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Clk2 and B56β mediate insulin regulated assembly of the PP2A phosphatase holoenzyme complex on Akt

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Summary

Akt mediates important cellular decisions involved in growth, survival and metabolism. The mechanisms by which Akt is phosphorylated and activated in response to growth factors or insulin have been extensively studied, but the molecular regulatory components and dynamics of Akt attenuation are poorly understood. Here, we show that a downstream target of insulin induced Akt activation, Clk2, triggers Akt dephosphorylation through the PP2A phosphatase complex. Clk2 phosphorylates the PP2A regulatory subunit B56β (PPP2R5B, B'β) which is a critical regulatory step in the assembly of the PP2A holoenzyme complex on Akt leading to dephosphorylation of both S473 and T308 Akt sites. Since Akt plays a pivotal role in cellular signaling, these results have important implications for our understanding of Akt regulation in many biological processes.

Introduction

One of the central mediators of growth factor and insulin signaling is the protein kinase Akt. Following stimulation of receptor tyrosine kinases and the PI3K pathway, Akt is rapidly phosphorylated on the activation loop residue T308 by PDK1 and in the hydrophobic region on S473. Phosphorylation of both T308 and S473 are well known to be required for full induction of Akt kinase activity (Alessi et al., 1996; Chan et al., 1999; Sarbassov et al., 2005). Once active, Akt targets a series of substrates to mediate biological processes involved in cell growth, survival, and metabolism (Manning and Cantley, 2007). Importantly, Akt activation is dynamic and though the mechanisms leading to Akt phosphorylation are well studied, little is known about the regulatory components of Akt dephosphorylation. Previous studies have identified that PP2A, PP1 and the PHLPP phosphatases can dephosphorylate the T308 and S473 sites on Akt (Beaulieu et al., 2005; Bertoli et al., 2009; Brognard et al., 2007; Gao et al., 2005; Ni et al., 2007; Padmanabhan et al., 2009; Rocher et al., 2007; Van Kanegan et al., 2005; Vereshchagina et al., 2008). However, the regulation of Akt dephosphorylation through these serine and threonine phosphatases remains enigmatic.

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Supplemental Information

Supplemental information includes Supplemental Experimental Procedures and four figures.

The authors declare no conflict of interest.

Results

Clk2 controls attenuation of Akt phosphorylation

A biological system where the regulation of Akt phosphorylation/activity is of important physiologic significance is the hepatic metabolic response to insulin fluctuations during fasting and feeding. In the liver, insulin activation of Akt plays an important role as the central regulator of glucose metabolism: Akt activates glycogen synthesis and glycolysis and suppresses gluconeogenesis (Cho et al., 2001; Cross et al., 1995; Nakae et al., 2001; Ono et al., 2003; Whiteman et al., 2002). In this context, we decided to investigate the mechanisms of insulin regulation and Akt dynamics using *in-vivo* and *in-vitro* models. As shown in Fig. 1A and Supp.Fig1A, immediately upon refeeding (after a fast) elevation of serum insulin levels caused rapid activation of the hepatic insulin receptor and Akt phosphorylation. Interestingly, at longer refeeding time points (8 and 24 hrs) hepatic Akt phosphorylation on both T308 and S473 became down regulated to nearly the level of fasted mice, even though the levels of serum insulin and insulin receptor tyrosine phosphorylation were high. We have recently identified that Cdc2-like kinase 2 (Clk2) is a downstream target of Akt signaling that mediates insulin suppression of hepatic glucose output (Rodgers et al., 2010). We noted a temporal correlation between attenuation of Akt phosphorylation and induction of Clk2 protein in the liver during the refeeding phase. Thus, following Akt phosphorylation, Clk2 activity is induced and stabilized, taking several hours to increase the protein levels (Fig.1A). This correlation between Clk2 protein induction and Akt attenuation also occurred in isolated primary hepatocytes following insulin stimulation (Supp.Fig.1B). Together, these findings led us to closely investigate if Clk2 could affect Akt phosphorylation.

To test this, we first knocked-down Clk2 protein levels *in vivo* in mouse liver using tailvein delivery of adenovirus. As expected, in the fasted state control mice showed low levels of Akt phosphorylation that was induced by refeeding and elevation of serum insulin (Fig.1B). However, in mice with Clk2 shRNA, Akt phosphorylation was strikingly increased in the fasted state and remained elevated throughout refeeding (Fig.1B). Induction of Akt phosphorylation by Clk2 shRNA in the liver was independent of serum insulin levels (Fig. 1B) and was not observed in other insulin responsive tissues such as white adipose and skeletal muscle (data not shown). Consistent with these results, adenovirus mediated expression of Clk2 in mouse liver caused the opposite effects and resulted in suppression of Akt phosphorylation, independent of serum insulin levels in the fasted and refed conditions (Fig.1C). Interestingly, this effect was entirely dependent on Clk2 kinase activity, expression of a kinase dead Clk2 (K192R) did not decrease but elevated levels of Akt phosphorylation (Fig.1C). In this case, Clk2 K192R may act as a dominant negative, mimicking the effects of Clk2 shRNA. These data demonstrate that hepatic Akt phophorylation can be controlled by *in vivo* modulation of Clk2 protein, suggesting that the insulin-mediated induction of Clk2 during refeeding plays a role in attenuation of Akt phosphorylation in the liver. This effect of Clk2 appears to be specific for Akt signaling, as AMPK signaling which is also regulated in the liver by fasting and feeding is not effected by Clk2 (Supp.Figs. 1C and 1D).

Next, we used mouse primary hepatoctyes to determine if Clk2 could regulate Akt phosphorylation in a cell autonomous manner. Following stimulation and removal of insulin from hepatocytes, Akt phosphorylation was quickly induced, then slowly started to decrease and is near baseline levels 2 hours after removal of insulin, coinciding with the point of maximal Clk2 induction in control cells (Fig.1D). Knock-down of Clk2 did not have a large effect on Akt phosphorylation in the basal state nor at early time points of insulin stimulation in this cell culture model. However, in the attenuation phase of insulin signaling (2 hours after insulin withdrawn), Akt phosphorylation remained elevated (Fig.1D). Consistent with the *in vivo* results, adenovirus mediated expression of Clk2 in hepatocytes

strongly decreased Akt phosphorylation (Supp.Fig.1E). Together, these results in primary hepatocytes indicate that the suppressive effects of Clk2 on Akt phosphorylation occur in a cell autonomous manner. Because Clk2 knockdown most strongly affected the attenuation phase of Akt signaling, these data suggested that Clk2 was not interfering with Akt phosphorylation *per se*, but Clk2 might be affecting the rate of Akt dephosphorylation.

B56β mediates PP2A-Akt interaction

Several phosphatases have been shown to regulate Akt dephosphorylation, among them PP2A is the most widely implicated. Treatment of hepatoctyes with okadaic acid (OA) following insulin stimulation strongly blocks attenuation of Akt phosphorylation, suggesting that PP2A may be an Akt phosphatase in the attenuation phase (Fig.2A). PP2A is a serine/threonine phosphatase formed by a heterotrimeric (also termed holoenzyme) complex of three subunits: scaffold (A), regulatory (B), and a catalytic subunit (C) (reviewed in (Virshup and Shenolikar, 2009)). Previous studies have shown that Akt phosphorylation can be regulated by virtue of the interaction between Akt and the PP2A catalytic subunit (from hereon PP2A-C) (Beaulieu et al., 2005;Bertoli et al., 2009). Since, it appeared both Clk2 and PP2A were regulating the rate of Akt dephosphorylation/attenuation, we tested if Clk2 affected the interaction between Akt and the PP2A complex. As shown in Figure 2B, immunoprecipitation of endogenous Akt from primary hepatoctyes pulled down PP2A-C as well as the scaffold subunit PP2A-Aα. In contrast, in hepatoctyes with Clk2 shRNA the interaction between Akt and PP2A-C and PP2A-Aα subunits was markedly decreased, suggesting a plausible mechanism to explain the effects of Clk2 on Akt phosphorylation through regulation of this interaction.

PP2A functions as a heterotrimeric complex but a large cellular pool also exists as free complex between the PP2A-C and PP2A-A subunits (termed PP2A core enzyme) (Kremmer et al., 1997). PP2A substrate specificity is determined by which regulatory-B subunit is incorporated into the PP2A holoenzyme complex (Virshup and Shenolikar, 2009). While there are four total genes encoding the PP2A-C and PP2A-A subunits (two apiece), there is considerably more diversity in the PP2A-B subunits. There are at least 4 gene families of PP2A-B subunits with around 20 genes total, most with multiple splice isoforms (list of PP2A components in Supp.Fig.2A). Recent work from several groups has centered around the B56 family of PP2A-B subunits, in particular B56β (also known as PPP2R5B, B'β, PR61B in mammals, pptr-1 in *C. elegans*, Wbd in *Drosophila*) which appears to mediate PP2A dephosphorylation of Akt (Padmanabhan et al., 2009; Rocher et al., 2007; Vereshchagina et al., 2008). To confirm these findings we expressed several HA-tagged B56 isoforms in HEK293 cells to determine which isoform would interact with Akt. Among the different B56 isoforms tested only B56β was able to co-immunoprecipitate endogenous Akt (Fig.2C), in addition Akt that was co-immunoprecipitated by B56β is almost completely de-phosphorylated (data not shown).

Next, we investigated if B56β could mediate the effects of Clk2 to alter the physical interaction between Akt and the PP2A core enzyme complex. Expression of HA-tagged B56β could co-immunoprecipitate the PP2A core enzyme complex along with Akt. Remarkably, coexpression of Clk2 increased the interaction between B56β and the PP2A core enzyme complex, but did not affect the interaction of B56β with Akt (Fig.2D and Supp.Fig.2B). In keeping with this data, knock-down of Clk2 in primary hepatoctyes did not alter the endogenous B56β-Akt interaction, but profoundly decreased the Akt interaction with PP2A-C and PP2A-Aα subunits (Fig.2B). In addition and similarly to Clk2 depletion, shRNA knock-down of B56β in primary hepatocytes (Supp.Fig 2C) disrupted the Akt interaction with PP2A-C and PP2A-Aα, indicating that B56β could mediate the interaction between Akt and the PP2A core enzyme complex (Fig.2B). Combined, these data suggest a

model in which Akt and B56β form a stable complex and Clk2 controls assembly of the PP2A core enzyme to this complex.

Clk2 phosphorylation of B56β is required for formation of Akt-PP2A complex

The fact that Clk2 is a protein kinase suggested that it may regulate the assembly of Akt-B56β and PP2A core enzyme complex via phosphorylation. Since we have previously shown that Clk2 interacts with Akt (Rodgers et al., 2010), we determined if Clk2 could phosphorylate the Akt-B56β complex. *In-vitro* kinase reactions using purified Clk2 kinase showed that Clk2 could not phosphorylate GST-tagged Akt1 (Supp.Fig.3A). In contrast, Clk2 could phosphorylate B56β but not the closely related B56α. Using deletion and mutation analysis we identified six serines (S32, S34, S44, S46, S47, S48) in the B56β N-terminus that were phosphorylated by Clk2 (Supp.Figs. 3B-D). In fact, several of these serines are predicted Clk2 phosphorylations sites (Fig.3A). Although single or several combinations of serine to alanine mutants were still phosphorylated (Supp.Fig.3B, C), mutating all of these serines to alanine completely abolished Clk2 phosphorylation of B56β (CPM: Clk2 phosphorylation mutant) (Figs. 3A, B). Additionally, B56β CPM was no longer phosphorylated in response to insulin stimulation in primary hepatocytes (Fig. 3C) and knock-down of Clk2 also blocked insulin stimulated phosphorylation of B56β (Fig.3D). Interestingly, alignment of the CPM residues with other B56 isoforms showed modest conservation (Fig.3A). Most of the CPM phosho-sites have been identified in phospho-proteome databases, however they have not been previously studied. In co-immunoprecipitation experiments B56β CPM showed decreased interaction with the PP2A core enzyme components, while a phospho-mimetic mutant CPM-D fully restored these interactions (Fig.3E and Supp.Figs.3E-F). In agreement with the results that co-expression of Clk2 stimulates the interaction of B56β with PP2A core enzyme complex, B56β CPM had profoundly decreased interaction with PP2A core enzyme complex and was completely resistant to Clk2 stimulation (Fig.3E and Supp.Fig.3E). Interestingly, B56β CPM displayed no defects in its interaction with Akt, indicating that the CPM sites are involved in the regulated assembly of the PP2A holoenzyme complex but not required for the Akt-B56β interaction. These data strongly supports our model proposed above that B56β and Akt are constitutively bound and Clk2 signals assembly of the PP2A core enzyme complex with the B56β-Akt complex through phosphorylation of the B56β N-terminus.

Clk2 and B56β are required for attenuation of insulin induced Akt phosphorylation

We next investigated the regulatory function of Clk2-induced assembly of the PP2A complex on Akt in response to insulin signaling as a potential mechanism for attenuation. Importantly, insulin in a time dependent-manner mimicked Clk2 to drive assembly of the PP2A core complex on Akt in primary hepatocytes. Furthermore, assembly of the PP2A holoenzyme complex on Akt correlated with progressive dephosphorylation of Akt (Fig.4A) and an increase in Akt associated phosphatase activity (Supp.Fig.4). Consistent with our previous results; B56β and Akt were in a constitutive complex, but upon insulin stimulation the PP2A core enzyme complex increased association with Akt. The insulin-induced assembly of Akt-PP2A holoenzyme complex required B56β and Clk2: primary hepatocytes with knock-down of Clk2 or B56β did not undergo insulin-regulated assembly of Akt-PP2A core complex (Fig.4B). Together, these data using endogenous proteins in primary hepatoctyes supported our proposed model that Akt and B56β are in a constitutive complex and insulin through Clk2 and B56β triggers assembly of the PP2A holoenzyme on Akt.

Finally, we determined the effects of disrupting the PP2A-Akt complex on Akt phosphorylation. We predicted that disruption of the Akt-PP2A complex would have strongest effects on Akt phosphorylation in the attenuation phase of insulin signaling when Clk2 levels are elevated and the Akt-PP2A complex is assembled. Knock-down of B56β in

primary hepatocytes caused a modest induction of basal Akt phosphorylation, with no large effects on maximal stimulation at 30 minutes. Importantly, after 7 hours of continuous insulin treatment, where the control cells have attenuated Akt phosphorylation, hepatocytes with B56β knock-down displayed high levels of Akt phosphorylation, with little decrease in phosphorylation from 30 minute stimulation (Fig.4C). In addition, expression of CPM largely blocks Akt dephosphorylation caused by Clk2 overexpression while Wt B56β had no effect (Fig 4D). Taken together, these data indicate that in the attenuation phase of insulin signaling elevation of Clk2 induces, through phosphorylation of B56, Akt dephosphorylation. Furthermore, these data also indicate that B56β is a required component in the attenuation of Akt phosphorylation after insulin stimulation by bridging Akt with the PP2A phosphatase core enzyme.

Discussion

In summary, here we provide evidence of an endogenous Akt attenuation pathway: following insulin stimulation of Akt phosphorylation and activity, Clk2 becomes activated and phosphorylates the N-terminus of B56β which is in a complex with Akt, phosphorylation of B56β signals assembly of the PP2A core enzymatic complex leading to dephosphorylation of Akt (Fig.4E). One of the important questions remaining in the field of intracellular signaling is the issue of substrate specificity and regulation of serine/threonine phosphatases. While the mammalian genome encodes over 400 serine/threonine kinases there are considerably fewer serine/threonine phosphatases (Virshup and Shenolikar, 2009). This is an especially compelling question in regard to PP2A, which is considered a major serine/threonine phosphatase in the cell. There are only a few mechanistic demonstrations of how PP2A achieves substrate specific regulation. One well described model PP2A regulation and substrate specificity is of the B56δ subunit targeting PP2A to Cdc25 (Forester et al., 2007;Margolis et al., 2006). Similar to our model, N-terminal phosphorylation of the B56δ subunit by Chk1 is critical for targeting PP2A to Cdc25. This, in combination with the data we present here suggest that the N-terminal regions of the B56 subunits are important regulatory regions. The physical/structural basis of how the N-terminal regions could regulate PP2A holoenzyme is very unclear. The N-terminal domains of the B56 proteins are variable and were not included in crystal structures of the PP2A holoenzyme they also seem to be dispensible for holoenzyme assembly (Cho and Xu, 2007;Xu et al., 2006). Our B56β CPM mutation, while having dramatically reduced binding to PP2A core enzyme, does not completely abolish binding like other residues on B56 proteins that are established to be involved in holoenzyme assembly (SS367DD in Supp.Fig.3F) (Rocher et al., 2007;Saraf et al., 2010). At this point, it remains to be investigated if other PP2A-B56β targets are regulated in a similar manner.

Previous studies of feedback and attenuation in the insulin/Akt pathway have identified that components of membrane signaling especially the IRS proteins play an important role (Canettieri et al., 2005; Copps et al., 2010; Morino et al., 2005; Shah et al., 2004; Um et al., 2004). While our work does not specifically address the role of these proteins, the fact remains that control of Akt dephosphorylation is just as critical a component of Akt activity as the upstream pathways. Integrating all these regulatory mechanisms together will yield valuable insights on the dynamics of insulin/Akt signal transduction in physiologic as well as diseased conditions. Moreover, given that Akt is a central regulator of cell growth, survival and metabolism, targeting the Clk2-B56β-PP2A pathway may be an approach to develop specific therapeutics to treat conditions with abnormal Akt activity such as Akt-driven tumors or metabolic diseases.

Experimental Procedures

Animals Procedures

All mouse experiments were performed with 6–10 week old male Balb/c mice purchased from Taconic. Adenovirus infections were performed by tail vein injection of 0.5×10^9 infectious particles per mouse. Animals were fed a standard diet (22.5% protein, 11.8% fat, 52% carbohydrate by mass) were sacrificed 5–7 days after infection by CO_2 asphyxiation; the livers were removed and snap frozen on liquid nitrogen until processing. In all mouse experiments, the feeding of the mice was synchronized by fasting 12–24 hrours prior to refeeding. The stomach contents of all mice were inspected upon dissection to verify food ingestion during refeeding. Glycemia was measured by tail bleeds using an Ascencia Elite (Bayer). Serum Insulin levels were measured using mouse insulin Elisa kit (Crystal Chem). All animal experiments were performed in at least two independent trials with similar results. All animals work was performed in accordance to Dana-Farber Cancer Institute and Beth Israel Deaconess animal care and use policies.

Cell Culture

Mouse primary hepatocytes were isolated from 6–10 week old male Balb/c mice using liver digest media (Invitrogen), the detailed protocol is available upon request. Adenovirus infections were performed using 1.0×10^7 infectious particles per well of a 6-well plate for 2.5 hours in maintenance media (DMEM 0.2% BSA, 1nM insulin, 2% Na-pyruvate, Pen/Strep). Cells were grown in starvation media (DMEM 0.2% BSA, 2% Na-pyruvate, Pen/Strep) overnight prior to insulin treatments. Cells were harvested 1–2 days after infection as indicated. All insulin treatments were done at a final concentration of 50nM.

HEK 293 cells were cultured in DMEM 10%FBS, Pen/Strep. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations. All transfection amounts were normalized with empty vector plasmid.

Bovine Insulin (I0516) was from Sigma and TG003 from CalBiochem.

Cell Extracts

Whole cell extracts for phosphorylation analysis were prepared by quickly removing growth media from plates, washing with cold PBS, adding RIPA buffer supplemented with 1mM PMSF, 1x Protease Inhibitors (Roche), 30mM glycerol 2-phosphate, 50mM NaF, 2mM Na orthovanadate and 50nM Okadaic Acid and snap freezing on liquid N_2. Plates were thawed at 4°C then cell extracts transferred to eppendorf tubes. Liver whole cell extracts were prepared using a TissueLyzer (Qiagen) for 5 minutes at 20/sec in RIPA buffer. Detailed protocols for immunoprecipitation, kinase and phosphatase experiments and a list of antibodies used can be found in Supplementary Materials.

Quantitation of Western Blotting

Image J was used to measure integrated density from films. Phospho-Akt signal was corrected to unphosphorylated/total levels of Akt, co-immunoprecipitations corrected to levels of the immunoprecipitated protein or as described in figure legend. When followed by plus/minus data is presented as average +/− stdev.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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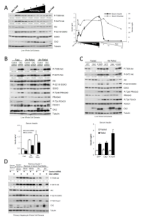

Figure 1. Attenuation of Akt phosphorylation by Clk2

(A) Western blot of liver whole cell extracts from mice sacrificed at indicated time points of fasting/feeding. Serum insulin and glucose measurements each point N=3.

(B) Western blot of liver whole cell extracts from mice infected with Control shRNA or Clk2 shRNA adenovirus fasted for 20 hours then refed for 2 or 4 hours. Serum insulin measurements each point N=4 average +/− SEM.

(C) Western blot of liver whole cell extract from mice infected with GFP, Flag-Clk2, or Flag-Clk2 K192R adenovirus following a 9 hours fast and 4 hours refed. Serum insulin measurements each point N=4, average +/− SEM.

(D) Western blot of whole cell extracts from primary hepatoctyes infected with Clk2 or Control shRNA treated with insulin as indicated.

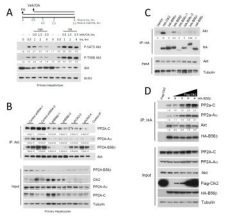

Figure 2. Clk2 B56β mediates PP2A-Akt interaction

(A) Okadaic Acid (OA) blocks attenuation of Akt phosphorylation. Western blot of whole cell extracts from primary hepatoctyes treated with insulin 30 minutes prior to addition of vehicle (DMSO) or 50nM OA as described.

(B) Co-immunoprecipitation of PP2A with Akt from primary hepatocytes infected with indicated control, shB56β, or shClk2 adenovirus. Quantitation was corrected to levels of immunoprecipitated Akt.

(C) Co-immunoprecipitation of Akt with HA-B56β. Plasmids encoding for indicated HA-tagged B56 isoform and Clk2 were transfected in HEK293 cells for 24 hours prior to harvest and immunoprecipitation using HA-agarose.

(D) Expression of Clk2 increases the interaction between B56β and PP2A core enzyme complex. HEK 293 cells were transfected with indicated plasmids 24 hours prior to harvest and immunoprecipitation with HA-agarose. Quantitation in (C) and (D was corrected to levels of immunoprecipitated HA-B56β.

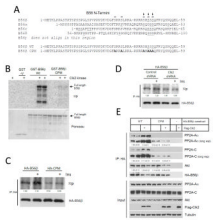

Figure 3. Clk2 phosphorylation of B56β is required for formation of Akt-PP2A complex

(A) N-terminal alignments of the human B56 family. Underlined residues indicate an identified phospho residue in PHOSIDA and/or PhosphoSitePlus databases. Predicted Clk2 phosphorylation sites by Scansite are indicated by arrows. N-terminus of CPM compared to Wt. B56β, mutations are indicated in bold.

(B) *In vitro* kinase assay using purified Clk2 kinase and GST-vector, B56β wt, or CPM mutants as substrate. All lanes have addition of ^{32}Pγ-ATP, indicated lanes have addition of Clk2.

(C) *In vivo* phosphorylation labeling of HA-B56β or HA-CPM in primary hepatoctyes +/− insulin as described in experimental procedures.

(D) *In vivo* phosphorylation labeling of HA-B56β in primary hepatoctyes with control or Clk2 shRNA +/− insulin. Quantitation of ^{32}P in (C) and (D) is correct to levels of HA-B56β.

(E) Co-immunoprecipitation of Akt and PP2A core enzyme with HA-B56β Wt or CPM mutant with/without Clk2. HEK 293 cells transfected as indicated 24 hours prior to harvest and immunoprecipitation with HA-agarose. All transfection amounts were corrected with empty vector plasmids. Quantitation of co-immunoprecipitation was correct to levels of HA-B56β.

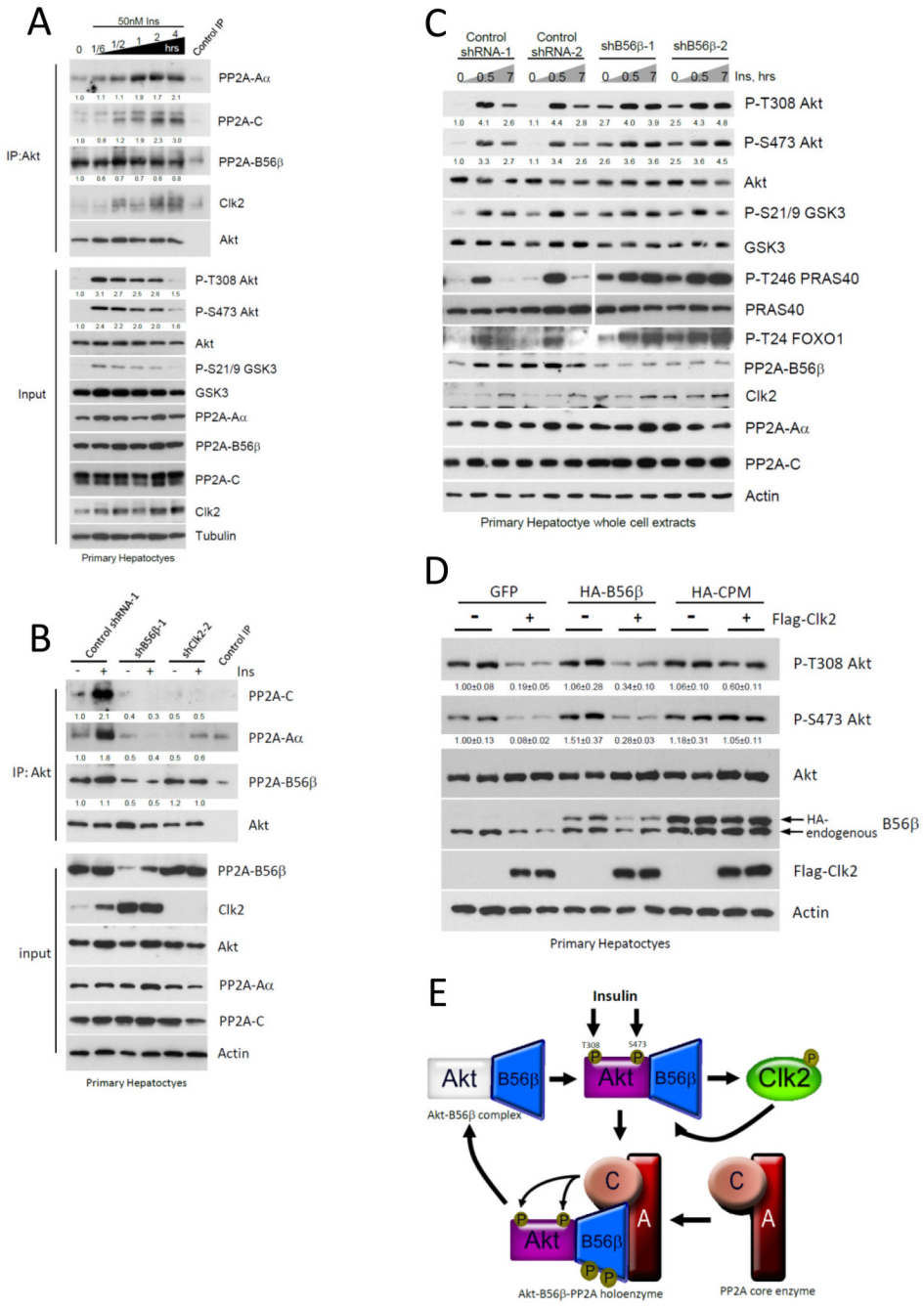

Figure 4. Insulin regulation of Akt-PP2A complex assembly and attenuation requires Clk2 and B56β

(A) Co-immunoprecipitation of PP2A complex with Akt in primary hepatocytes treated with insulin for indicated time.

(B) Insulin regulation of Akt-PP2A complex requires B56β and Clk2. Co-immunoprecipitation of PP2A with Akt from primary hepatocytes infected with Control shRNA, shB56β, or shClk2 adenovirus. Quantitation of co-immunoprecipitation in (A) and (B) was corrected to levels of immunoprecipitated Akt.

(C) B56β is required of attenuation of Akt phosphorylation. Western blot of whole cell extracts from primary hepatocytes infected with indicated adenovirus 48hrs prior to insulin treatment for indicated time.
(D) Expression of B56 CPM rescues Clk2 stimulated Akt dephosphorylation. Western blot of whole cell extracts from primary hepatoctyes infected as indicated, all viral loads were corrected with GFP.
(E) Proposed model of attenuation of Akt phosphorylation through Clk2 and B56β-PP2A following insulin stimulation.