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Insulin/insulin-like growth factor (IGF) stimulation abrogates an association between a deubiquitinating enzyme USP7 and insulin receptor substrates (IRSs) followed by proteasomal degradation of IRSs

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ABSTRACT

Insulin receptor substrates (IRSs) play central roles in insulin/insulin-like growth factor (IGF) signaling and mediate a variety of their bioactivities. IRSs are tyrosine-phosphorylated by activated insulin receptor/IGF-I receptor tyrosine kinase in response to insulin/IGF, and are recognized by signaling molecules possessing the SH2 domain such as phosphatidylinositol 3-kinase (PI3K), leading to the activation of downstream pathways. Recent studies have suggested that degradation of IRSs by the ubiquitin–proteasome pathway leads to impaired insulin/IGF signaling, but the precise mechanism underlying the process is still unclear. In this study, we identified deubiquitinating enzyme ubiquitin specific protease 7 (USP7) as an IRS-2-interacting protein and demonstrated that deubiquitinase activity of USP7 plays important roles in IRS-2 stabilization through the ubiquitin–proteasome pathway. In addition, insulin treatment dissociated USP7 from IRS-2, leading to degradation of IRS-2. This dissociation was prevented by treatment with LY294002, a PI3K inhibitor, indicating that insulin activation of the PI3K pathway leads to dissociation of IRS-2 from USP7 and IRS-2 degradation. We obtained similar results for IRS-1 in cells treated with insulin and for IRS-2 in cells treated with IGF-I. Taken together, this is the first report demonstrating that USP7 is an IRS-1/2 deubiquitinating enzyme forming a negative feedback loop in insulin/IGF signaling.

1. Introduction

Insulin stimulates glucose uptake and utilization in muscle and adipose tissues and suppresses glucose production in liver, controlling the maintenance of glucose homeostasis [1]. On the other hand, insulin-like growth factors (IGFs) induce cell proliferation, differentiation and survival of various cell-types, coordinating the growth and development *in vivo* [2]. Insulin receptor substrate (IRS)-1 and IRS-2 are important mediators of insulin/IGF signaling. Tyrosine residues of IRSs are phosphorylated by insulin receptor/IGF-I receptor tyrosine kinase in response to insulin/IGFs, followed by the association with SH2 domain-containing proteins such as phosphatidylinositol 3-kinase (PI3K) and Grb2. These events cause the activation of the downstream kinases such as Akt (also called protein kinase B) and extracellular signal-regulated kinase (Erk), leading to the induction of a variety of insulin/IGF bioactivities [1,2].

Protein levels of IRSs often dramatically change under various conditions such as diabetes [3–6]. Recent studies have shown that several ubiquitin ligases ubiquitinate IRSs and induce their

Abbreviations: IRS, insulin receptor substrate; IGF, insulin-like growth factor; PI3K, phosphatidylinositol 3-kinase; SH2, src homology region 2; USP7, ubiquitin specific protease 7; Grb2, growth factor receptor-bound protein 2; Erk, extracellular signal-regulated kinase; DUB, deubiquitinating enzymes; IRSAP, IRS-associated protein; PVDF, polyvinylidene difluoride; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; KI, kallikrein-inactivating; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MATH, meprin associated TRAF homology; MEK, mitogen-activated protein kinase kinase; CIAP, calf intestinal alkaline phosphatase.

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degradation by the 26 S proteasome [7–10]. In addition, it is well known that prolonged insulin/IGF stimulation induces ubiquitination of IRSs followed by their degradation, which is an important mechanism for the desensitization of insulin/IGF signaling [11,12]. Protein ubiquitination is regulated not only by ubiquitin ligases but also by deubiquitinating enzymes (DUB), however, it is unknown how IRS-1/2 are deubiquitinated.

We have previously shown that IRS-1/2 generally form high-molecular-mass complexes over 1000 kDa containing IRS-associated proteins (IRSAPs) that modulate insulin/IGF signals [13]. In this study, we show that deubiquitinating enzyme USP7 is one of the IRSAPs and it prevents IRS-1/2 ubiquitination and proteasomal degradation. We also demonstrate that insulin or IGF-I treatment stimulates USP7 dissociation from IRSs via activation of PI3K pathway, leading to degradation of IRSs. These results reveal USP7 as an IRS-1/2 deubiquitinating enzyme, forming a negative feedback loop in insulin/IGF signaling.

2. Materials and methods

2.1. Cell culture and reagents

HEK293 cells, HEK293T cells, FRTL-5 thyroid cells, and L6 myoblasts were cultured and transfected as previously described [13–15]. H4IIE hepatoma cells were cultured with Dulbecco's modified Eagle medium containing 10% fetal bovine serum.

2.2. Immunoprecipitation and immunoblotting analysis

These procedures were carried out as described previously [13], except for modifications as described in Supplementary methods.

2.3. LC–MS/MS analysis

LC–MS/MS analysis was performed as described previously [16].

2.4. Purification of GST fused proteins and GST pull-down analysis

These procedures were performed as previously described [17], except for modifications as described in Supplementary methods.

2.5. Far-Western blotting analysis

Proteins were resolved by SDS–PAGE, transferred to polyvinylidene difluoride (PVDF) membranes. After the membranes were incubated with blocking buffer (10 mM Tris–HCl pH 7.2, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 3% bovine serum albumin) for 30 min, they were incubated with blocking buffer containing 1 mg/ml GST or GST-fused proteins overnight, followed by the immunoblotting with anti-GST antibody conjugated with horseradish peroxidase.

2.6. Subcellular fractionation into cytoplasm and nuclei

Cells were lysed in fractionation buffer (10 mM Tris–HCl pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% TritonX-100, 20 μg/ml calpain inhibitor I (Roche, Basel, Switzerland), 500 μM Na_3VO_4, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 20 μg/ml phenylmethylsulfonyl fluoride (PMSF), and 100 kallikrein-inactivating (KI) U/ml aprotinin). The cell lysates were passed through a 25 G needle syringe four times and centrifuged at 4000*g* for 10 min at 4 °C. The supernatants were saved as the cytoplasmic fraction. The pellets were resuspended in fractionation buffer without TritonX-100, sonicated three times for 5 s, and centrifuged at 15,000*g* for 10 min at 4 °C. The supernatants were saved as the nuclei fraction. The protein assay of the each fractions was performed using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of proteins subjected to immunoprecipitation and immunoblotting analysis.

2.7. RNA interference

This procedure was performed as previously described [18], except for modifications as described in Supplementary methods.

2.8. In vitro tyrosine phosphorylation and dephosphorylation of IRSs

Phosphorylation/de-phosphorylation of IRSs with insulin receptor kinase/alkaline phosphatase was performed as described in Supplementary methods.

Additional experimental materials and methods are described in Supplementary materials.

3. Results

3.1. USP7 associates with IRS-1/2

In the initial screening to identify IRSs-associated proteins, FLAG-IRS-1 or FLAG-IRS-2 was over-expressed in HEK293 cells, and the cell lysates were subjected to the immunoprecipitation with anti-FLAG antibody-conjugated beads. Mixtures of co-immunoprecipitated proteins were digested with lysyl endopeptidase, and then subjected to LC–MS/MS analysis. As a result, we identified a deubiquitinating enzyme USP7 as one of the IRSs-associated proteins.

Co-immunoprecipitation and immunoblotting analysis validated the initial screening and confirmed that USP7 associated with IRS-1/2 in HEK293T cells over-expressing IRS-1/2 (Fig. 1A and B). We also found that USP7 associated with endogenous IRS-1 and IRS-2 in H4IIE hepatoma cells (Fig. 1C) and in L6 myoblasts (Fig. S1).

We then determined the regions of each protein that are required for their association. Pull-down analysis using USP7 deletion mutants revealed that the MATH domain of USP7 was responsible for the association with IRS-1/2 (Fig. 1D–F). Far-western blotting analysis using the MATH domain of USP7 fused with GST as a probe revealed that this domain directly binds to IRS-1/2 (Fig. 1G). On the other hand, pull-down analysis using IRS-1/2 deletion mutants indicated that USP7 binds to multiple regions in IRS-1/2 (Figs. S2A and B). We also investigated the subcellular localization of USP7-IRS-2 complex. Co-immunoprecipitation analysis after subcellular fractionation into cytoplasm and nuclei indicated that a large part of the complex is detected in the cytoplasm in H4IIE cells (Fig. 1H).

3.2. USP7 deubiquitinates IRS-2 and prevents its proteasomal degradation

We next investigated the physiological roles of USP7 for IRS-2. We hypothesized that USP7 deubiquitinates IRS-1/2 and prevents their proteasomal degradation. In HEK293 cells, over-expression of USP7 resulted in the increase in IRS-2 protein levels (Fig. 2A). By contrast, over-expression of dominant negative USP7 (USP7 CS, where the single cysteine residue in the catalytic center was substituted with serine [19]) decreased IRS-2 protein levels, an effect prevented by pre-treatment with a proteasome inhibitor MG132 (Fig. 2B, left panel). Importantly, over-expression of dominant negative USP7 CS increased ubiquitination of IRS-2 in the presence of MG132 (Fig. 2B, right panel). Moreover, in H4IIE cells,

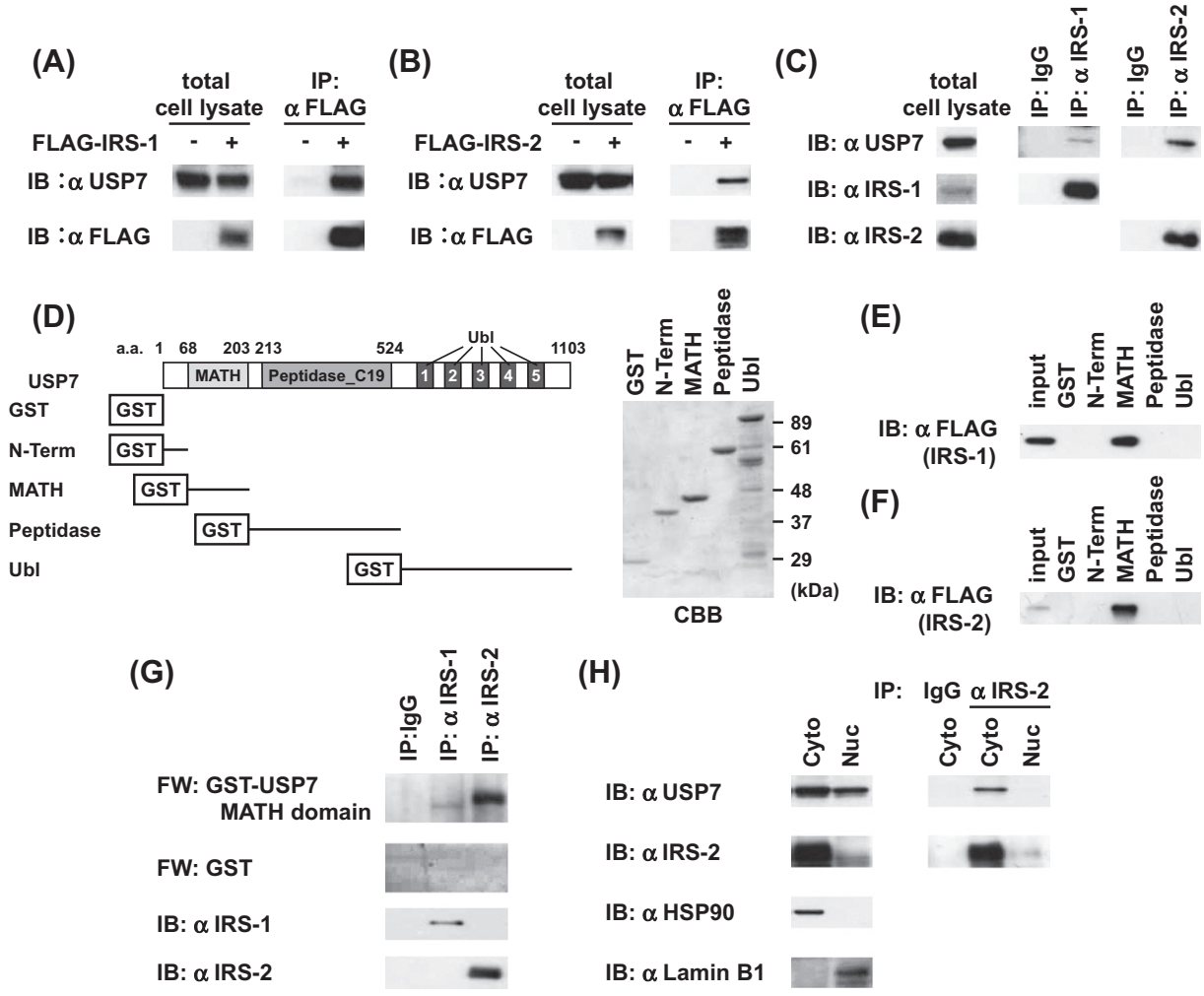

Fig. 1. The association of USP7 with IRS-1/2. (A–C) HEK293T cells over-expressing FLAG-IRS-1 (A) or FLAG-IRS-2 (B), or H4IIE cells (C) were cultured under serum-free conditions for 24 h. The lysates were subjected to immunoprecipitation (IP) and immunoblotting (IB) analyses. (D) Schematic representation of rat USP7 and its deletion mutants used in the following experiments. MATH, meprin associated TRAF homology domain. Ubl, ubiquitin-like domain. The purity of GST-fused proteins was validated by CBB staining. (E and F) HEK293T cells over-expressing FLAG-IRS-1 (E) of FLAG-IRS-2 (F) were cultured under serum-free condition for 24 h. The lysates were subjected to pull-down analysis using GST-fused USP7 deletion mutants. Input, 3/100 volume of HEK293T cell lysates. (G) Lysates of serum-starved H4IIE cells were immunoprecipitated with anti-IRS-1/2 antibodies. The immunoprecipitates were resolved by SDS–PAGE and subjected to far- western blotting (FW) analyses using purified GST or GST-fused MATH domain of USP7 (upper panels) as probes, and immunoblotting analysis using anti-IRS-1/2 antibodies (lower panels). (H) Lysates of serum-starved H4IIE cells were fractionated into cytoplasm (Cyto) and nuclei (Nuc), followed by immunoprecipitation with anti-IRS-2 antibody. The fractions and immunoprecipitates were subjected to immunoblotting analysis. Lamin B1, a nuclear protein. HSP90, a cytoplasmic protein. Data are representative of at least three independent experiments.

knockdown of USP7 decreased IRS-2 protein levels (Fig. 2C). All of these results support our hypothesis that USP7 prevents IRS-2 degradation by deubiquitinating activity. In addition, we found that over-expression of dominant-negative USP7 CS decreased IRS-1 protein levels (Fig. 2D), indicating that USP7 also plays roles in the regulation of IRS-1 protein levels.

3.3. USP7 dissociates from IRS-1/2 in response to insulin/IGF stimulation, leading to the proteasomal degradation of IRS-1/2

In several cell-types, insulin/IGF stimulation causes the association of some ubiquitin ligases with IRS-1/2, followed by their proteasomal degradation [7,8,10], which is thought to be one of the feedback inhibition mechanisms in insulin/IGF signaling [7,8,10,11]. We examined the effects of insulin stimulation on the association of USP7 with IRS-2 in H4IIE cells. Surprisingly, co-immunoprecipitation analysis revealed that the amounts of USP7 bound to IRS-2 were dramatically decreased within 1 min after insulin stimulation (Fig. 3A). We also found that insulin stimulation gradually decreased electrophoretic mobility of IRS-2 at 1–60 min after insulin stimulation reflecting its phosphorylation state [20] followed by the decrease in IRS-2 protein levels (Fig. 3A). In addition, we found that USP7 also dissociated from IRS-1 in response to insulin stimulation followed by decrease of IRS-1 protein levels (Fig. 3B). In FRTL-5 cells, IGF-I stimulation dissociated USP7 from IRS-2 followed by decrease of IRS-2 protein levels (Fig. 3C). These results clearly indicate that the rapid dissociation of USP7 from IRS-1/2 in response to insulin/IGF-I stimulation may enable the subsequent ubiquitination and proteasomal degradation of IRS-1/2.

3.4. USP7 dissociates from IRS-2 followed by its degradation through activation of the PI3K pathway in response to insulin stimulation

Finally, we investigated molecular mechanisms of the USP7 dissociation. Insulin-induced dissociation was prevented in the presence of LY294002, a PI3K inhibitor, but not of PD98059, a MEK inhibitor (Fig. 4A and B). We also performed pull-down analysis using the MATH domain of USP7 as a bait and IRS-2 derived from H4IIE cells treated with or without insulin as a prey. The result

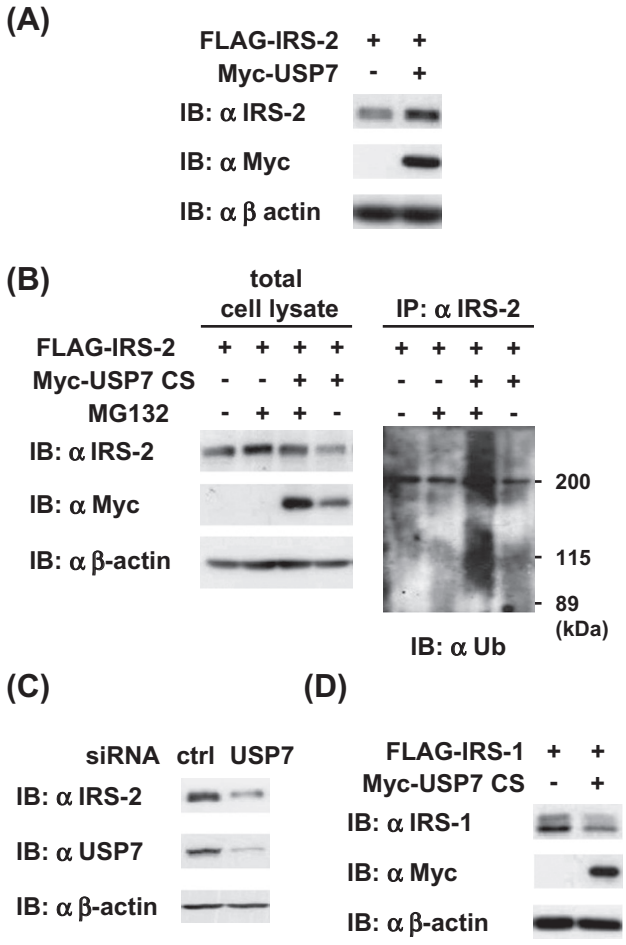

Fig. 2. Roles of USP7 in the regulation of IRS-1/2 protein levels. (A) HEK293 cells over-expressing indicated proteins were cultured under serum-free condition for 24 h. The cell lysates subjected to immunoblotting analysis. β-Actin, loading control. (B) HEK293 cells over-expressing FLAG-IRS-2 and myc-USP7 CS (dominant-negative mutant) were cultured under serum-free condition for 24 h, and treated with 10 μM MG132 for the last 6 h. The lysates were subjected to immunoprecipitation and immunoblotting analysis. (C) H4IIE cells transfected with control RNA or USP7 siRNA were cultured under serum-free condition for 24 h. The lysates were subjected to immunoblotting analysis. (D) HEK293 cells over-expressing FLAG-IRS-1 and myc-USP7 CS were cultured under serum-free condition for 24 h. The lysates were subjected to immunoprecipitation and immunoblotting analysis. Data are representative of at least three independent experiments.

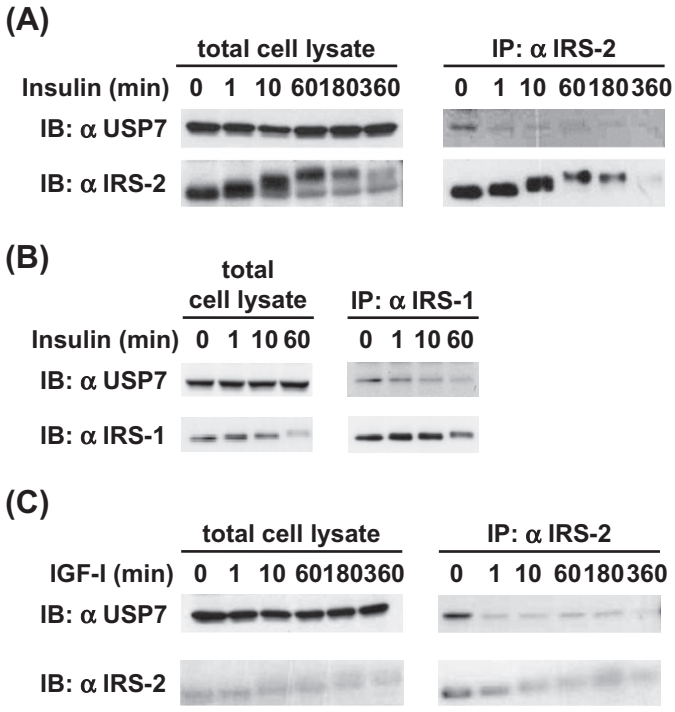

Fig. 3. The dissociation of USP7 from IRS-1/2 in response to insulin/IGF stimulation. (A–C) Serum-starved H4IIE cells (A and B) or FRTL-5 cells (C) were treated with 100 nM insulin (A and B) or 100 ng/ml IGF-I (C) for the indicated durations. The lysates were subjected to immunoprecipitation with anti-IRS-1 antibody (B) or anti-IRS-2 antibody (A and C) and immunoblotting analysis.

indicated that insulin stimulation decreased the affinity of IRS-2 for the MATH domain, and this decrease was prevented by the treatment of cells with LY294002 (Fig. 4C), suggesting that phosphorylation of IRS-2 via PI3K pathway triggers the USP7 dissociation.

IRS-2 is phosphorylated by some serine/threonine kinases downstream of PI3K [21]. To examine the roles of the serine/threonine phosphorylation of IRS-2 in the association with the MATH domain, IRS-2 derived from cells stimulated with serum was treated with alkaline phosphatase *in vitro*, and then subjected to the pull-down analysis. The result indicated that phosphorylation of IRS-2 decreased the affinity of IRS-2 for the MATH domain (Fig. 4D). In contrast, tyrosine phosphorylation of IRS-2 by insulin receptor kinase *in vitro* prior to the pull down analysis did not affect the affinity of IRS-2 for the MATH domain (Fig. 4E).

4. Discussion

In this study, we identified deubiquitinating enzyme USP7 as an IRSs-associated protein and examined the physiological significance of this association in the regulation of insulin/IGF signaling. Based on our results, USP7 deubiquitinated IRS-1/2 resulting in increase in their stabilization. In addition, insulin/IGF treatment caused dissociation of USP7 from IRS-1/2 followed by induction of their degradation, suggesting that USP7 formed a negative feedback loop in insulin/IGF signaling.

Since the human genome encodes 95 different DUBs [22], many fewer than the more than 600 E3 ligase molecular species [23], DUBs are considered to be low specificity enzymes. Actually, numerous proteins have been identified as potential substrates of USP7 [24] including p53, a tumor suppressor, and Mdm2, an E3 ligase of p53 [19,25]. USP7 associates with these proteins through the MATH domain and deubiquitinates them [26,27]. In addition, USP7 regulates these protein levels through alteration of affinity for these proteins [28] and phosphorylation of serine residues within the MATH domain-binding motif of p53 or Mdm2 which abrogated binding to USP7 [29]. We demonstrated that USP7 directly associated with IRS-2 through the MATH domain (Fig. 1G), and that dephosphorylation of IRS-2 increases the interaction while tyrosine phosphorylation of IRS-2 by insulin receptor kinase did not increase the interaction (Fig. 4D and E), suggesting the common mechanisms for IRS-2 and p53/Mdm2 that serine/threonine phosphorylation of their binding regions reduces their USP7 association. Different phosphorylation status of each substrate may enable USP7 to regulate their protein stabilities specifically.

Recent studies have shown that several serine/threonine kinases downstream of PI3K such as Akt, GSK-3, mTOR and p70S6K phosphorylate IRS-1/2, and the phosphorylation triggers IRS-1/2 ubiquitination and degradation [21]. In addition, particular serine residues of IRS-1 such as S307, S527, S636 and S639 were reported to be key phosphorylation sites required for IRS-1 degradation [8,21]. In the present study we demonstrated that IRS-2 modification induced by insulin stimulation via PI3K pathway activation releases USP7 from IRS-2 (Fig. 4B). Taken together with the data

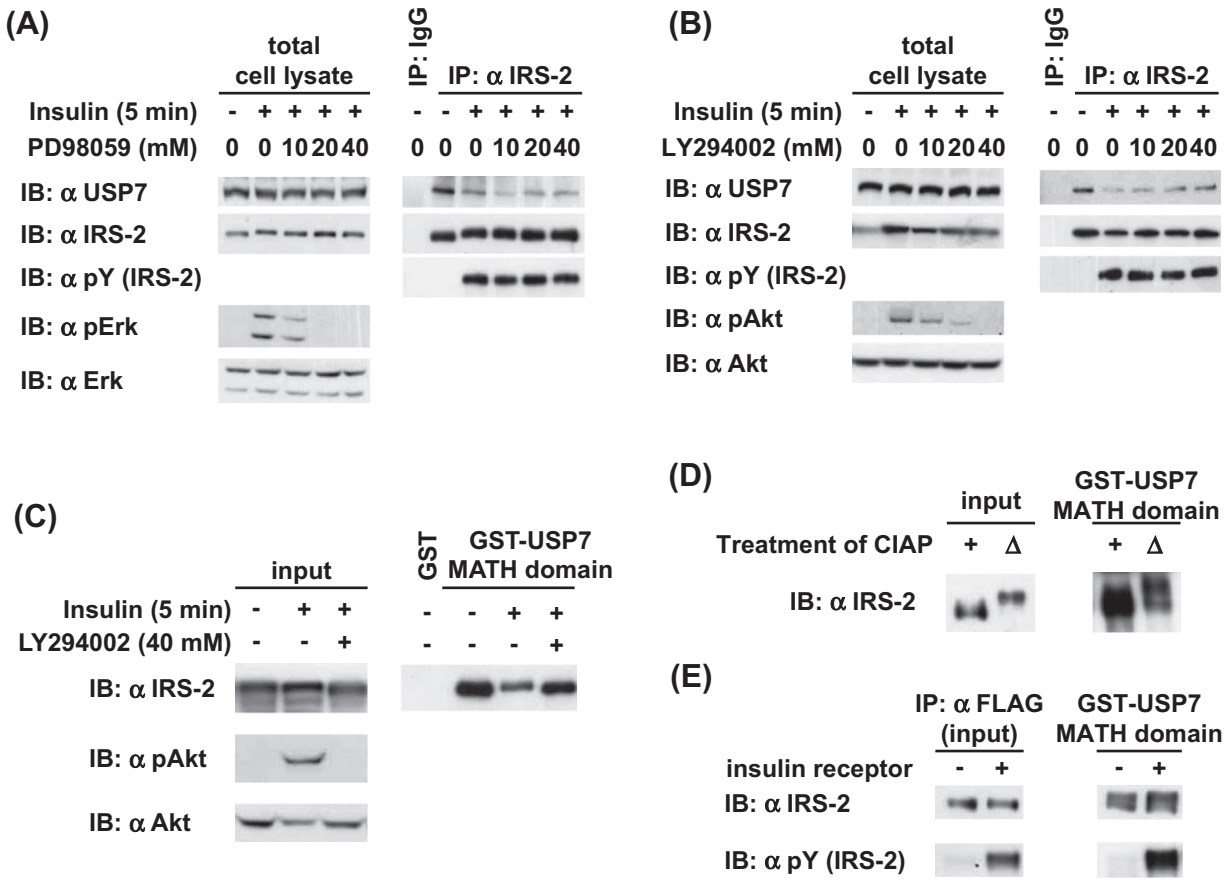

Fig. 4. The molecular mechanisms of dissociation of USP7 from IRS-2. (A and B) Serum-starved H4IIE cells were pretreated with indicated concentrations of PD98059 (A) or LY294002 (B) for 30 min. Cells were treated with 100 nM insulin for 5 min. The lysates were subjected to immunoprecipitation and immunoblotting analysis. (C) Serum-starved H4IIE cells were pretreated with 40 μM LY294002 for 30 min and then treated with 100 nM insulin for 5 min. The lysates were subjected to pull-down analysis using GST-fused MATH domain of USP7. (D) Lysates of HEK293T cells over-expressing FLAG-IRS-2 were subjected to immunoprecipitation with anti-FLAG antibody-conjugated beads. The precipitates on beads were treated with calf intestine alkaline phosphatase (CIAP) or heat-denatured CIAP (Δ), washed with lysis buffer, and then eluted with FLAG peptide. Eluates were subjected to pull-down analysis. (E) HEK293T cells over-expressing FLAG-IRS-2 were cultured under serum-free condition for 24 h. The lysates were subjected to immunoprecipitation with anti-FLAG antibody-conjugated beads. Precipitates were subjected to *in vitro* tyrosine phosphorylation assay using insulin receptor kinase, washed with lysis buffer, and then eluted with FLAG peptide. Eluates were subjected to pull-down analysis. Data are representative of at least three independent experiments.

indicating serine phosphorylation of IRS-1/2 triggered dissociation of USP7 from IRS-1/2 (Fig. 4D and E), these results suggest that there are key phosphorylation site(s) and kinase(s) downstream of PI3K involved in IRS-1/2 degradation and required for dissociation of USP7 from IRS-1/2.

The ubiquitin–proteasome-mediated degradation of IRS-1/2 induced by insulin/IGF stimulation is a component of the cellular feedback inhibition that modulates insulin/IGF action by limiting the magnitude and duration of insulin/IGF signals [21]. We demonstrated that USP7 associates with IRS-1/2 and prevents proteasomal degradation of IRS-1/2 (Figs. 1C and 2B and D). Moreover, we showed that insulin/IGF stimulation releases USP7 from IRS-1/2 (Fig. 3A and B). Taking these data together, we concluded that the dissociation of USP7 from IRS-1/2 induced by insulin/IGF stimulation may be involved in the feedback inhibition mechanism of insulin/IGF signaling by allowing the ubiquitin dependent degradation of IRS-1/2.

It was reported that prolonged insulin/IGF stimulation decreases IRS-1/2 levels in several cell-types, whereas the detailed time courses of their decreases are often different for different IRSs isoforms as well as different cell-types [21,30]. In addition to insulin/IGF stimulation, various stimulation factors and physiological conditions affect IRS-1/2 levels. Obesity and inflammatory cytokines secreted from adipose tissues induce IRS-1/2 degradation in insulin target tissues [7], which is thought to be one of the mechanisms for the onset of insulin resistance and type 2 diabetes. We had previously reported that dietary protein deprivation increased IRS-1/2 in liver, leading to the potentiation of hepatic insulin action [31]. Unloading stress induces IRS-1 degradation in muscle, which causes unloading-mediated muscle atrophy [9]. So far, several E3 ligases had been identified as responsible factors for the degradation of IRS-1/2 in some conditions. However, since our study revealed USP7 as a novel DUB targeting IRS-1/2 and we recently identified other DUBs as IRSAPs (data not shown), there is an urgent need to elucidate roles of DUBs containing USP7 in the regulation of IRS-1/2 protein levels in IRSs-isoform-, cell-type-, and stimulation factor-dependent manner.

Protein degradation through proteasome-system is regulated by the balance between ubiquitination and deubiquitination of target proteins. Several E3 ligases have been shown to ubiquitinate IRS-1/2. On the other hand, there is no study showing how IRS-1/2 are deubiquitinated. To elucidate the degradation mechanism of IRS-1/2, it is necessary to reveal not only how E3 ligases ubiquitinate IRS-1/2 but also how DUB (s) deubiquitinate IRS-1/2.

In conclusion, we identified deubiquitinating enzyme USP7 as a novel regulator of IRS-1/2 stability and showed that activation of the PI3K pathway induced by insulin leads to dissociation of IRS-1/2 from USP7 and degradation of IRS-1/2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.093.

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