin-2 by caveolin-2 siRNA arrested specifically a long term activation of IR. The attenuation of IR activation resulted in retardation of rapamycin-sensitive pS727-STAT3 activation. As caveolin-2 tyrosine mutants were examined, Y27A-caveolin-2 explicitly impeded the long term IR activation by insulin, enhanced tyrosine dephosphorylation of IR, impaired tyrosine phosphorylation of IRS-1, and exerted the interaction between activated IR and SOCS-3. Together, we propose that pY27-caveolin-2 prolongs IR activation by its interaction with IR, thereby preventing IR interaction with SOCS-3.



## Introduction

Insulin receptor (IR) is a member of a large family of ligand-activated tyrosine kinases [1]. Insulin binding to its receptor results in the autophosphorylation of tyrosine residues within the β subunit of the receptor, increasing the tyrosine kinase activity of the IR, which in turn phosphorylates cellular substrates [2]. These substrates act as docking sites for Src homology 2 (SH2) domain-containing signaling molecules via their phosphotyrosine motifs [3,4]. Internalization of IR constitutes the major mechanism for insulin degradation and down-regulation of the cell surface receptor [5,6]. Upon dissociation of insulin from the receptor, both the IR and IR substrates undergo a rapid dephosphorylation, implicating protein–tyrosine phosphatases (PTPs) in signal termination [7–9].

Suppressor of cytokine signaling-3 (SOCS-3) proteins binds to the activated IR and interferes with the phosphorylation of insulin receptor substrate (IRS) without blocking phosphorylation of IR [10,11]. Overexpression of SOCS-3 decreases insulin-stimulated phosphorylation of IRS-1 and -2 by blocking the ability of IRS proteins to interact with the IR [12]. Signal transducer and activator of transcription factor 3 (STAT3) activates the transcription of SOCS-3 [13] and its expression is induced by insulin [10]. Interleukin-6-induced hepatic insulin resistance is regulated by STAT3-SOCS-3 pathway through mTOR-dependent signaling [14]. Down-regulation of SOCS-3 expression reverses insulin resistance in obese diabetic mice [11,15]. Thus, these reports support a role for SOCS-3 in insulin resistance and suggest that SOCS-3 functions as a key regulator in the insulin-induced STAT3 signaling pathway.

Caveolins are principal component of caveolae and function as scaffolding proteins [16,17]. Interestingly, the scaffolding domain of caveolins shares primary sequence similarities with the SOCS-3 pseudosubstrate domain [18]. As SOCS-3 has been reported to negatively regulate the insulin signaling pathway through an association with IR or IRS [10,11], caveolin-1 has been shown to negatively regulate several proliferative signaling molecules, such as Ha-Ras, c-Src, ERK1/2, and cyclinD1 [19,20]. Our recent investigation [21–23], however, has demonstrated that caveolin-2 positively regulates cellular mitogenesis in insulin signaling. Further, we have shown that phosphorylation of caveolin-2 at tyrosines 19 and 27 is required for the insulin-induced mitogenesis [22,23].

The present study was conducted to investigate the role of tyrosine phosphorylation of caveolin-2 in IR activation. Here, we report that pY27-caveolin-2 persists a long term tyrosine kinase activation of IR.

## Materials and methods

*Materials.* Anti-caveolin-2, anti-caveolin-1, anti-phosphotyrosine-PY20, and anti-IRS-1, anti-Akt, and anti-STAT3 antibodies were obtained from BD Transduction Laboratories; Anti-F-actin, anti-IR, and anti-SOCS-3 antibodies from Santa Cruz Biotechnology; Anti-phosphoserine antibody from Abcam. Anti-p-Akt and anti-pS727-STAT3 antibodies, and rapamycin were purchased from Cell Signaling; Human insulin from Novo Nordisk.

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*Plasmids.* A full-length caveolin-2 cDNA (NM_131914) was subcloned into pcDNA3 vector (Invitrogen). Constructs encoding WT-caveolin-2 and point mutants including Y19A, Y27A, and Y19A/Y27A were generated by PCR mutagenesis as described [22,23].

*Cell culture and transfection.* Human insulin receptor-overexpressed rat 1 (Hirc-B) and Rat1 fibroblasts and 3T3L1 adipocytes were grown in DMEM (Gibco/BRL) containing 5 mM D-glucose supplemented with 10% (v/v) FBS (Cambrex Bio Science) as described [21–23]. Cells were transfected with plasmids by using Lipofectamine LTX transfection reagent (Invitrogen).

*Immunoprecipitation and immunoblotting.* Cells were lysed in buffer containing 60 mM *n*-octylglucoside (Calbiochem). Lysates were immunoprecipitated with the indicated antibodies and subjected to immunoblot analyses as described [22,23].

*Down-regulation of the caveolin-2.* The caveolin-2-siRNA duplexes (D-010958-01) were synthesized and purified by Dharmacon Research Inc. A control siRNA was used as a nonsilencing control (D-001136-01-05). Transfection of siRNA duplexes was carried out using DhamaFECT Transfection Reagents (Dharmacon).

*RT-PCR analysis.* The cDNA was used as the template for the subsequent PCR amplification. PCR primers for GAPDH were 5′-ACCACCATGGAGAAGGCTGG-3′ and 5′-CTCAGTGTAGCCCAGGATGCC-3′, and for SOCS-3 were 5′-ATGGTCACCCACAGCAAGTTTC-3′ and 5′-CTGGTCCAGGAACTCCCGAA-3′. PCR was performed using AccuPower PCR PreMix (Bioneer) kit.

## Results

### *Caveolin-2 is required for prolonged tyrosine kinase activation of IR*

Hirc-B cells have been our model system to explore the regulatory role of caveolin-2 in insulin signaling due to their high expression of IR, the well-characterized downstream signaling triggered by insulin treatment [24,25], and having caveolin-2 as an endogenous caveolin with no caveolin-1 gene expression [21–23]. When cells were treated with insulin for a detailed time-course experiment, the caveolin-2 interaction with IR was observed as early as 5 min after insulin incubation (Fig. 1A). Of interest, the interaction and tyrosine kinase activation of IR were sustained until 180 min of insulin stimulation (Fig. 1A). The interaction of caveolin-2 with IR occurred in Rat1 fibroblasts and 3T3L1 adipocytes, and was increased by insulin (Fig. 1B).

To verify if caveolin-2 is required for IR activation by insulin, the effect of caveolin-2 siRNA was examined. The caveolin-2 siRNA effectively depleted caveolin-2 protein by over 90% of levels observed in the scrambled siRNA-transfected control cells (Fig. 1C).

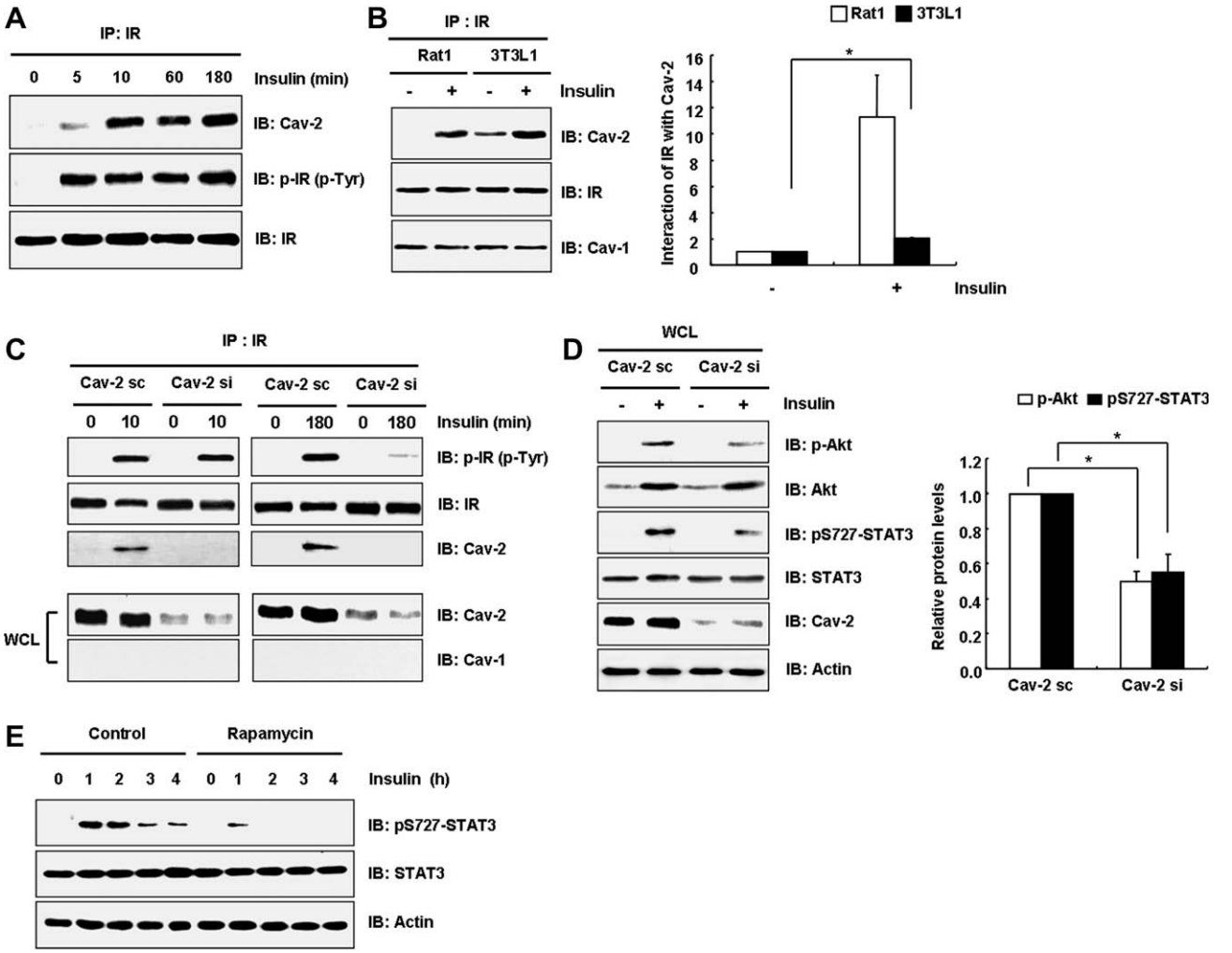


**Fig. 1.** Caveolin-2 is required to prolong IR phosphorylation and signaling. (A) Hirc-B cells were treated with insulin (100 nM) for different time periods. Whole cell lysates (WCL) were immunoprecipitated with anti-IR antibody and immunoblotted as indicated ($n = 3$). (B) Rat1 fibroblasts and 3T3L1 adipocytes were treated with or without insulin (100 nM) for 10 min. WCL were immunoprecipitated with anti-IR antibody and subjected to immunoblotting as indicated (mean ± SE, $n = 3$. $^{*}P < 0.05$). (C) Hirc-B cells were transfected with scramble or caveolin-2-siRNAs for 48 h and treated with insulin for 10 or 180 min. WCL were immunoprecipitated with anti-IR antibody and analyzed by immunoblotting. ($n = 3$). (D) Caveolin-2-siRNA-transfected cells were treated with insulin for 180 min. WCL were analyzed by immunoblotting as indicated (mean ± SE, $n = 3$. $^{*}P < 0.05$). (E) Cells were treated with rapamycin (10 nM) for 1 h before insulin treatment for different time periods.

Knockdown of caveolin-2 caused no changes in tyrosine phosphorylation of IR at 10 min after insulin incubation. However, at 180 min, the tyrosine kinase activation of IR was markedly decreased (Fig. 1C). These data suggest that caveolin-2 is required for a long term IR activation. As previously demonstrated [21–23], caveolin-1 was not detected and exhibited no changes by insulin and caveolin-2 siRNA.

*Caveolin-2 siRNA attenuates rapamycin-sensitive pS727-STAT3 activation*

We investigated if the attenuation of a long term IR activation by caveolin-2 siRNA affects insulin signaling. Insulin-induced phosphorylation of Akt was reduced 2-fold and activation of pS727-STAT3 was decreased 1.8-fold by caveolin-2 siRNA (Fig. 1D). When cells were treated with rapamycin, the activation of pS727-STAT3 was a 6.7-fold decreased as compared to control after 1 h of insulin incubation (Fig. 1E). Although insulin-stimulated pS727-STAT3 activation of controls without rapamycin treatment was gradually decreased from 2 to 4 h insulin time course by 1.6 to 6.2-fold as compared to 1 h after insulin incubation, the activation was completely inhibited by rapamycin as early as 2 h (Fig. 1E). Thus, these results collectively reveal that caveolin-2 regulates a long term tyrosine phosphorylation of IR, thereby promoting activation of pS727-STAT3 via rapamycin-sensitive, possibly Akt/mTOR-mediated signaling.

*pY27-caveolin-2 is essential for persisting tyrosine kinase activation of IR*

To determine whether the phosphorylation of caveolin-2 at tyrosines 19 and 27 influences the IR activation, we mutated the caveolin-2 tyrosines to alanine. As shown in Fig. 2, transfection of the caveolin-2 variants exhibited tyrosine phosphorylation of IR almost the same degree as observed by vector control at 10 min after insulin incubation. Of interest, the IR activation was 3-fold attenuated by Y27A-caveolin-2 at 180 min as compared to 10 min. The effect of phosphorylation of caveolin-2 on tyrosine 27 was reconfirmed by expression of double mutant, Y19A/Y27A-caveolin-2 exhibiting the same retardation in the IR activation at 180 min. Y19A-caveolin-2, however, caused no detectable changes. Thus, the data indicate that pY27-caveolin-2 is required for the tyrosine activation of IR till 180 min of insulin stimulation.

*pY27-caveolin-2 impedes tyrosine dephosphorylation of IR*

We investigated further the effect of the phosphorylation of caveolin-2 at tyrosines 19 and 27 on the rate of IR dephosphorylation. Cells transfected with the caveolin-2 variants were stimulated with insulin for 180 min and then maintained in serum-free medium for various periods of chasing time to detect IR dephosphorylation rate. In vector control, insulin-induced tyrosine phosphorylation of IR was rapidly decreased with a maximum dephosphorylation at 120 min chase (Fig. 3A). WT caveolin-2 prolonged the insulin-induced tyrosine phosphorylation of IR till 60 min. Y19A-caveolin-2 exhibited the same IR dephosphorylation kinetics as observed by WT caveolin-2 (Fig. 3A). Consistent with 2.7 and 2.4-fold decreases of IR activation at 180 min of insulin incubation observed in Fig. 2, Y27A- and Y19A/Y27A-caveolin-2 decreased the phosphorylation of IR at 0 time chase by 2.7 and 2.9-fold, respectively, as compared to WT caveolin-2. The reduced tyrosine phosphorylation of IR was rapidly dephosphorylated as early as 15 min with a complete dephosphorylation at 60 min chase. These results suggest that pY27-caveolin-2 maintains the

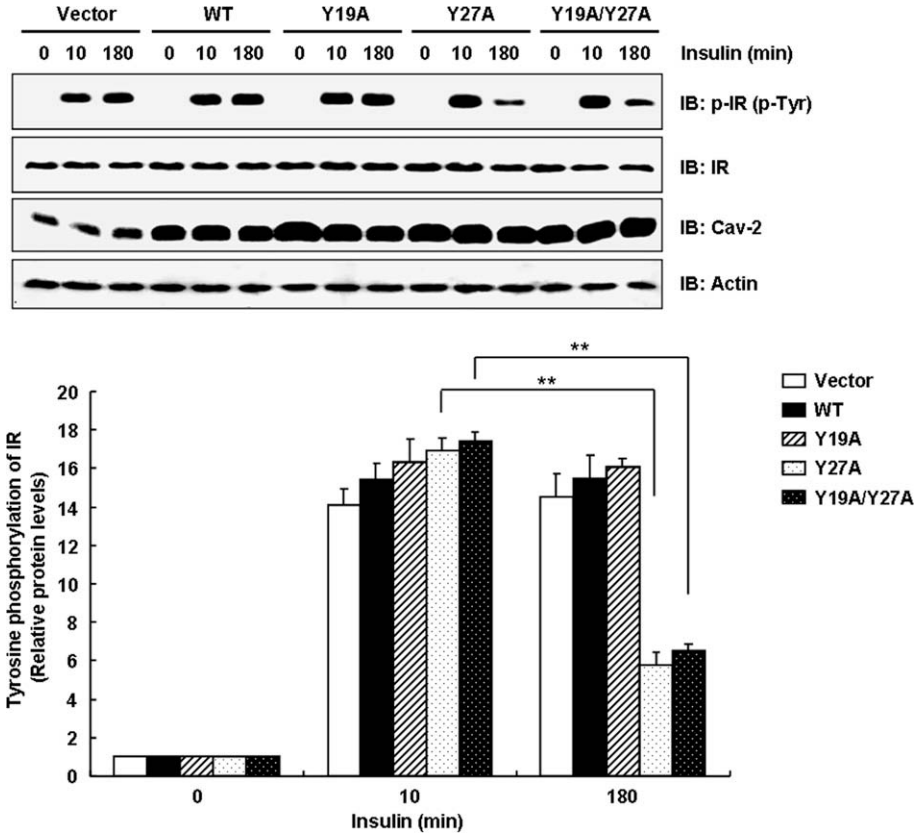


**Fig. 2.** Y27A-caveolin-2 mutation attenuates a long term IR activation. Hirc-B cells were transfected with caveolin-2-variants and treated with insulin for 10 or 180 min. WCL were analyzed by immunoblotting (mean ± SE, $n = 4$, $^{**}P < 0.01$.).

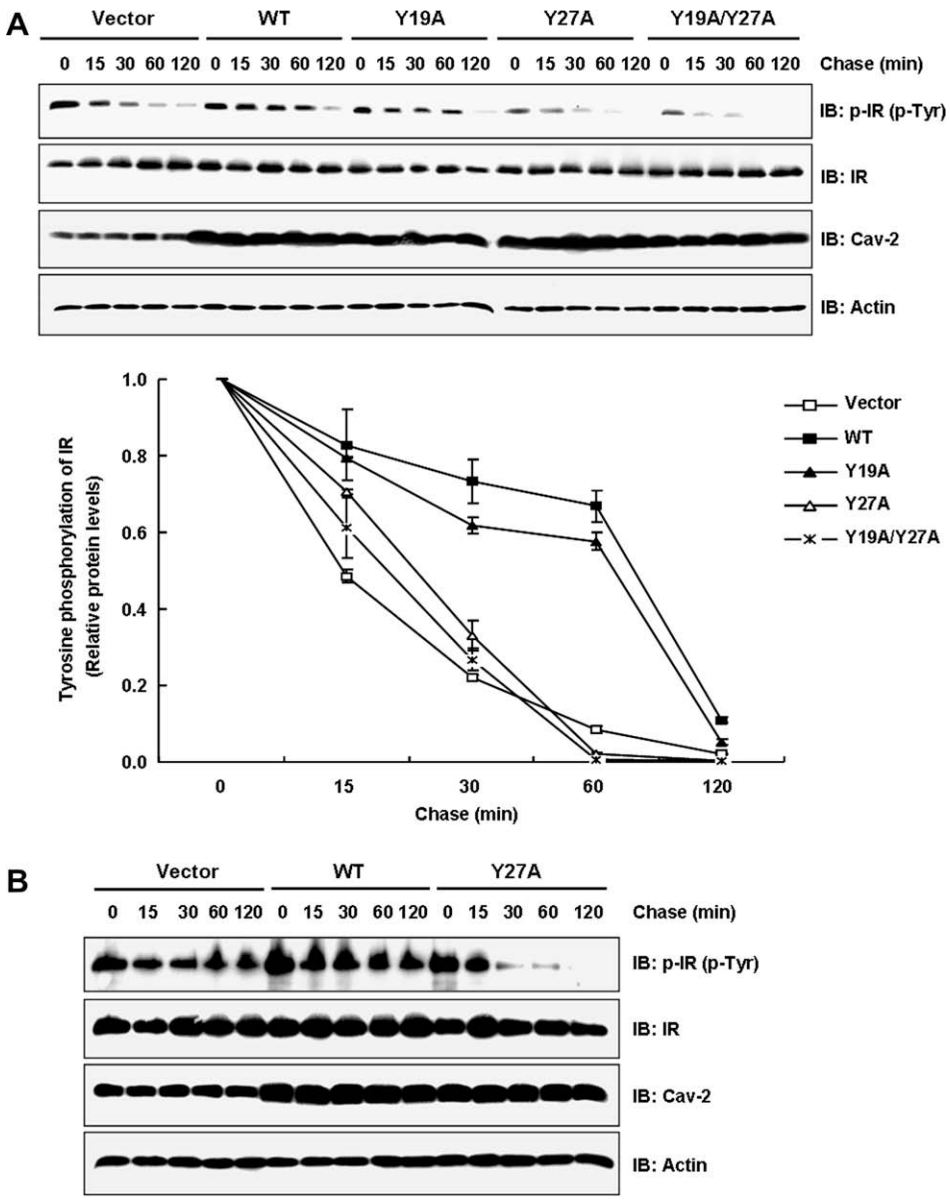


**Fig. 3.** Y27A-caveolin-2 mutant enhances IR dephosphorylation. (A) Hirc-B cells expressing the caveolin-2-variants were treated with insulin for 180 min, rinsed twice, and chased for the indicated times in serum-free medium. WCL were analyzed by immunoblotting (mean ± SE, $n = 3$). (B) The transfected cells were incubated with ortho-vanadate (200 μM) for 30 min followed by insulin incubation for 180 min. The cells were rinsed twice and chased.

prolonged tyrosine phosphorylation of IR by preventing tyrosine dephosphorylation of IR.

Since tyrosine phosphorylation of IR is dephosphorylated by PTPs [9], we investigated if PTPs involve in the long term IR activation regulated by pY27-caveolin-2. Cells were pretreated with protein–tyrosine phosphatase inhibitor, ortho-vanadate, stimulated with insulin for 180 min and IR dephosphorylation rate was detected by chasing various periods of time in serum-free medium (Fig. 3B). Ortho-vanadate kept the tyrosine kinase activation of IR all the chasing time till 120 min in both vector control- and WT caveolin-2-transfected cells. Of interest, ortho-vanadate restored the decrease in tyrosine phosphorylation of IR by Y27A-caveolin-2 observed at 0 time chase (Fig. 3A) to the levels by vector control and WT caveolin-2, and inhibited the tyrosine dephosphorylation of IR till 15 min chase (Fig. 3B). However, striking dephosphorylation of IR was detected as early as 30 min with the complete dephosphorylation of IR at 120 min chase in Y27A-caveolin-2-transfected cells. These results suggest that pY27-caveolin-2 persists a long term IR activation by a mechanism involving prevention of IR dephosphorylation partially by ortho-vanadate-sensitive PTPs and additionally by another mechanism dependent on ortho-vanadate-insensitive PTPs.

*pY27-caveolin-2 interferes interaction between IR and SOCS-3*

Transcription of SOCS-3 mRNA is mediated by STAT3 activation [13] and SOCS-3 negatively regulates insulin signaling through an association with activated IR [10,11]. Since the prolonged tyrosine kinase activation of IR by caveolin-2 was required for the rapamycin-sensitive pS727-STAT3 activation (Fig. 1), we investigated if the pS727-STAT3-mediated SOCS-3 expression negatively regulates the IR activation. Induction of SOCS-3 mRNAs was detected as early as 10 min until 180 min of insulin stimulation (Fig. 4A) as caveolin-2 mRNA levels were also up-regulated [21]. Of interest, SOCS-3 interacted with neither tyrosine kinase-inactivated nor -activated IR at both 10 and 180 min after insulin incubation (Fig. 4B). These

results show that SOCS-3 exerted no effect on the prolonged IR activation by insulin.

Although SOCS-3 did not interact with activated IR (Fig. 4B), caveolin-2 interacts with IR and pY27-caveolin-2 sustains tyrosine activation of the IR till 180 min of insulin stimulation (Figs. 1–3). To test whether pY27-caveolin-2 persists the IR activation by interfering the interaction of SOCS-3 with IR, we examined the effect of Y27A-caveolin-2 mutation in the association between SOCS-3 and activated IR. Consistent with the results in Fig. 4B, SOCS-3 did not interact with tyrosine-phosphorylated IR in vector control- and WT caveolin-2-transfected cells. Y27A-caveolin-2, however, induced interaction between SOCS-3 and tyrosine-phosphorylated IR (Fig. 4C). Tyrosine phosphorylation of IRS-1 by tyrosine kinase activation of IR is crucial event for insulin-induced signal transduction to down-stream targets, whereas serine/threonine phosphorylation of IRS-1 is discussed as a major mechanism for the termination of the insulin signaling [26,27]. Of interest, Y27A-caveolin-2 completely inhibited tyrosine phosphorylation of IRS-1 after 180 min of insulin stimulation with no effect on serine phosphorylation of IRS-1 (Fig. 4D). The tyrosine phosphorylation of IRS-1 interacting with IR was prevented by SOCS-3 presence in the complex with IR in Y27A-caveolin-2 expressing cells (Fig. 4E). Taken together, these data reveal that pY27-caveolin-2 keeps the tyrosine phosphorylation of IR and IRS-1 by interfering the negative regulation of SOCS-3.

## Discussion

The tyrosine kinase activity of IR regulates substrate protein interactions and enzymatic activities [2]. The IR phosphorylates IRS, the phosphorylated IRS then bind to SH2 domain-containing proteins [3,4]. The phosphorylated IRS serves as docking molecule that binds to and activates cellular kinases. Like IRS, caveolin-2 contains a conserved sequence for recognition by SH2 domain-containing proteins (*pYADP*) at tyrosine 27. pY27-caveolin-2 has been shown to interact with Ras-GAP, c-Src, and Nck in c-Src-overexpressed cells [28]. It has been reported that the scaffolding domain of caveolin-1 and -3, but not caveolin-2, binds to a conserved caveolin binding motif ($\Phi X\Phi XXXX\Phi$; where $\Phi$ is an aromatic residue) in the tyrosine kinase domain of insulin and EGF receptors [29,30]. Our results show that caveolin-2 interacts with IR. Thus, it would appear that pY27-caveolin-2 binds to the IR indirectly through interactions with SH2 domain-containing proteins such as Ras-GAP, c-Src, or Nck. When we tested whether c-Src is involved in pY27-caveolin-2-regulated duration of IR activation with PP2, an inhibitor of Src family members, PP2 treatment did not affect the long term IR activation (data not shown). Thus, our findings suggest that IR activation is prolonged through IR interaction with pY27-caveolin-2, which is possibly mediated by Ras-GAP or Nck, but not c-Src, in response to insulin.

PTPs act as negative regulators of insulin action by dephosphorylating the tyrosine kinases of IR [8,9]. To define the regulatory

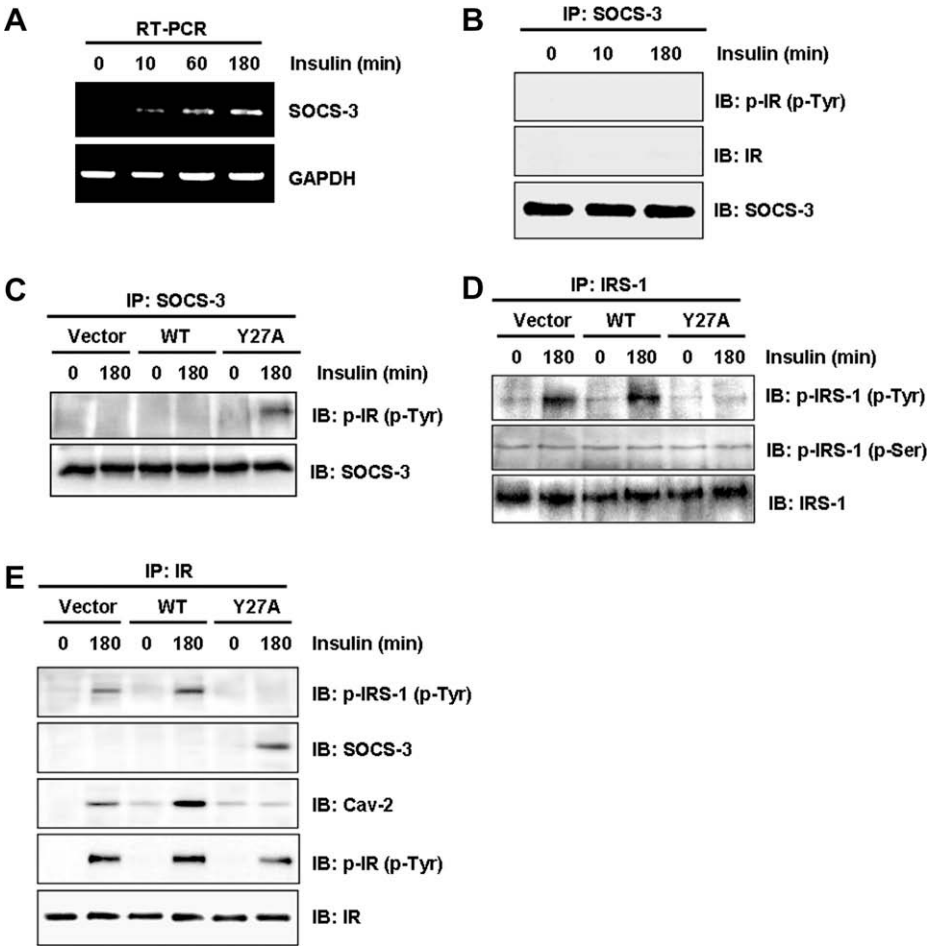


**Fig. 4.** Y27A-caveolin-2 interferes interaction of SOCS-3 with activated IR. (A) SOCS-3 mRNA levels in Hirc-B cells were analyzed by RT-PCR following insulin time course. (B) After insulin incubation, WCL were immunoprecipitated with anti-SOCS-3 antibody and subjected to immunoblot analysis. (C–E) Cells transfected with the caveolin-2-variants were treated with insulin for 180 min. WCL were immunoprecipitated with anti-SOCS-3 (C), anti-IRS-1 (D), or anti-IR (E) antibodies and subjected to immunoblot analysis as indicated ($n = 3$).

mechanism by PTPs involved in the prolonged IR activation by pY27-caveolin-2, we investigated the effect of ortho-vanadate in Y27A-caveolin-2-transfected cells. The present results show that ortho-vanadate inhibited dephosphorylation of the tyrosine-activated IR after 180 min of insulin stimulation (0 time chase) in Y27A-caveolin-2-transfected cells. Ortho-vanadate treatment, however, fails to inhibit dephosphorylation of the IR starting as early as 30 min with a complete dephosphorylation of the tyrosine-activated IR at 120 min chase. Since the level of IR protein was unchanged throughout the chase, it is unlikely that IR degradation plays a role in the reduction of IR tyrosine phosphorylation. Thus, our finding suggests that the dephosphorylation of IR by Y27A-caveolin-2 mutant is dependent on ortho-vanadate-insensitive PTPs.

Akt/mTOR [31,32] activation of STAT3 is declined by SOCS-3, a negative feedback modulator in the signaling of STAT3 activation [33]. Insulin-induced SOCS-3 expression inhibits insulin signaling through its binding to activated IR [10,11]. Our data show that activation of pS727-STAT3 is increased by 180 min of insulin stimulation and the activation is abolished by rapamycin. A recent report showed that induction of SOCS-3 gene by STAT3 activation is regulated by mTOR [14]. Thus, the activation of pS727-STAT3 seems likely dependent on mTOR signaling. Although SOCS-3 mRNA is induced at 180 min after insulin incubation, SOCS-3 association with activated IR is not detected. In contrast, caveolin-2 interacts with IR after 180 min of insulin stimulation. Y27A-caveolin-2 mutation, which attenuates the prolonged tyrosine kinase activation of IR, allows association of SOCS-3 with activated IR. And the interaction leads to inhibition of tyrosine phosphorylation of IRS-1. These results indicate that pY27-caveolin-2 maintains the long term tyrosine phosphorylation of IR by a mechanism interfering the interaction between SOCS-3 and activated IR.

Micro-ribonucleic acid 29 (miR-29), which regulates Akt-mediated glucose uptake in adipocytes, is up-regulated in skeletal muscles of type 2 diabetic Goto-Kakizaki rats. Of interest, caveolin-2 was one of the target genes of the miR-29 [34]. Indeed, caveolin-2 expression is significantly decreased in skeletal muscle and liver tissue of diabetic rats [34,35]. In contrast, SOCS-3 is up-regulated in liver tissue of obese diabetic mice [15]. In addition, SOCS-3 has been suggested to function as tumor suppressor genes [18]. Inactivation of SOCS genes by hypermethylation of CpG island, caveolin promoter region [36], was observed in various human cancers [37,38]. Further, caveolin-2 is widely presented and up-regulated in tumor cells [39–41]. Thus, our findings imply that insulin resistance and tumorigenesis might be controlled by expression of caveolin-2 status and tyrosine phosphorylation of caveolin-2 and potentially provide a new therapeutic approach to diabetes and cancers.

## Acknowledgments

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