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Cellular Phosphatase Activity of C1-Ten/Tensin2 is Controlled by Phosphatidylinositol-3,4,5-triphosphate Binding through the C1-Ten/Tensin2 SH2 Domain

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Abstract

Regulation of tyrosine phosphorylation on insulin receptor substrate-1 (IRS-1) is essential for insulin signaling. The protein tyrosine phosphatase (PTP) C1-Ten/Tensin2 has been implicated in the regulation of IRS-1, but the molecular basis of this dephosphorylation is not fully understood. Here, we demonstrate that the cellular phosphatase activity of C1-Ten/Tensin2 on IRS-1 is mediated by the binding of the C1-Ten/Tensin2 Src-homology 2 (SH2) domain to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3). We show that the role of C1-Ten/Tensin2 is dependent on insulin-induced phosphoinositide 3-kinase activity. The C1-Ten/Tensin2 SH2 domain showed strong preference and high affinity for PtdIns(3,4,5)P_3. Using site-directed mutagenesis, we identified three basic residues in the C1-Ten/Tensin2 SH2 domain that were critical for PtdIns(3,4,5)P_3 binding but were not involved in phosphotyrosine binding and PTP activity. Using a PtdIns(3,4,5)P_3 binding-deficient mutant, we showed that the specific binding of the C1-Ten/Tensin2 SH2 domain to PtdIns(3,4,5)P_3 allowed C1-Ten/Tensin2 to function as a PTP in cells. Collectively, our findings suggest that the interaction between the C1-Ten/Tensin2 SH2 domain and PtdIns(3,4,5)P_3 produces a negative feedback loop of insulin signaling through IRS-1.

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Author contributions

E.K. designed and performed experiments. D.K. performed TIRF imaging and edited the manuscript. I.S. performed the peptide binding assay. E.K. and H.J. performed the *in vitro* PTP assay. E.K., H.J., A.K., and J.L. interpreted the data. E.K., W.C., and S.H.R. wrote the manuscript. All authors reviewed the paper.

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Keywords

C1-Ten/Tensin2; Src-homology 2 domain; Insulin receptor substrate-1; Phosphatidylinositol-3,4,5-triphosphate binding; Protein tyrosine phosphatase

1. Introduction

Insulin signaling involves an integrated network, whose metabolic, transcriptional, and mitogenic roles are controlled by reversible tyrosine phosphorylation. Aberrant regulation of insulin signaling causes pathological conditions such as insulin resistance, which may progress to type 2 diabetes [1]. Insulin receptor substrate-1 (IRS-1), a key component of insulin signaling, becomes rapidly tyrosine-phosphorylated in response to insulin [2]. Phosphorylated IRS-1 generates docking sites for downstream effector molecules, which then mediate different signaling pathways. Thus, the steady state level of IRS-1 tyrosine phosphorylation determined by protein tyrosine kinases and protein tyrosine phosphatases (PTPs) dictates the degree of insulin signaling, which must be tightly controlled. PTPs are important negative regulators of insulin signaling that catalyze the dephosphorylation of tyrosine-phosphorylated proteins [3]. However, in contrast to our extensive knowledge of the catalytic functions of PTPs, the mechanism underlying their regulation has remained unclear.

C1-Ten/Tensin2 (also known as TENC1; referred to as C1-Ten hereafter) belongs to the tensin family, which is a group of focal adhesion molecules that interact with integrin [4], receptor tyrosine kinases [5], and actin [6]. It has been suggested that C1-Ten functions as a biological regulator for the Akt/PKB pathway [7–9] and its interaction with deleted in liver cancer 1 (DLC-1) provides tumor-suppressive function [10, 11]. We identified the metabolic role of C1-Ten as an active PTP of insulin signaling components, which regulate the phosphoinositide 3-kinase (PI3K) binding site, and contributes to pathological conditions such as diabetes [12, 13]. In addition, it has been suggested that C1-Ten may be an interesting therapeutic target in diabetes, because its expression appears upregulated under such conditions and may lead to insulin resistance [14]. C1-Ten is a relevant PTP in insulin signaling that directly interacts with IRS-1, without affecting insulin receptor tyrosine phosphorylation [13]. However, little is known at present regarding the mechanism by which C1-Ten PTP activity is regulated in response to insulin at the molecular level.

In this study, we examined a novel regulatory mechanism for C1-Ten PTP activity mediated by PI3K. We suggest that the C1-Ten Src-homology 2 (SH2) domain is a lipid-binding domain with specificity for phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3). Furthermore, we determined the role of PtdIns(3,4,5)P_3 in the regulation of the C1-Ten PTP activity through mutation of residues involved in PtdIns(3,4,5)P_3 binding. Collectively, our data provide the first evidence for PtdIns(3,4,5)P_3 binding activity of the SH2 domain of C1-Ten and its importance in regulating the phosphorylation of IRS-1 in insulin signaling pathways.

2. Materials and methods

2.1. Antibodies and reagents

Insulin (I5500, Lot 79H0566), LY294002 (440202, Lot 2825363), and anti-Flag antibody (F7425, Lot 001M4789) were purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-Tensin 2 (11990, Lot 1), anti-phospho-Akt (T308) (9275, Lot 20), and anti-phospho-Akt (S473) (9271S, Lot 13) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against IRS-1 (06–248, Lot 32682) and phospho-IRS-1 (Y612) (09–432, Lot 2382120) were obtained from Millipore (Billerica, MA, USA). Anti-Akt1/2 (sc-1619, Lot E1013) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The following lipids were used for surface plasmon resonance (SPR) analysis: 1-palmitoyl-2-oleoyl-sn-glycero-3phosphocholine (POPC, 850457P), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS, 840034P, both Avanti Polar Lipids, Alabaster, AL, USA), 1,2-dipalmitoyl derivatives of Ptdins(3,4,5)P_3 (64920), phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2, 10008115), phosphatidylinositol-3,4-bisphosphate (PI(3,4)P_2, 64922), phosphatidylinositol-3,5bisphosphate (PI(3,5)P_2, 10008398), phosphatidylinositol-3-monophosphate (PI(3)P, 64921), phosphatidylinositol-4-monophosphate (PI(4)P, 64923), and phosphatidylinositol-5monophosphate (PI(5)P, 64925, all Cayman Chemical, Ann Arbor, MI, USA).

2.2. Plasmids

Full-length plasmids bearing Flag-C1-Ten and EGFP-C1-Ten were generated as described previously [13, 15]. The SH2 domain (amino acids 1120–1253) of C1-Ten was inserted between the *Xho*I and *Eco*RI restriction sites of pRSET-B vector (Invitrogen, Carlsbad, CA, USA) with a His_6 tag at the N-terminus and an EGFP epitope at the C-terminus for SPR analysis. Full-length C1-Ten was sub-cloned into the *Eco*RI and *Xba*I restriction sites of the p3XFlag-CMV10 vector (Sigma Aldrich) [14] for stable expression in HEK293 cells. Point mutations of C1-Ten were generated with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

2.3. Cell culture and transfection

L6 myoblasts were grown and maintained in low-glucose α-minimal essential medium (α-MEM LM 008–01, Welgene, Gyeongsan, Korea) supplemented with 10% (v/v) fetal bovine serum (FBS, 16000–044, Gibco, Life Technologies, Carlsbad, CA, USA). For the C1-Ten knockdown experiment, AllStars negative-control small interfering RNA (siRNA) (siCon, Qiagen, Valencia, CA, USA), C1-Ten siRNA1 (targeting the C1-Ten sequence TCAGTGGATTACAACATGACA) and siRNA2 (targeting the C1-Ten sequence CCAGTGGACACAGCACACTG) (Bioneer, Daejeon, South Korea) were used, as described previously [13]. L6 myoblasts were transfected with either 100 nM siCon, siRNA1, or siRNA2 using Lipofectamine RNAiMax (13778–075, Invitrogen) according to the manufacturer's instructions. HEK293 cells were grown and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, 12–604F, Lonza, Basel, Switzerland) supplemented with 10% (v/v) FBS (14–506F, Lonza). Transfection of HEK293 cells was performed using Lipofectamine (18324–020, Invitrogen) according to the manufacturer's instructions.

2.4. Cell lysis and western blotting

Harvested cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 20 mM NaF, 10 mM glycerophosphate, 1 mM PMSF, 10% glycerol, 1% triton X-100, and a protease inhibitor cocktail. Samples were centrifuged at 14,000 rpm for 15 min at 4 °C; the resulting supernatants were separated by SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare, Little Chalfont, UK), and probed using various antibodies. The blots were then reacted with secondary antibodies, followed by enhanced chemiluminescence (ECL; Thermo Fisher Scientific). Positive immunoreactive bands were quantified by densitometric analysis using ImageJ software (NIH, Bethesda, MD, USA) and compared with those of the control.

2.5. Protein expression and purification

A recombinant construct of the C1-Ten SH2 domain was expressed in *Escherichia coli* BL21(DE3)pLysS (Novagen, Madison, WI, USA) at 16 °C in the presence of 1 mM isopropyl beta-D-thiogalactopyranoside (Sigma Aldrich). Cell pellets were lysed and sonicated in a buffer containing 50 mM Tris-HCl (pH 7.9), 300 mM NaCl, 10 mM imidazole, and 10% (v/v) glycerol. The lysates were centrifuged at 61,000 × *g* for 30 min at 4 °C. The supernatant was then mixed with nickel-nitrilotriacetic acid-agarose beads (Qiagen, Hilden, Germany) for 1 h at 4 °C. The resin was washed with washing buffer 1 (50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole [pH 7.9]), followed by washing buffer 2 (20 mM Tris-HCl, 160 mM NaCl, 20 mM imidazole [pH 7.9]). Bound proteins were eluted with elution buffer (20 mM Tris-HCl, 160 mM NaCl, 300 mM imidazole [pH 7.9]). Eluted fractions were then exchanged to the SPR elution buffer (20 mM Tris-HCl [pH 7.4], 160 mM NaCl) using a PD-10 gel filtration column (GE Healthcare), and equilibrated with the same buffer. Protein purity was checked on a 20% polyacrylamide gel, and protein concentration was determined by a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Lipid vesicle preparation and SPR analysis

Large unilamellar vesicles were prepared using a Liposofast microextruder (Avestin, Ottawa, ON, Canada) with a 100-nm polycarbonate filter. Phosphoinositide (PtdIns*P*) vesicles were prepared by mixing POPC, POPS, and PtdIns*P* at a molar ratio of 77:20:3 [16]. All SPR measurements were performed on a Biacore T100 system (GE Healthcare) at 25 °C in SPR running buffer containing 20 mM Tris-HCl (pH 7.4) and 160 mM NaCl. PtdIns*P* vesicles were immobilized onto the active surface, and POPC vesicles were immobilized onto the control surface of L1 chips to yield identical response units and ensure an equal concentration of coated lipids. Equilibrium SPR measurements were done at a flow rate of 5 μl/min to allow sufficient time for the response in the association phase to reach near-equilibrium values (R_{eq}) [17]. Different protein concentrations were injected at a flow rate of 10 μl/min to generate a set of R_{eq} values to measure the apparent dissociation constant (K_d). K_d values were fitted by nonlinear least squares analysis of the binding isotherm using the following equation: $R_{eq} = R_{max}/(1 + K_d/P_o)$, where R_{max} indicates the maximal R_{eq} value, and P_o indicates protein concentration [18].

2.7. SH2 peptide binding assay by fluorescence anisotropy

A fluorescein-6-aminohexanoyl (F-Ahx)-labeled pY-containing peptide, F-Ahx-NEpYDNV, was used for binding studies, as described previously [19]. The peptide was dissolved in 20 mM Tris buffer (pH 7.4) containing 160 mM NaCl and 5% dimethyl sulfoxide (DMSO). To each well of a 96-flat bottom black polystyrol plate, 100 μl solution was added, containing each peptide (50 nM) and SH2 domain (10 nM to 100 μM). After 30-min incubation, the plate was inserted into a BioTek Synergy Neo microplate reader and the fluorescence anisotropy (r) was measured with excitation and emission wavelengths set at 485 and 535 nm, respectively. Since $P_o >> Pep_o$ under our conditions, the K_d for the SH2 domain-peptide binding was determined by non-linear least-squares analysis of the binding isotherm using the following equation:

$$Pep_{bound}/Pep_o = \Delta r/\Delta r_{max} = \frac{1}{1 + K_d/P_o}$$

where Pep_{bound}, Pep_o, and P_o indicate the concentration of bound peptide, total peptide, and total SH2 domain, respectively, and Δr and Δr_{max} are the anisotropy change for each P_o and the maximal Δr, respectively.

2.8. In vitro PTP assay

A PTP assay was performed and its activity was calculated as previously described [13, 14]. Briefly, three 10-cm dishes containing HEK293 cells were transfected with 3 μg Flag C1-Ten plasmids. After 48 h, the cells were lysed in lysis buffer (50 mM Tris-HCI [pH 7.4], 150 mM NaCI, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 20 mM NaF, 0.025% mercaptoethanol, 1% Triton X-100, 0.2% sodium deoxycholate, and a protease inhibitor cocktail). The soluble fractions of the cell lysates were isolated by centrifugation at 14,000 rpm for 15 min at 4 °C. The supernatant was subjected to immunoprecipitation with FLAG M2 affinity Gel for 4 h at 4 °C. After incubation, the immunoprecipitates were washed three times with lysis buffer and once with wash buffer (50 mM Tris-HCI [pH 7.4], 50 mM NaCI, 10% glycerol, 5 mM DTT). Bound proteins were eluted with elution buffer (50 mM Tris-HCI [pH 7.4], 50 mM NaCI, 10% glycerol, 5 mM dithiothreitol, and 100 μg/ml 3× FLAG peptide). After purification, the concentration and activity of purified Flag protein were confirmed ahead of each experiment. Approximately 17 ng/μl C1-Ten wild-type (WT) and C1-Ten K1142Q/K1155Q/K1157Q (3KQ) mutant were obtained. A malachite green assay kit (Millipore) was used to measure PTPase activity. Enzyme reactions were performed in a final volume of 50 μl reaction buffer (50 mM Tris-HCI [pH 7.4], 50 mM NaCI, 5 mM dithiothreitol, 2 mM $MgCI_2$) in 96-well plates at 30 °C for 90 min. The reaction was terminated by adding malachite green detection solution. Absorbance was measured at 620 nm.

2.9. Total internal reflection fluorescence (TIRF) microscopy

Plasma membrane localization of WT and 3KQ mutant forms of C1-Ten were imaged using an objective-type TIRF microscope built on an inverted microscope (IX-81, Olympus, Tokyo, Japan) equipped with an XY-axis automated stage (MS-2000, Applied Scientific

Instrumentation, Eugene, OR, USA). A 488-nm laser (35-LAL-415–220R, Melles Griot, NY, USA) was aligned and collimated to focus on the back focal plane of an oil-immersion 60× objective lens (APON 60×OTIRF/1.49, Olympus). A dichroic mirror (ZTUV-405/488/561RPC, Chroma, Taoyuan City, Taiwan) and an emission filter (ZET405/488/561M, Chroma) were used. Images were collected with an electron multiplying charge-coupled device (EM-CCD) camera (iXon3 897, Andor Technology, Belfast, UK). For higher magnification, we used a 1.6× amplifier and inserted a 1.43× tube lens. The images were obtained before and after insulin treatment (2 min, 5 min, 7 min, and 9 min) at a frame rate of 20 Hz. At the indicated time point, the fluorescence intensity of each cell was subtracted from the background fluorescence measured outside the cell. Fluorescence intensity was determined for each condition by normalizing fluorescence intensity of the image in the NT condition.

2.10. Stable cell line preparation

The full-length coding regions of WT and 3KQ C1-Ten were subcloned into the p3XFlag-CMV10 vector (Sigma Aldrich) and transfected into HEK293 cells to establish stable cell lines. Transfected HEK293 cells were selected following addition of 400 μg/ml geneticin sulfate G418 (Gibco) to the complete medium. G418-resistant cells were then analyzed for the presence of WT and 3KQ C1-Ten by western blotting using an anti-Flag antibody. We confirmed the parallel expression of WT and 3KQ C1-Ten.

2.11. Statistical analysis

All results are presented as the mean ± standard error of the mean (SEM). Experimental data were compared using analysis of variance (ANOVA) with Tukey's post-hoc test or an unpaired two-tailed Student's *t*-test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. C1-Ten attenuates IRS-1 phosphorylation in a PI3K-dependent manner

We previously reported that C1-Ten preferentially dephosphorylated IRS-1 Y612 during insulin signaling [13]. To further investigate the molecular mechanism underlying this function of C1-Ten, we examined the insulin-induced time-course effect of phosphorylation in L6 myoblasts. C1-Ten-silenced cells showed significantly increased levels of phosphorylation on IRS-1 Y612 (Fig. 1A) during insulin stimulation. The amount of total IRS-1 was not altered by C1-Ten knockdown and the phospho-IRS-1 Y612 level increased throughout insulin stimulation and decreased 60 min after stimulation. Phosphorylation of Akt at T308 and S473, a downstream target of IRS-1, increased significantly after 5 min and 15 min, respectively, of insulin treatment in C1-Ten-silenced cells. This result suggests that C1-Ten-mediated dephosphorylation of IRS-1 Y612 contributed to reduced phosphorylation of Akt at both sites. To corroborate the observed increase of phospho-IRS-1 Y612 and phospho-Akt (T308 and S473) in C1-Ten-silenced cells, distinct non-overlapping siRNAs against C1-Ten (siC1-Ten 2) were tested (Fig. S1). We confirmed a consistent increase in phospho-IRS-1 Y612 and phospho-Akt T308 and S473 during an insulin-induced time-course in C1-Ten-silenced cells using distinct siRNA. These results suggest that a reduction of IRS-1 Y612 phosphorylation in response to insulin is not due to other factors (e.g., off-

target effects of the siRNA), but is linked to the function of C1-Ten. To determine the putative signaling pathways affected by C1-Ten and involved in IRS-1 Y612 phosphorylation, we pretreated the endogenous C1-Ten-expressing L6 myoblasts with a PI3K inhibitor, LY294002. Interestingly, insulin-induced IRS-1 Y612 phosphorylation was upregulated in the presence of LY294002 (Fig. 1B). These results suggest that IRS-1 Y612 dephosphorylation by C1-Ten is regulated by PI3K activity. To further test this notion, we measured the effect of overexpressing C1-Ten in the presence and absence of LY294002 in the HEK293 cell line, which expresses low levels of C1-Ten [20]. As shown in Fig. 1C, C1-Ten overexpression in HEK293 cells resulted in the successful dephosphorylation of IRS-1 Y612. Conversely, IRS-1 Y612 phosphorylation was restored in the presence of LY294002 in C1-Ten-overexpressing HEK293 cells. Collectively, these results indicate that PI3K activity is involved in the regulation of C1-Ten, which appears to function as a PTP in insulin signaling.

3.2. C1-Ten SH2 domain affects PtdIns(3,4,5)P_3 binding

Our present results indicate that the dephosphorylation of IRS-1 Y612 by C1-Ten is PI3K-dependent. We previously reported that most human SH2 domains can bind lipids with high affinity and specificity, which is essential for cellular signaling activities of their host proteins [19, 21]. We thus tested if PI3K-dependent dephosphorylation of IRS-1 Y612 by C1-Ten was dependent on the lipid-binding activity of its SH2 domain. In particular, we examined if the C1-Ten SH2 domain could specifically interact with the products of PI3K by quantitatively measuring PtdIns*P* selectivity for the C1-Ten SH2 domain. Indeed, the C1-Ten SH2 domain exhibited selectivity for two lipid products of PI3K, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. In particular, its ability to distinguish among three iso-electric bisphophates, PtdIns(3,4)P_2, PtdIns(4,5)P_2 and PtdIns(3,5)P_2, underscored the specific nature of its lipid binding. For PtdIns(3,4,5)P_3-containing vesicles, C1-Ten-SH2 showed a K_d of 260 ± 40 nM (Fig. 2A and B), which was comparable to that of the Akt1 PH domain [22], a canonical PtdIns(3,4,5)P_3-and PtdIns(3,4)P_2-binding domain [23]. Collectively, these results show that the C1-Ten SH2 domain is a genuine PtdIns(3,4,5)P_3- (and PtdIns(3,4)P_2-)binding domain and suggest that PI3K modulates C1-Ten activity through PtdIns(3,4,5)P_3-SH2 domain interaction during insulin signaling.

3.3. The lipid-binding site of C1-Ten SH2 is topologically distinct from its canonical pY-binding site

We previously reported that most SH2 domains contained surface cationic patches and grooves involved in lipid binding. The location of these sites is highly variable but in general spatially separated from their canonical pY-binding pockets [19, 21]. Examination of a solubilized NMR structure of C1-Ten-SH2 revealed that it also possessed a prominent cationic patch containing K1147, K1155, and K1157, which was distant from its pY-binding pocket [24] (Fig. 3A) and might be involved in PtdIns(3,4,5)P_3 binding. We thus sequentially mutated these cationic residues to K1157Q, K1155Q/K1157Q, K1142Q/K1157Q, and K1142Q/K1155Q/K1157Q to determine if they were involved in PtdIns(3,4,5)P_3 binding (Fig. 3B). In general, mutation of these residues reduced the affinity of C1-Ten SH2 for PtdIns(3,4,5)P_3-containing vesicles, with a triple-site mutant (K1142Q/K1155Q/K1157Q; referred to as 3KQ hereafter) showing the lowest affinity. Equilibrium

SPR analysis revealed that 3KQ had 6-fold lower affinity for PtdIns(3,4,5)P_3 vesicles than the WT (Fig. 3C). Also, 3KQ had much lower selectivity for PtdIns(3,4,5)P_3 over PtdIns(4,5)P_2 vesicles than the WT (Fig. 3D), supporting the notion that the mutated residues were involved in specific PtdIns(3,4,5)P_3 binding. As a negative control, we mutated cationic residues in other parts of the C1-Ten SH2 domain (i.e., K1209, K1212, and K1214) (Fig. S2A) and none significantly affected its binding to PtdIns(3,4,5)P_3 vesicles (Fig. S2B). We also mutated a conserved cationic residue in the pY-binding pocket of C1-Ten SH2 (R1165A) but no significant difference was observed between WT and the mutant in terms of binding to PtdIns(3,4,5)P_3containing vesicles (Fig. 3E), confirming that the pY pocket was not involved in lipid binding. Taken together, these data indicate that K1142, K1155, and K1157 in the main cationic patch are involved in specific PtdIns(3,4,5)P_3 binding to the C1-Ten SH2 domain.

The 3KQ mutation of C1-Ten SH2 is not expected to alter its primary function as a pY motif-binding module, because the lipid-binding sites and pY-binding site are spatially separated. Prior to performing cell studies using 3KQ, we thus checked if this mutation might have affected its binding to a pY motif [25]. Binding analysis using a fluorescein-labeled pY peptide by fluorescence anisotropy showed that the WT and 3KQ C1-Ten SH2 domains exhibited essentially the same affinity for the peptide while R1165A showed much reduced peptide affinity (Fig. 3F), confirming the spatial and functional orthogonality of the lipid- and pY-binding sites.

3.4. The lipid-binding motif of C1-Ten is essential for its biological function

Lipids regulate cellular activities of cytosolic proteins either by controlling their subcellular localization [26, 27] or by modulating their conformation and function [28]. To determine whether PtdIns(3,4,5)P_3 controls in the membrane translocation of C1-Ten, we measured the plasma membrane localization of WT and 3KQ C1-Ten in response to insulin signaling, using TIRF imaging. TIRF microscopy is well suited for analysis of the localization and dynamics of molecules near the plasma membrane [29]. As shown in Fig. 4A, both WT and 3KQ C1-Ten are significantly pre-localized in the plasma membrane before insulin localization, and thus showed little to no change in localization after insulin stimulation. In contrast, the Akt PH domain, used as a positive control, showed a significant increase in TIRF signal after insulin stimulation.

We then investigated whether or not PtdIns(3,4,5)P_3 modulates the functional activity of C1Ten in cells. To determine whether the C1-Ten 3KQ mutant was catalytically intact, we first performed an *in vitro* PTP assay. WT and 3KQ C1-Ten displayed similar PTP activity (Fig. 4B), indicating that the 3KQ mutation of C1-Ten does not alter its intrinsic PTP activity. We then compared the activity of WT and 3KQ C1-Ten in a stable expression system. As shown in Fig. 4C, the phosphorylation levels of IRS-1 Y612, Akt T308 and S473 upon insulin stimulation were much lower in WT C1-Ten-expressing cells than in C1-Ten 3KQ-expressing cells. This means that despite its fully intact PTP activity, 3KQ with compromised PtdIns(3,4,5)P_3 binding activity cannot perform its signaling function in the cells. These results thus show that recognition of PtdIns(3,4,5)P_3 by the C1-Ten SH2 domain is essential for the cellular signaling function of C1-Ten.

4. Discussion

Strength and duration of insulin signaling are determined largely by the coordinated actions of tyrosine kinases and phosphatases. Indeed, abnormal regulation of tyrosine phosphorylation at the molecular level can lead to diseases such as diabetes. IRS-1 is phosphorylated at multiple tyrosine residues upon insulin stimulation; these sites serve as docking points to recruit downstream molecules, many of which contain SH2 domains [30]. Phosphorylation of IRS-1 Y612 generates a major docking site for PI3K, which is associated with its full activity [31] and plays a central role in recruitment of important downstream signal transduction cascades (e.g., PI3K-PKB/Akt pathway) [32]. C1-Ten is the only physiologically relevant PTP for the dephosphorylation of IRS-1 Y612 [13]. However, it remains unclear how C1-Ten regulates IRS-1 Y612 phosphorylation in the insulin signaling pathway. In this study, we determined the effect of PI3K activity on IRS-1 Y612 phosphorylation in the absence or presence of C1-Ten in different cell lines (Fig. 1). Inhibition of the PI3K activity increased phosphorylation of IRS-1 Y612 in L6 myoblasts, which contain a high level of endogenous C1-Ten, but not in HEK293 cells, in which endogenous C1-Ten levels are low. To determine whether C1-Ten's role was dependent on PI3K activity, we overexpressed C1-Ten in HEK293 cells and observed a significant decrease in IRS-1 Y612 phosphorylation. Also, inhibition of PI3K activity restored the phosphorylation level of IRS-1 Y612 in C1-Tenoverexpressing HEK293 cells. These results suggest a negative feedback mechanism for PI3K-dependent IRS-1 Y612 dephosphorylation mediated by C1-Ten. Additionally, increased intensity of IRS-1 Y612 phosphorylation in C1-Ten knockdown L6 myoblasts indicates that insulin dependency was improved with the decrease of C1-Ten level. Collectively, these results establish that IRS-1 serves as a phosphorylation-sensitive rheostat to control the strength of insulin signaling and that its tyrosine dephosphorylation is regulated by C1-Ten in a PI3K-dependent manner.

PTPs play a key role in regulating the steady-state of reversible tyrosine phosphorylation events in the insulin pathway [3]. Many studies have shown that human PTP-based protein interaction networks transduce and regulate a wide range of PTP-related cellular functions [33–35]. For example, PTP1B and SHP-2 are known to regulate the insulin signaling pathway by modulating tyrosine phosphorylation of the insulin receptor itself and/or IRS-1, as well as the binding of SH2 domain-containing adaptor proteins to IRS-1 [36–38]. Lipids play important roles in modulating cellular activities [19, 21, 39–41]. However, lipid-mediated regulation of PTP activity is not well understood. This study shows that the PtdIns(3,4,5)P_3-binding activity of C1-Ten is important for its cellular PTP activity. C1-Ten affects IRS-1 Y612 phosphorylation only through direct interaction with IRS-1 [13]. Because the catalytic domain of PTPs lacks substrate specificity, their substrate specificity is typically achieved through specific interaction with a substrate protein individually or within a multi-protein signaling complex [42], Likewise, specific interaction between the two molecule would be necessary for the specificity of C1-Ten towards IRS-1. The exact mechanism of the direct interaction between C1-Ten and IRS-1 is not fully elucidated, but such interaction is reduced in the presence of vanadate, a phosphotyrosine mimetic [13], suggesting the involvement of pY binding. Interestingly, the lipid-binding-deficient C1-Ten 3KQ mutant retained pY-binding activity and intrinsic phosphatase activity but did not

exhibit intracellular PTP activity, suggesting that the C1-Ten-IRS-1 interaction is affected by lipid binding. In response to insulin stimulation, IRS-1 approaches the plasma membrane, where it binds to the insulin receptor [43, 44]. For C1-Ten to interact with IRS-1, both should come close to the plasma membrane. However, C1-Ten is pre-localized in the plasma membrane before insulin stimulation and thus shows no further increase in TIRF signal after insulin stimulation (Fig. 4A). Accordingly, the main role of PtdIns(3,4,5)P_3 is likely to coordinate the IRS-1-C1-Ten interaction through allosteric effects as shown in our previous report [21]. Undoubtedly, further studies are required to elucidate the complex mechanism by which PtdIns(3,4,5)P_3 regulates C1-Ten PTP activity by specifically facilitating its interaction with IRS-1.

C1-Ten has two phosphotyrosine binding modules, the SH2 and PTB domain. Studies on the role of C1-Ten in signaling are based mainly on work about the C-terminal region of C1-Ten, which includes these two domains [5, 10, 45, 46]. These domains are essential for interaction and regulation of their binding partners. Subsequent studies have shown that, while binding of the SH2 domain to target proteins is strictly regulated by tyrosine phosphorylation, most PTB domains bind to their nonphosphorylated targets in a constitutive manner [47]. Unlike other tensin family, C1-Ten uses the PTB domain predominantly to regulate DLC-1 activity [11] and bind to the pY motif on integrin [6] whereas it employs the SH2 domain to regulate DLC-1 in a pY-independent manner [48]. Thus, the C1-Ten SH2 domain may have other properties beyond passive pY-binding. Accumulating evidence suggests that SH2 domains are dual-specificity lipid- and protein-binding modules utilizing alternative cationic patches that are structurally and functionally independent from their canonical pY-binding pockets [19, 21, 49, 50]. Our study shows that the C1-Ten SH2 domain interact with lipids—especially PtdIns(3,4,5)P_3—via its prominent alternate cationic patches rather than its pY-binding pocket. This may enable C1-Ten to simultaneously interact with lipids and the pY motif using two separate sites, allowing lipids to allosterically coordinate and modulate its pY-binding activity and specificity [19]. The C1-Ten SH2 domain has been implicated in binding to a number of signaling proteins that regulate cell migration [48, 51, 52]; however, its involvement in insulin signaling, through specific interactions with the components of the major signaling pathways has not been thoroughly investigated. In this study, a good correlation between *in vitro* lipid-binding properties of the C1-Ten SH2 domain and various cellular activities of C1-Ten shows that the lipid-binding activity of the SH2 domain is essential for the PTP activity of C1-Ten in insulin signaling. Although further studies are required to elucidate the interplay of the SH2 domain and other parts of C1-Ten in its signaling activities, our results suggest that dual lipid- and pY-binding activity of the C1-Ten SH2 domain is a crucial element in the spatiotemporal regulation of the cellular C1-Ten activity.

5. Conclusions

Our study identified a novel type of C1-Ten regulation mediated by interactions with PtdIns(3,4,5)P_3 via the SH2 domain, which allows for its interaction with tyrosine-phosphorylated IRS-1, as summarized in Fig. 4D. Our results suggest that C1-Ten acts as a spatiotemporal negative regulator in insulin signaling. The delicate regulation of C1-Ten

potentially improves the specificity for IRS-1 and tightly controls its phosphorylation level in the insulin signaling pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

PTP	protein tyrosine phosphatase
IRS-1	insulin receptor substrate-1
PI3K	phosphoinositide 3-kinase
C1-Ten	C1 domain-containing phosphatase and tensin homolog
SH2	Src-homology 2
PtdIns(3,4,5)P_3	phosphatidylinositol-3,4,5-triphosphate
SPR	surface plasmon resonance

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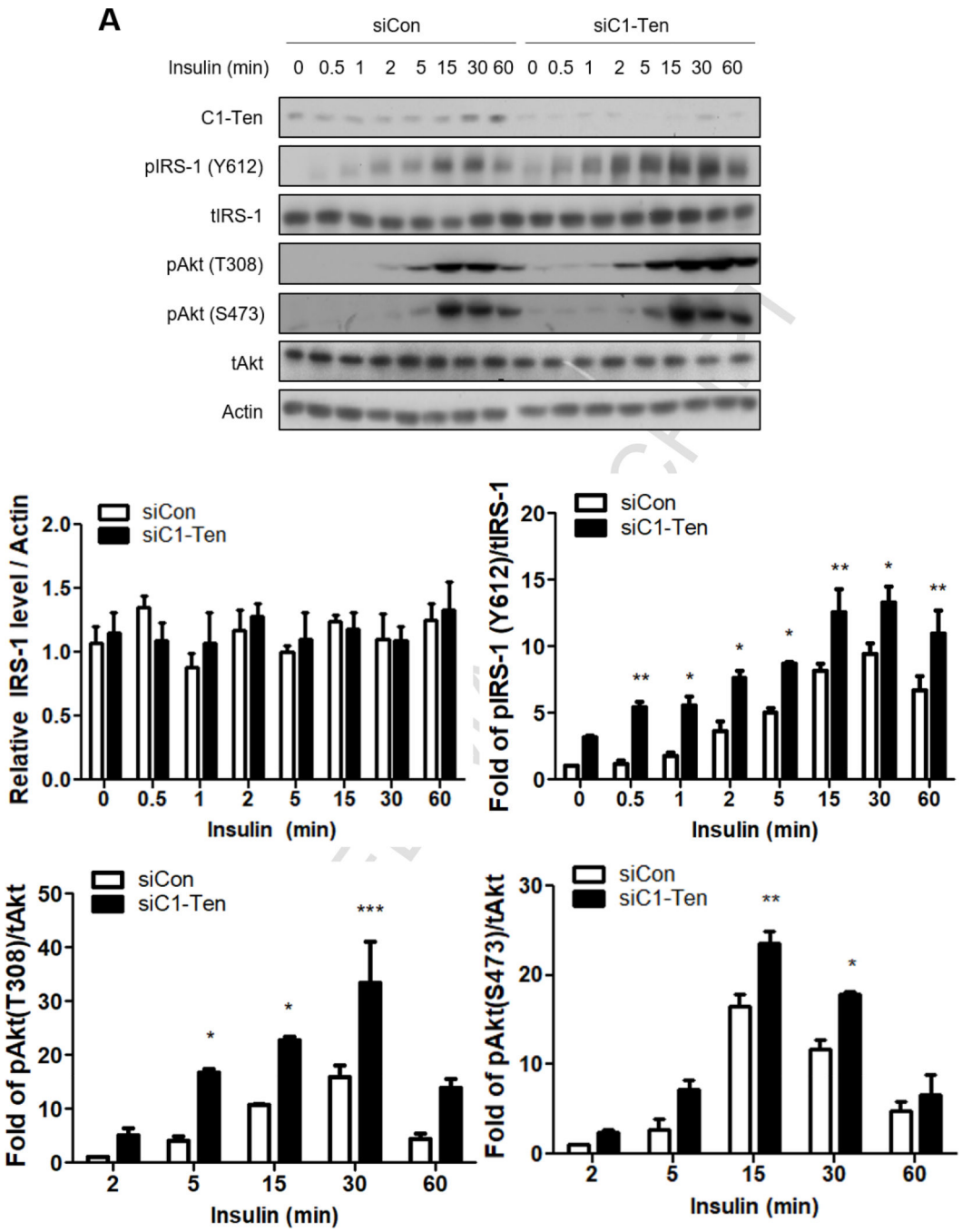
Highlights

- C1-Ten's function as a PTP for IRS-1 in insulin signaling is controlled by PI3K.
- The C1-Ten SH2 domain binds to PtdIns(3,4,5)P_3 with high affinity.
- The PtdIns(3,4,5)P_3 binding of C1-Ten is necessary for its function as PTP.



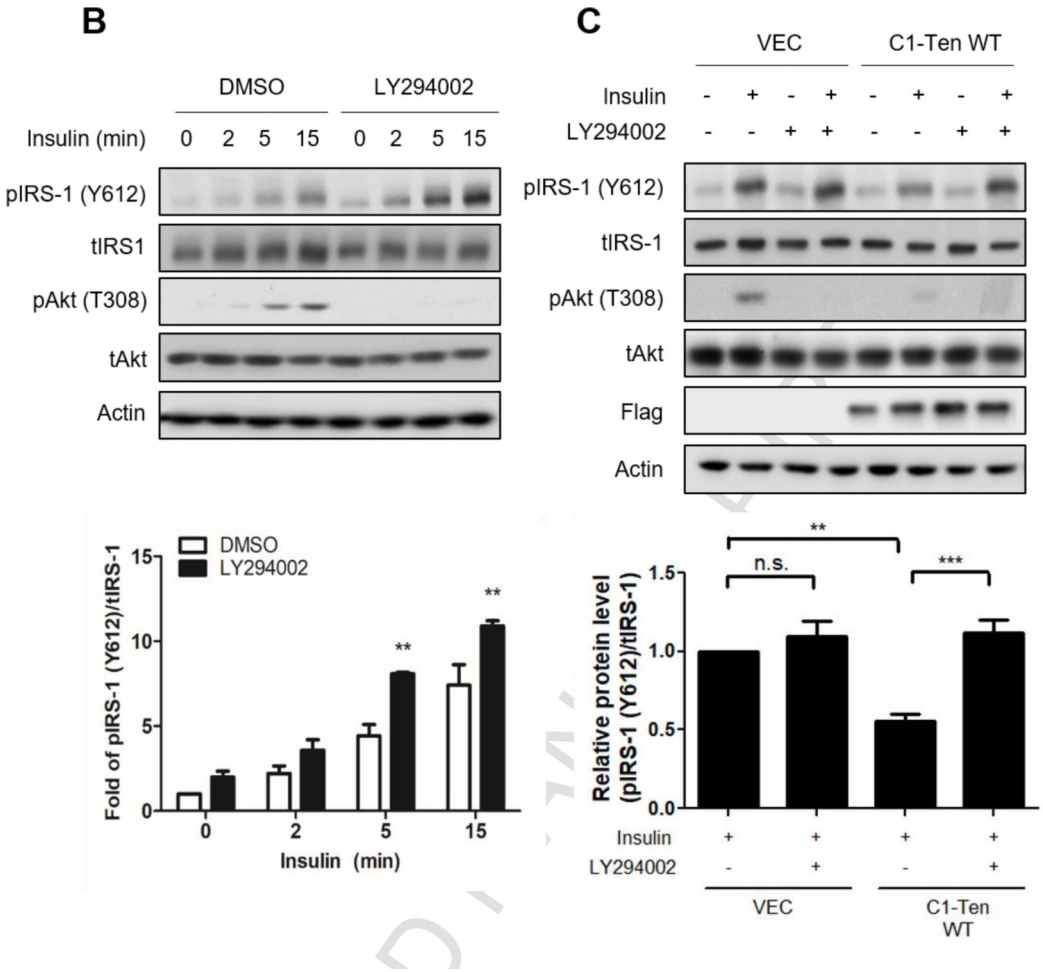


Figure 1. The role of C1-Ten in insulin signaling is phosphoinositide 3-kinase (PI3K)-dependent.

(A) Time-course effect of C1-Ten knockdown on insulin receptor substrate-1 (IRS-1)-mediated insulin signaling in L6 myoblasts. Cells were transfected with 100 nM siRNA control (siCon) or targeting C1-Ten siRNA1 (siC1-Ten). After 24 h, cells were serum-starved for 4 h and stimulated with 50 nM insulin for the indicated time periods. Cell lysates were analyzed by immunoblotting with the indicated antibodies. Levels of total IRS-1 protein are relative to those of actin. The phospho-immunoblot was normalized to the corresponding total protein immunoblot and quantified as the relative fold change compared to siCon's initial values. Data are presented as mean ± SEM (n = 4). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (B) Time-course effect of LY294002 on IRS-1-mediated insulin signaling in L6 myoblasts. Cells were serum-starved for 4 h and pretreated with dimethyl sulfoxide (DMSO) or 50 μM LY294002 for 30 min prior to stimulation with 50 nM insulin for the indicated time periods. Cell lysates were analyzed by immunoblotting with the indicated antibodies. Data are presented as mean ± SEM (n = 3). $**P < 0.01$. (C) Effect of LY294002 on IRS-1-mediated insulin signaling in HEK293 cells. Cells were transfected with Flag-VEC or Flag-C1-Ten wild-type (WT) and serum-starved for 18 h, and pretreated with DMSO or 10 μM LY294002 for 2 h prior to stimulation with 5 nM insulin for 5 min. Cell

lysates were analyzed by immunoblotting with the indicated antibodies. Data are presented as mean ± SEM (n = 4). $^{**}P < 0.01$, $^{***}P < 0.001$.

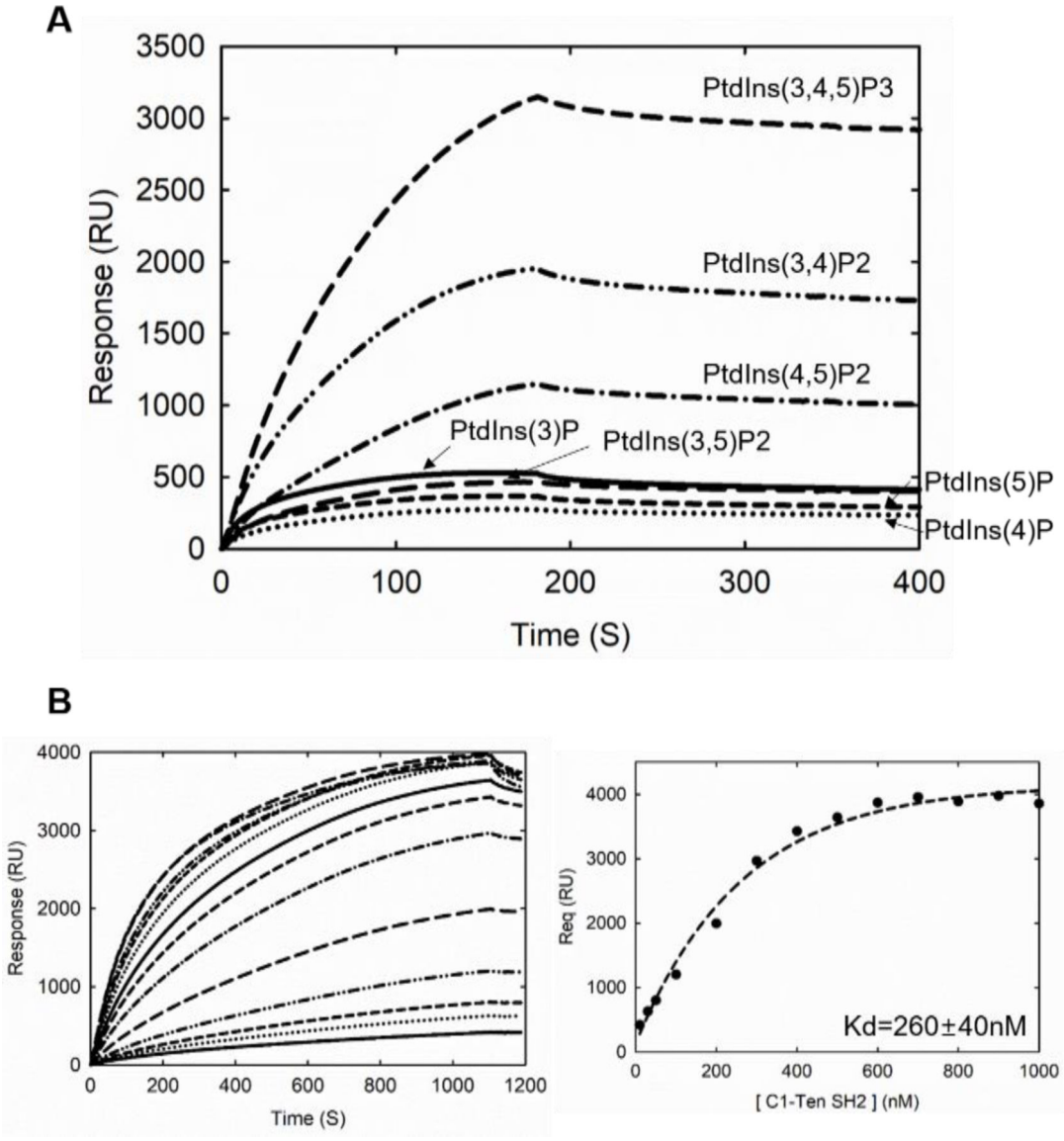
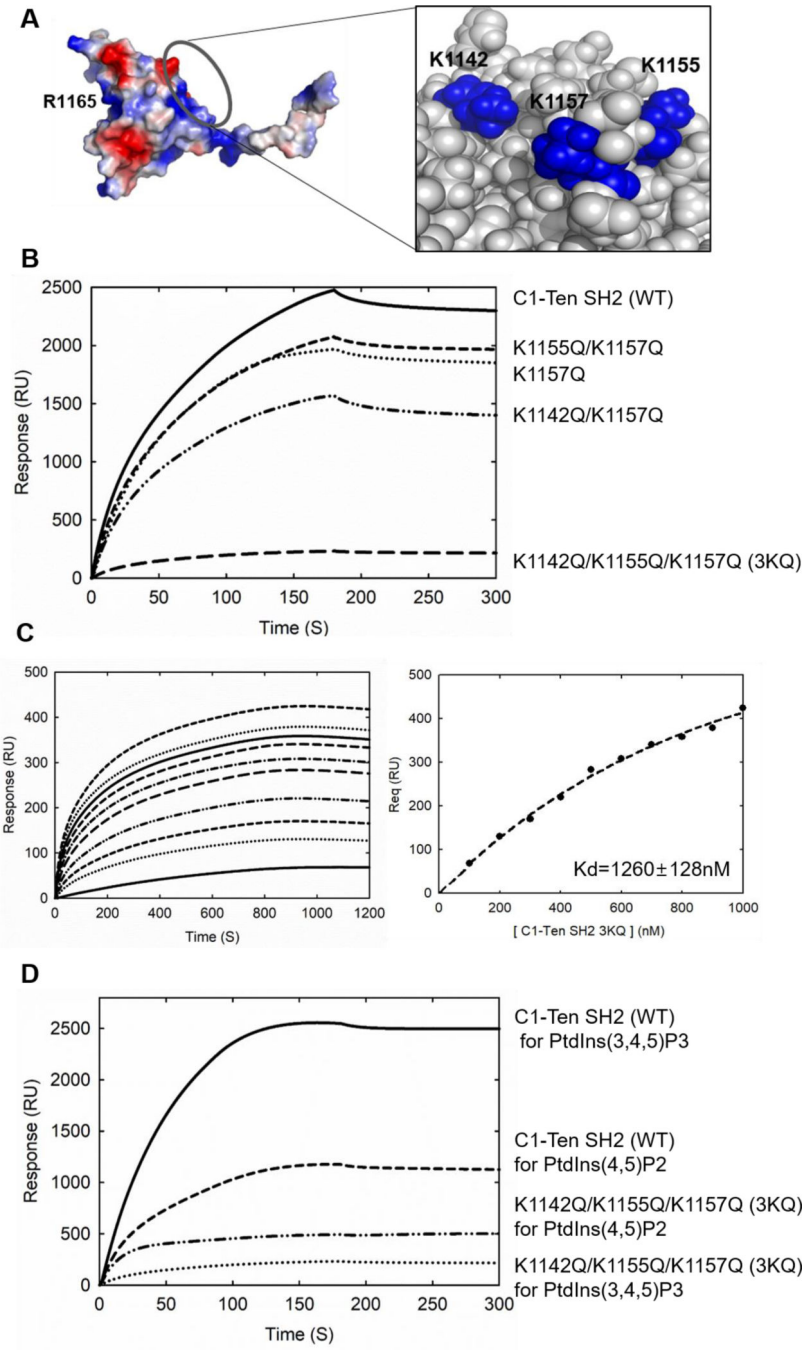


Figure 2. C1-Ten SH2 domain binds specifically to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3).

(A-B) All SPR measurements were performed at 25 °C in SPR running buffer containing 20 mM TrisHCl (pH 7.4) and 160 mM NaCl. POPC vesicles were used for the control surface. (A) Phosphoinositide specificity of the C1-Ten SH2 domain. The C1-Ten SH2 domain (500 nM) was allowed to interact with POPC/POPS/PtdIns*P* (77:20:3) vesicles using the L1 chip. Sensorgrams show PtdIns(3,4,5)P_3 > PtdIns(3,4)P_2 > PtdIns(4,5)P_2 > PtdIns(3)P ~ PtdIns(3,5)P_2 ~ PtdIns(5)P ~ PtdIns(4)P (from top to bottom). (B) Determination of K_d for PtdIns(3,4,5)P_3 vesicles of the C1-Ten SH2 domain. The C1-Ten SH2 domain was injected at 5 μl/min at varying concentrations (10, 30, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 nM, from bottom to top) over the L1 chip coated with PtdIns(3,4,5)P_3 vesicles (left). Binding isotherms were generated from the R_{eq} versus the concentration (P_o) of the C1-Ten SH2 domain plot (right). Solid lines indicate the theoretical curve constructed from R_{max} (= 5234 ± 285) and K_d (= 260 ± 40) values.



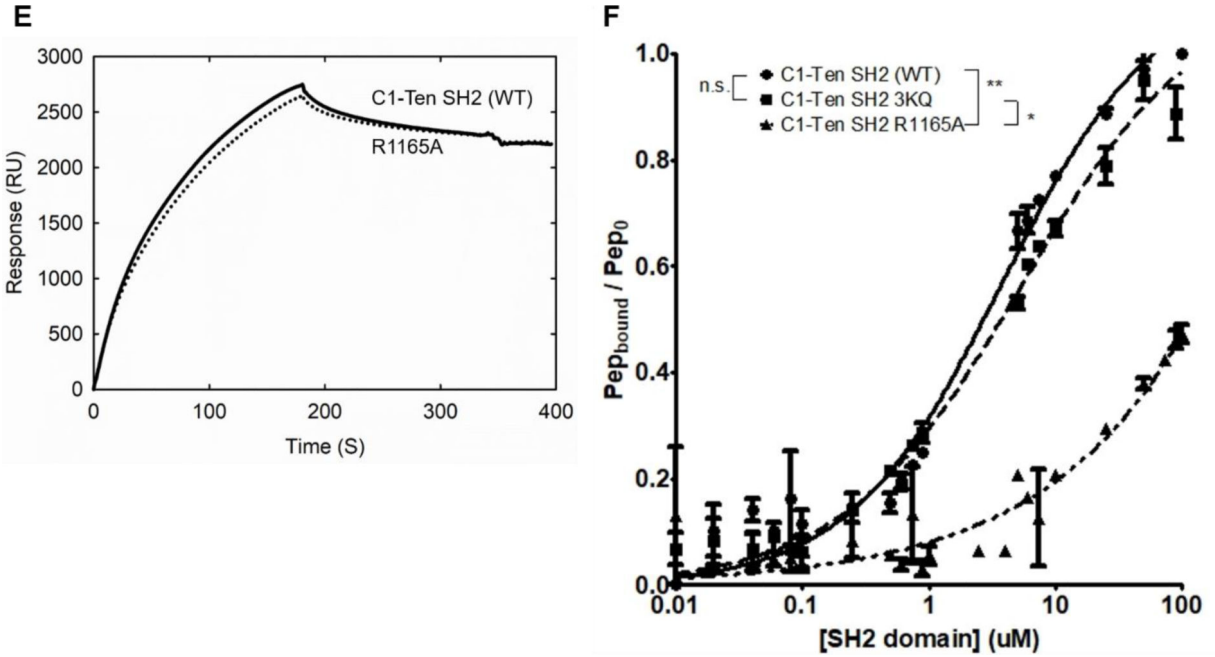
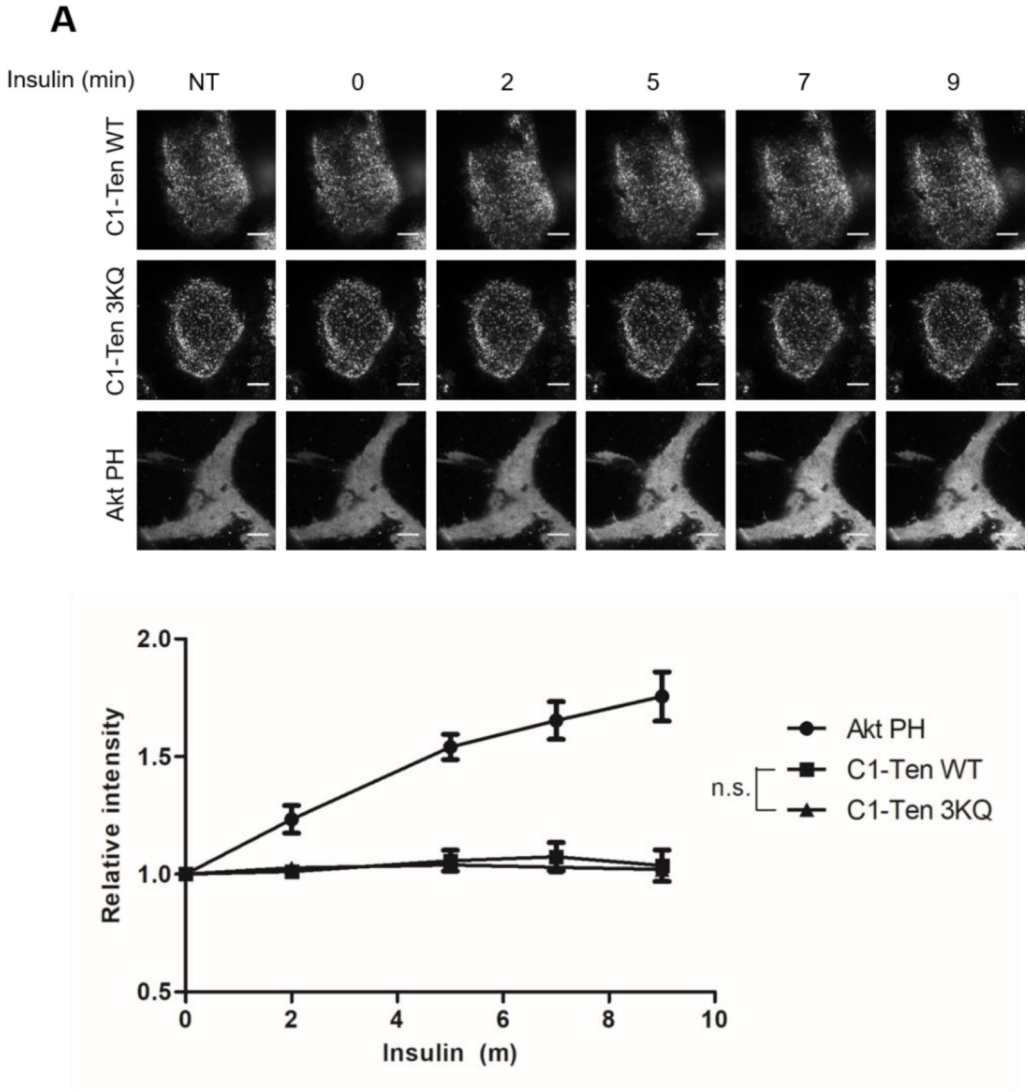


Figure 3. The K1142, K1155, and K1157 residues of the C1-Ten SH2 domain are involved in PtdIns(3,4,5)P_3 binding.

(A) Structure of the C1-Ten SH2 domain according to an electrostatic surface model. The model shows the pY-binding pocket containing R1165 and lipid-binding residues K1142, K1155, and K1157. Note that the putative lipid-binding residues are topologically distinct from the pY-binding pocket. (B) PtdIns(3,4,5)P_3 binding values of the cationic patch of C1-Ten SH2 domain as determined by SPR analysis. The WT, K1157Q, K1142Q/K1157Q, K1155Q/K1157Q, and K1142Q/K1155Q/K1157Q (3KQ) mutant C1-Ten SH2 domain was allowed to interact with POPC/POPS/PtdIns(3,4,5)P_3 (77:20:3) vesicles. (C) Determination of K_d for PtdIns(3,4,5)P_3 vesicles of the 3KQ mutation of C1-Ten SH2 domain by SPR analysis. The C1-Ten SH2 3KQ was injected at 5 μl/min at varying concentrations (10, 30, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 nM, from bottom to top) over the L1 chip coated with PtdIns(3,4,5)P_3 vesicles (left). Binding isotherms were generated from R_{eq} versus concentration (P_o) of the C1-Ten SH2 3KQ plot (right). Solid lines indicate the theoretical curve constructed from R_{max} (941 ± 79) and K_d (1260 ± 128) values. (D) PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 binding of the WT and 3KQ C1-Ten SH2 by SPR analysis. The WT and 3KQ mutant C1-Ten SH2 domains were allowed to interact with POPC/POPS/PtdInsP (77:20:3) vesicles. (E) PtdIns(3,4,5)P_3 binding values for the C1-Ten SH2 domain in WT and R1165A mutant as determined by SPR analysis. WT and R1165A C1-Ten SH2 were allowed to interact with POPC/POPS/PtdIns(3,4,5)P_3 (77:20:3) vesicles. (F) Affinity of WT, 3KQ, and R1165A C1-Ten SH2 to a pY-containing peptide (F-Ahx-NEpYDNV; 50 nM) was measured by fluorescence anisotropy. WT (K_d = 2.5 ± 1.3 μM) and 3KQ (K_d = 3.6 ± 1.4 μM) showed comparable affinities, whereas R1165A exhibited a ~36-fold decrease in affinity (K_d = 90.9 ± 11.4 μM). Pep_{bound} and Pep_o indicate the concentration of bound peptide and total peptide, respectively. *P < 0.05, **P < 0.01, n.s., not significant.



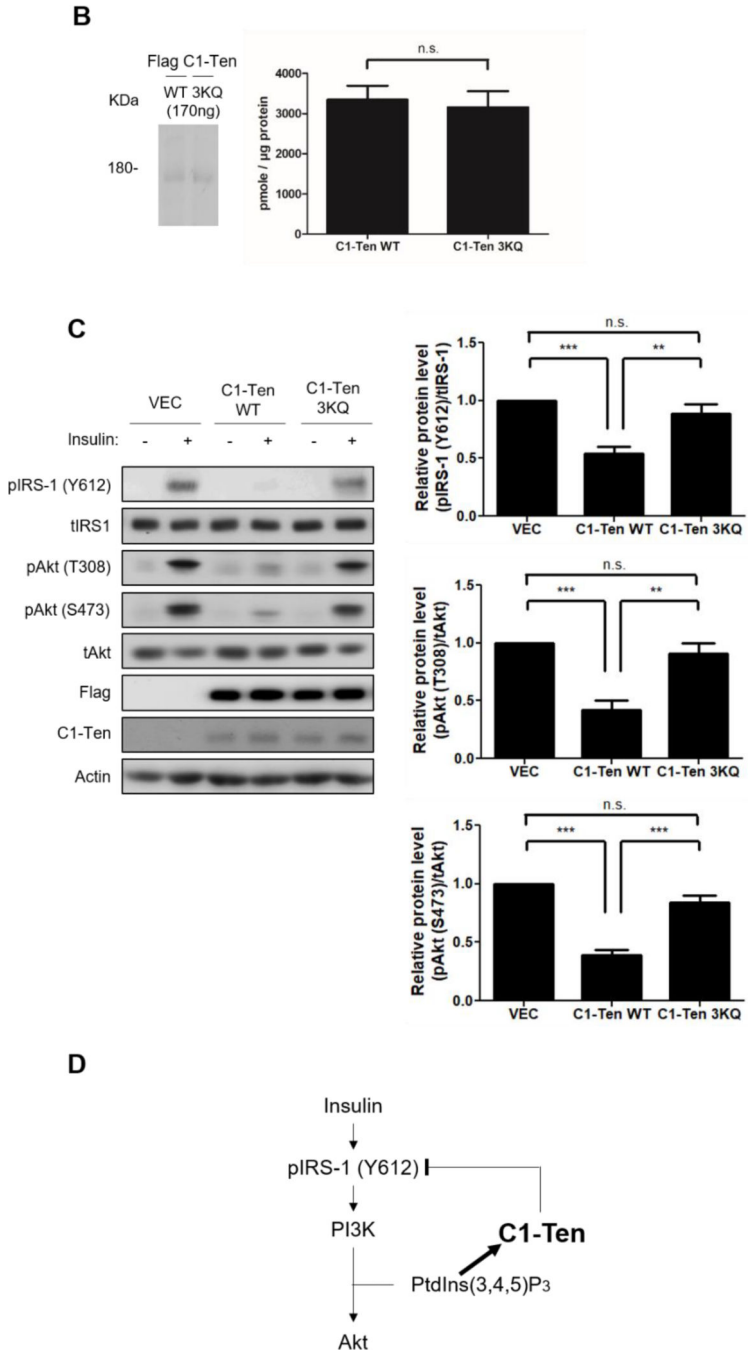


Figure 4. The PtdIns(3,4,5)P_3 binding motif of C1-Ten is essential for cellular phosphatase activity.

(A) Localization of WT and 3KQ mutant C1-Ten in HEK293 cells was determined by TIRF microscopy. HEK293 cells transfected with EGFP-tagged C1-Ten WT, C1-Ten 3KQ mutant, and Akt PH domain (as positive control) were starved for 18 h. The time elapsed after stimulation with 10 nM insulin is shown on each image. Scale bars: 20 μm. Fluorescence intensity changes at different times after insulin stimulation are quantified. Graphs represent average fluorescence intensity from each cell normalized to the non-stimulation time point. Data are presented as mean ± SEM from three independent experiments. n.s., not significant.

(B) *In vitro* PTP activity of WT and 3KQ C1-Ten. Ponceau S staining revealed the quantity, integrity, and purity of full-length WT and 3KQ C1-Ten (left). A malachite green assay was performed with C1-Ten WT and 3KQ mutant and 400 μM EEEEpYEEEE peptide. Assays were repeated three times in duplicate. Data are presented as mean ± SEM (n = 3) (right). (C) Effect of PtdIns(3,4,5)P_3 binding on IRS-1-mediated insulin signaling in HEK293 cell lines stably expressing Flag-C1-Ten WT or Flag-C1-Ten 3KQ. Cells were serum-starved for 18 h and stimulated with 5 nM insulin for 5 min. Cell lysates were subjected to immunoblotting with the indicated antibodies. Data are presented as mean ± SEM (n = 4). $^{**}P < 0.01$, $^{***}P < 0.001$. (D) A proposed model: interaction of C1-Ten with PtdIns(3,4,5)P_3 mediates IRS-1 dephosphorylation.