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# Insulin Receptor Kinase-Independent Signaling Via Tyrosine Phosphorylation of Phosphatase PHLPP1

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## ABSTRACT

Most insulin responses correlate well with insulin receptor (IR) Tyr kinase activation; however, critical exceptions to this concept have been presented. Specific IR mutants and stimulatory IR antibodies demonstrate a lack of correlation between IR kinase activity and specific insulin responses in numerous independent studies. IR conformation changes in response to insulin observed with various IR antibodies define an IR kinase-independent signal that alters the C-terminus. IR-related receptors in lower eukaryotes that lack a Tyr kinase point to an alternative mechanism of IR signaling earlier in evolution. However, the implied IR kinase-independent signaling mechanism remained obscure at the molecular level. Here we begin to define the molecular basis of an IR-dependent but IR kinase-independent insulin signal that is equally transmitted by a kinase-inactive mutant IR. This insulin signal results in Tyr phosphorylation and catalytic activation of phosphatase PHLPP1 via a PI 3-kinase-independent, wortmannin-insensitive signaling pathway. Dimerized SH2B1/PSM is a critical activator of the IR kinase and the resulting established insulin signal. In contrast it is an inhibitor of the IR kinase-independent insulin signal and disruption of SH2B1/PSM dimer binding to IR potentiates this signal. Dephosphorylation of Akt2 by PHLPP1 provides an alternative, SH2B1/PSM-regulated insulin-signaling pathway from IR to Akt2 of opposite polarity and distinct from the established PI 3-kinase-dependent signaling pathway via IRS proteins. In combination, both pathways should allow the opposing regulation of Akt2 activity at two phosphorylation sites to specifically define the insulin signal in the background of interfering Akt-regulating signals, such as those controlling cell proliferation and survival. J. Cell. Biochem. 107: 65–75, 2009. 

**KEY WORDS:** CELL-PERMEANT PEPTIDE; DIMERIZATION; INSULIN ACTION; PI 3-KINASE-INDEPENDENT; PH; PSM; SCOP; SH2-B; SH2B1

The concept of insulin-induced insulin receptor (IR) tyrosine kinase activation and the resulting IR autophosphorylation and substrate protein phosphorylation to provide binding sites for numerous signaling mediators has led to an explosion of our knowledge of molecular insulin signaling over the last two decades. In many studies, insulin-stimulated activation of the IR kinase was found to correlate with the physiologic insulin response [Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987; Russell et al., 1987; Wilden et al., 1992; Wilden and Kahn, 1994]. However, in a number of alternative studies various IR cellular responses were observed in the absence of significant IR kinase activity. This was repeatedly and independently reported for various IR mutants or in response to cell treatment with stimulatory IR antibodies [Simpson and Hedo, 1984; Zick et al., 1984; Ponzio et al., 1988; Baron et al., 1989; Soos et al., 1989; Sung et al., 1989; Moller et al., 1991; Sung, 1992a,b; Rolband et al., 1993]. A complementary lack of correlation was observed in alternative studies where IR mutants or Tyr kinase inhibitor treatment resulted in the inhibition of insulin responses despite an observed normal IR kinase activity [Abler et al., 1992; Quon et al., 1992]. After the IR cDNA had been cloned and sequenced, IR mutants were created in vitro that were lacking Tyr kinase activity including single amino acid substitutions such as K1018A to more formally address the role of the IR Tyr kinase. The majority of these studies reported the resulting loss of insulin action and confirmed the importance of the IR kinase [Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987; Russell et al., 1987]. On the other hand, several independent studies reported physiologic insulin responses that were transmitted by kinase-defective in vitro IR mutants [Gottschalk, 1991; Rolband et al., 1993; Wong et al., 1995]. Despite their importance, the findings that demonstrated IR kinase-

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independent insulin action resulted in few follow-up studies in large part since they did not offer any molecular explanation that, in contrast, is so convincingly available for the IR kinase-mediated insulin signal.

A comparison of insulin action in more simple eukaryotes helps elucidate its evolution and sheds light on an alternative mechanism of IR signaling. A single IR-related receptor has been reported in various insects, mollusks, cnidarians, cephalochordates, and protochordate tunicates, including *Drosophila melanogaster*, *Bombyx mori*, *Caenorhabditis elegans*, *Branchiostoma californiensis*, and *Ciona intestinalis* [De Meyts, 2004; Sherwood et al., 2006]. Only in some of these models functional information is available, most importantly in *C. elegans* and in *D. melanogaster* where a receptor Tyr kinase-mediated signaling mechanism has been described involving orthologs of several key mediators implicated in signaling pathways described in mammals [Claeys et al., 2002]. In more primitive, lower eukaryotes a cell surface receptor protein with high affinity (at 66 kDa in *Neurospora crassa*) or low affinity (at 53 kDa in *Saccharomyces cerevisiae*) for mammalian insulin has been characterized [Fawell and Lenard, 1988; Muller et al., 1998b]. In *N. crassa* detectable protein kinase activity could not be demonstrated for the receptor [Kole et al., 1991]; however, at least 14 discrete proteins including 6 membrane proteins were found to be phosphorylated on Ser, Thr, or Tyr in response to insulin [Kole and Lenard, 1991]. In *S. cerevisiae* the receptor was found phosphorylated on Ser but not Tyr and in the same complex as a 70 kDa phospho-Tyr-containing protein [Muller et al., 1998a]. In this model, insulin was found to modulate protein phosphatase 2A, protein kinase A, cAMP-specific phosphodiesterase, and glycosyl-phosphatidylinositol-specific phospholipase C activities at insulin concentrations correlating well with those required for half-saturation of the specific binding sites as well as for stimulation of protein phosphorylation and glycogen accumulation [Muller et al., 2000]. This suggests the existence of a signaling network related to the insulin-signaling network described in mammalian cells as well as an IR kinase-independent insulin signal that may have evolved early in evolution likely prior to the acquisition of a Tyr kinase activity by the IR in higher eukaryotes. Numerous studies as summarized above indicate that an IR kinase-independent signaling mechanism still co-exists today with catalytic IR-signaling in mammals.

Here we begin to define the molecular basis of an IR-dependent but IR kinase-independent insulin signal that is equally transmitted by a kinase-inactive mutant IR. This insulin signal results in Tyr phosphorylation and catalytic activation of phosphatase PHLPP1 via a PI 3-kinase-independent, wortmannin-insensitive, signaling pathway. Dimerized SH2B1/PSM is a critical activator of the IR kinase and the resulting established insulin signal [Zhang et al., 2008b]. However, here we show that it is also an inhibitor of the IR kinase-independent insulin signal, and disruption of SH2B1/PSM dimer binding to IR potentiates this signal. The resulting dephosphorylation of Akt2 by PHLPP1 provides an alternative, SH2B1/PSM-regulated insulin-signaling pathway from IR to Akt2 of opposite polarity and distinct from the established PI 3-kinase-dependent signaling pathway via IRS proteins. In combination, both pathways should allow the opposing regulation of Akt2 activity at two phosphorylation sites to specifically define the insulin signal in the background of interfering Akt-regulating signals, such as those controlling cell proliferation and survival.

## MATERIALS AND METHODS

Bar graphs have been typically shown based on duplicate measurements with the error indicated while for immunoblots one representative experiment has been displayed.

### CELL-PERMEANT SH2B1/PSM DOMAIN-SPECIFIC FUSION PEPTIDES

Cell-permeant peptides representing the SH2B1/PSM amino-terminal Pro-rich sequence or SH2 domain had been prepared by fusion with a sequence of the *D. melanogaster* antennapedia homeoprotein as described earlier [Riedel et al., 2000; Deng et al., 2007]. The SH2 domain had been produced as a fusion peptide in *E. coli*, and the Pro-rich region was produced as a synthetic peptide (American Peptide Company) composed of the transduction sequence (RQIKIWFQNRRMKWKK) of the *D. melanogaster* antennapedia homeodomain followed by the Pro-rich amino terminal PSM sequence FPSPPALPPPPPPSWQ [Riedel et al., 2000]. An additional synthetic (Genscript, Inc.) cell-permeant peptide was composed of a fragment FCESHARAAALDFA of the amino terminal PSM dimerization domain (DD) [Nishi et al., 2005] fused with the transduction sequence RKKRRQRRR [Wadia and Dowdy, 2005] derived from the HIV TAT protein at the amino terminus via a linker sequence AA. A synthetic transduction peptide lacking PSM sequences or a dialyzed column eluate of a control *E. coli* cell extract served as the respective peptide control. Peptides were prepared as reported earlier [Deng et al., 2007].

### ANTIBODIES, PEPTIDE HORMONES, INHIBITORS, AND CELL CULTURE

Rabbit polyclonal antibody against IR and goat polyclonal antibody against SH2B1/PSM were obtained from Santa Cruz Biotechnology, rabbit polyclonal antibody against phospho-Ser473/474 Akt from Cell Signaling Technology, mouse monoclonal antibody against phospho-Tyr from BD Biosciences, rabbit polyclonal antibody against PHLPP1 from Bethyl Laboratories, and horseradish peroxidase-coupled immunoglobulin G (IgG) antibody from Kirkegaard and Perry Laboratories. Human recombinant insulin was obtained from Upstate Cell Signaling Solutions. PI 3-kinase inhibitor wortmannin was obtained from Calbiochem and dissolved in DMSO. Chinese hamster ovary (CHO) cells or NIH 3T3 fibroblasts stably expressing human IR were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% (v/v) penicillin/streptomycin solution in a 5% $CO_2$ environment at 37°C.

### PEPTIDE TRANSDUCTION AND cDNA/siRNA TRANSFECTION

Subconfluent CHO cells or NIH 3T3 fibroblasts stably expressing IR were cultured to quiescence for 20 h in serum-free DMEM supplemented with 0.1% BSA (RIA grade, Sigma). To study cellular responses cultures were typically incubated with 10 μg/ml of cell-permeant peptide for 1 h in serum-free medium followed by addition of 100 nM insulin for 15 min. Mouse SH2B1/PSM variants [Yousaf et al., 2001], normal human IR or mutant K1018A [McClain et al., 1987], or PHLPP1 (kindly provided by Alexandra Newton, UCSD)

[Gao et al., 2005] were expressed transiently from their respective cDNA plasmids. Sub-confluent cell cultures on 6 cm plates were rinsed with antibiotic-free DMEM before 0.5 ml transfection mix including 2 μg of expression plasmid or control plasmid, 6 μl Lipofectamine and 4 μl Plus reagent (Invitrogen) were added according to the instructions of the manufacturer. After 5 h the transfection medium was replaced with DMEM including 10% FBS or, alternatively, for subsequent insulin stimulation cells were cultured to quiescence for about 20 h in serum-free DMEM. siRNA pools of three SH2B1/PSM sequences (gene accession # NM_011363) were obtained from Santa Cruz Biotechnology: (GUAGGGCAUUGGCUAAUGA, GAAGUCGCUUGGAGUUCUU, CCCGAGCCAUUAAUAAUCA). CHO cells at 50–70% confluence in six-well cell culture plates were transfected with 40–80 μmol siRNA and 5 μl Lipofectamine 200 in 250 μl of Opti-MEM I (Invitrogen). Cells were subsequently analyzed as described below.

### IMMUNOPRECIPITATION AND IMMUNOBLOTTING

To prepare detergent extracts, cell cultures were rinsed twice with ice-cold PBS and were lysed in buffer containing 1% Triton X-100, 50 mM HEPES, pH 7.4, 10% glycerol, 137 mM NaCl, 10 mM NaF, 100 mM $Na_3VO_4$, 10 mM $Na_4P_2O_7$, 2 mM EDTA, 10 μg/ml leupeptin, and 1 mM PMSF. Cell extract containing 200–500 μg of total protein was incubated at 4°C with the respective specific antibody for 2 h and subsequently with added 25 μl of 50% protein A-Sepharose slurry for 1.5 h. Immunocomplexes were sedimented by centrifugation and rinsed three times in the same buffer at 4°C. Proteins were re-suspended in 25 μl of 2× Laemmli loading buffer, boiled, separated by SDS–PAGE, and transferred to a nitrocellulose membrane. Proteins were identified by immunoblotting with the respective specific antibody, and visualized by enhanced chemiluminescence detection (ECL; Amersham).

### IR OR PHLPP1 Tyr PHOSPHORYLATION

CHO cells were transiently transfected with the indicated expression plasmids, propagated to quiescence for 20 h in serum-free DMEM supplemented with 0.1% BSA (RIA grade; Sigma), and stimulated with 100 nM of insulin for 15 min. Cultures were rinsed twice with ice-cold PBS, detergent cell extracts were prepared with lysis buffer, and aliquots containing 250 μg of total protein were immunoprecipitated with IR- or PHLPP1-specific antibody as described in the preceding section. The level of IR or PHLPP1 Tyr phosphorylation was identified by immunoblotting with phospho-Tyr antibody.

### PHLPP1 PHOSPHATASE ACTIVITY

CHO cell detergent extracts were prepared and PHLPP1 was immunopreciptated with specific antibody. Immunocomplexes were sedimented by centrifugation and rinsed twice with lysis buffer, twice with phosphatase assay buffer (0.05 M Tris, 0.05 M Bis–Tris, 0.1 M NaAc, pH 6.5), were resuspended in phosphatase assay buffer containing 10 mM DTT, and 50 μl aliquots were transferred to 96-well plates. The phosphatase activity assay was initiated by addition of *para*-nitrophenol phosphate to each reaction to a final concentration of 50 mM. After 30 min of incubation on an orbital microplate shaker, the $OD_{405}$ was measured with a microplate reader to quantify the production of *para*-nitrophenol.

## RESULTS

### PSM REGULATION OF INSULIN- AND IR-DEPENDENT BUT WORTMANNIN-INSENSITIVE PHLPP1 ACTIVITY

We had earlier reported the essential role of SH2B1/PSM variants in the activation of the IR kinase and the resulting metabolic insulin response that included the regulation of the central mediator Akt2 [Zhang et al., 2008b], the major isoform involved in insulin action [Cho et al., 2001; Bae et al., 2003]. A *PH* domain *l*eucine-rich repeat protein phosphatase termed PHLPP1/2 had been reported to regulate Akt by dephosphorylating its hydrophobic motif and for Akt2 (Ser 474) PHLPP1 was identified as the preferred isoform [Gao et al., 2005; Brognard et al., 2007; Mendoza and Blenis, 2007]. Since regulation of this site is of considerable interest in Akt2 signaling, we addressed the question whether PHLPP1 regulation would play a role in insulin action. We tested such a role in CHO cells transfected with IR by measuring phosphatase activity in PHLPP1 immunoprecipitates towards substrate *para*-nitrophenol phosphate in response to insulin (Fig. 1). We observed substantial accumulation of *para*-nitrophenol in response to insulin indicating stimulation of phosphatase activity that correlated with the level of PHLPP1 expression (Fig. 2B) and was highly increased after PHLPP1 cDNA transfection (Fig. 1). The observed insulin stimulation clearly pointed to a role of PHLPP1 in insulin action. Intriguingly, insulin-mediated PHLPP1 activation was highly potentiated by PSM DD or SH2 domain peptide mimetics that disrupt the established IR-PSM signaling complex and inactivate the IR kinase [Zhang et al., 2008b], whereas a control Pro-rich domain PSM peptide mimetic had no effect. In contrast, increased levels of the PSM variants that potentiate IR kinase activity as reported earlier [Zhang et al., 2008b] highly attenuated insulin-meditated PHLPP1 activation with an inversely proportional pattern of activity. No wortmannin sensitivity was observed for insulin-mediated PHLPP1 activation under any of the tested conditions (Fig. 1). Our observations suggest an alternative insulin-signaling mechanism via PHLPP1 that is inversely regulated by PSM when compared to IR kinase activation and the established IR downstream responses.

### PSM REGULATION OF INSULIN-DEPENDENT PHLPP1 Tyr PHOSPHORYLATION

The endogenous protein levels of PSM and PHLPP1 are frequently quite low [Yousaf et al., 2001; Brognard et al., 2007] and not easily detected in immunoblots of cell extracts (Fig. 2B). In contrast, the transfected PHLPP1 and PSM variant expression levels are about 10-fold higher [Brognard et al., 2007] and easily visualized in immunoblots (Fig. 2B). Consequently, the 10-fold increase in PHLPP1 activity that we observed in response to PHLPP1 cDNA transfection or the 10-fold decrease of that signal that we observed in response to PSM cDNA transfection is consistent with the relative cellular level of either protein when comparing transfected and endogenous levels (Figs. 1 and 2B). Since protein phosphatases or kinases are frequently regulated by phosphorylation, we tested whether the PHLPP1 catalytic activation observed in Figure 1 would correlate with Tyr phosphorylation under the same experimental

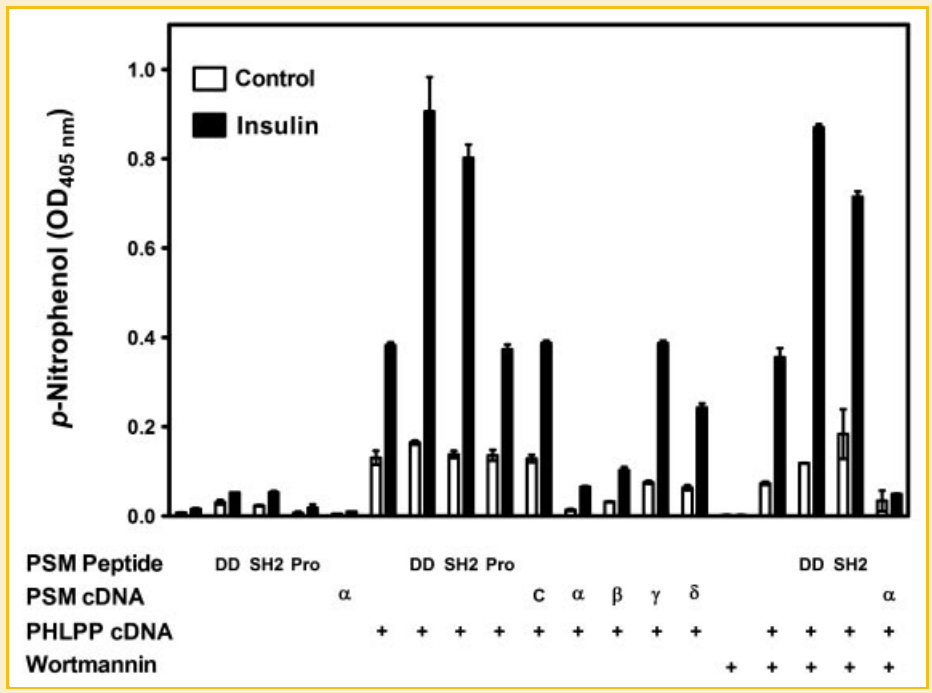


Fig. 1. PSM regulation of insulin-dependent but wortmannin-insensitive PHLPP1 activity. CHO cells were transfected with expression plasmids carrying cDNA encoding normal human IR and, as indicated, PHLPP1 cDNA (+) and PSM cDNA (variant alpha, beta, gamma, or delta) or with control plasmid (C). Cells were incubated, where indicated, with insulin, PI 3-kinase inhibitor wortmannin (+), and with cell-permeant PSM peptide mimetic either representing the amino terminal Pro-rich sequence (Pro), dimerization domain (DD), or SH2 domain. PHLPP1 was immunoprecipitated from detergent cell extracts with specific antibody, and its phosphatase activity was quantified by assaying the production of *para*-nitrophenol. For each condition, two data bars (control, insulin) have been shown.

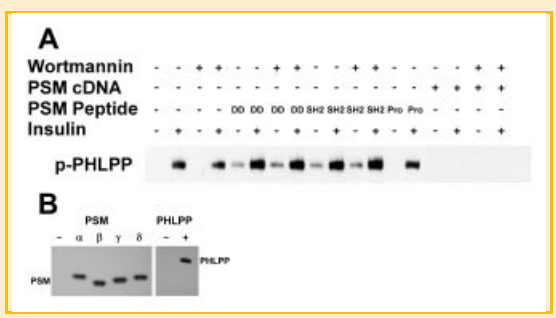


Fig. 2. PSM regulation of insulin-dependent PHLPP1 Tyr phosphorylation. A: CHO cells were transfected with expression plasmids carrying cDNAs encoding normal human IR, PHLPP1, and, where indicated, alpha variant PSM cDNA (+/−). Cells were incubated, where indicated, with insulin (+/−), PI 3-kinase inhibitor wortmannin (+/−) and with cell-permeant peptide mimetics (PSM peptide) either representing the PSM amino terminal Pro-rich sequence (Pro), dimerization domain (DD), SH2 domain, or no peptide (−). PHLPP1 was immunoprecipitated from detergent cell extracts with specific antibody and evaluated in immunoblots with phospho-Tyr antibody. The position of Tyr-phosphorylated PHLPP1 (p-PHLPP) is indicated. B: Cells were transfected with expression plasmids carrying PHLPP1 cDNA (+) or PSM cDNA (variant alpha, beta, gamma, or delta) or with control plasmid (−), as indicated. Proteins were analyzed in cell extracts by immunoblotting with PSM or PHLPP antibody and the respective expression products have been identified.

conditions. For this purpose, PHLPP1 expressed from cDNA was immunoprecipitated from detergent cell extracts with specific antibody and evaluated in immunoblots with phospho-Tyr antibody. Tyr phosphorylation of PHLPP1 was specifically observed in response to insulin that was significantly potentiated by PSM DD or SH2 domain but not by control Pro-rich peptide treatment (Fig. 2A). PHLPP1 Tyr phosphorylation was insensitive to wortmannin treatment but was attenuated below the level of detection by increased cellular levels of PSM alpha through cDNA expression (Fig. 2A). Overall, PHLPP1 Tyr phosphorylation correlated well with PHLPP1 catalytic activity as measured under similar experimental conditions (Fig. 1). In most samples in Figure 1 and exclusively in Figure 2A, PHLPP1 was consistently expressed from transfected cDNA at comparable levels as represented in Figure 2B. The observed change in the level of Tyr-phosphorylated PHLPP1 (Fig. 2A) was due to the regulation of PHLPP1 Tyr phosphorylation and not due to changes in PHLPP1 protein level. The full level of regulation was observed after temporary peptide treatment or only 15 min of insulin treatment that is too short to provide for a significant change in PHLPP1 protein expression. In addition, any potential cellular mechanism to control PHLPP1 gene expression cannot regulate the expression of transfected PHLPP1 due to the absence of the required regulatory sequences on the PHLPP1 cDNA expression plasmid.

### PSM REGULATION OF INSULIN- AND IR-DEPENDENT BUT IR KINASE-INDEPENDENT PHLPP1 ACTIVITY

We had earlier reported that PSM DD or SH2 domain mimetics inactivated the IR kinase, reflecting an essential requirement of PSM dimer binding for IR kinase activity [Zhang et al., 2008b]. The observation that these same conditions actually potentiated PHLPP1 Tyr phosphorylation and activity (Figs. 1 and 2A) indicated that PHLPP1 is activated under conditions that inactivate the IR kinase. To formally confirm this conclusion, we compared a kinase-inactive IR mutant K1018A with normal IR after transfection of CHO cells for PHLPP1 activation. A comparable insulin response was observed for either receptor. It was highly potentiated by cDNA transfected, increased levels of PHLPP1. Little if any response was observed in control transfected cells in the absence of IR cDNA (Fig. 3). Most importantly, the kinase-inactive IR mutant K1018A mediated PHLPP1 activation in response to insulin essentially equally when compared to normal IR (Fig. 3). PHLPP1 activity was highly potentiated by PSM DD or SH2 domain peptide mimetics and highly attenuated by PSM transfection. Alternatively, eliminating PSM protein as much as possible by PSM siRNA knockdown maximally potentiated PHLPP1 activation in response to insulin to levels comparable to DD or SH2 domain peptide treatment (not shown). We confirmed comparable expression levels of normal IR and mutant K1018A in immunoblots with IR-specific antibody (Fig. 4A). We compared the resulting Tyr phosphorylation in response to insulin in immunoblots with phospho-Tyr antibody. For cDNA expression of normal IR, a major phosphoprotein corresponding to its beta subunit was demonstrated at 95 kDa in response to insulin (Fig. 4A). In contrast, no change in the level of phospho-Tyr was observed in mutant IR K1018A-transfected cells when compared to control-transfected CHO cells—compatible with a completely inactive mutant IR kinase (Fig. 4A) as predicted. We formally confirmed in a dose–response experiment that mutant IR K1018A expression levels despite its inactive Tyr kinase correlated with PHLPP1 activation. Insulin-mediated PHLPP1 activation clearly increased with increased transfected levels of IR K1018A cDNA (not shown) confirming mutant IR K1018A as the mediator of this response.

### INSULIN-DEPENDENT BUT PHOSPHO Tyr-INDEPENDENT, SH2 DOMAIN-MEDIATED PSM INTERACTION WITH KINASE-INACTIVE IR K1018A

As mediated by transfected mutant IR K1018A in response to insulin, PSM DD or SH2 domain peptide mimetics highly potentiated whereas increased levels of PSM highly attenuated PHLPP1 activity (Fig. 3). This observation indicated that the PSM SH2 domain peptide mimetic still associated with mutant IR K1018A to displace cellular PSM binding despite the fact that IR K1018A does not carry Tyr-phosphorylated sequence motifs (Fig. 4A). PSM association was directly confirmed in co-immunoprecipitation experiments comparing CHO cells transfected with either normal or mutant IR. Remarkably, cellular PSM association was observed with either receptor in response to insulin and a PSM SH2 domain peptide mimetic disrupted the interaction (Fig. 4B). The observed interaction was reduced for mutant IR K1018A lacking Tyr phosphorylation

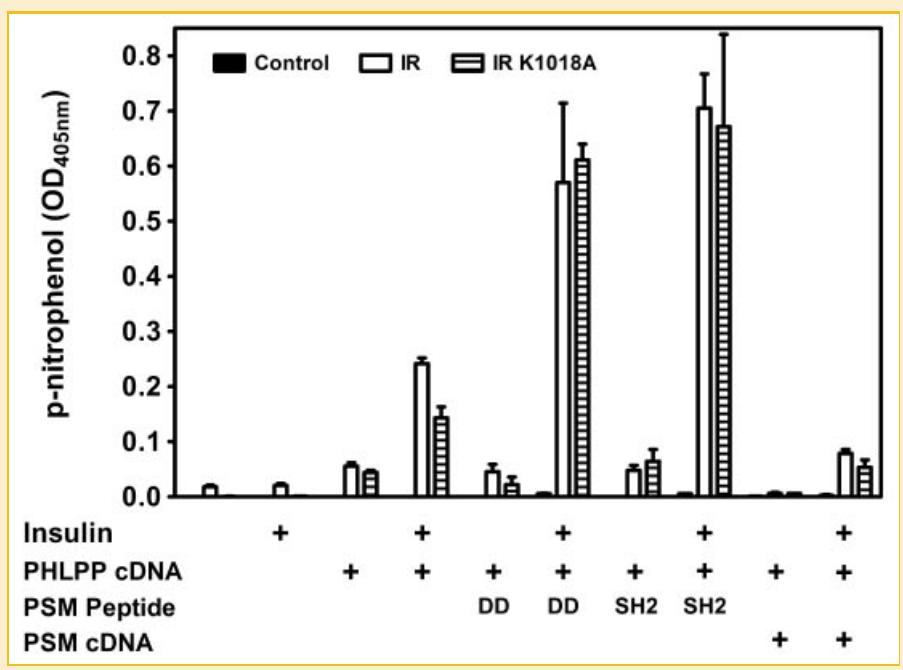


Fig. 3. PSM regulation of insulin- and IR-dependent but IR kinase-independent PHLPP1 activity. CHO cells were transfected with expression plasmids carrying cDNA encoding normal human IR, a kinase inactive mutant human IR K1018A, or with control plasmid and, where indicated, with expression plasmid carrying PHLPP1 cDNA (+) or variant alpha PSM cDNA (+). Cells were incubated, where indicated, with insulin (+) and with cell-permeant PSM peptide mimetic either representing the dimerization domain (DD) or SH2 domain. PHLPP1 was immunoprecipitated from detergent cell extracts with specific antibody and its phosphatase activity was quantified by assaying the production of *para*-nitrophenol. For each condition, three data bars (control, IR, IR K1018A) have been shown.


Fig. 4. A: Normal IR and kinase-inactive IR K1018A cDNA expression and Tyr phosphorylation. CHO cells were transfected with expression plasmids carrying cDNA encoding normal human IR, a kinase-inactive mutant human IR K1018A, or with control plasmid. Cells were stimulated with insulin, and proteins were immunoprecipitated with IR antibody from detergent cell extracts and analyzed by immunoblotting with phospho-Tyr or IR antibody. The position of p-IR or total IR is indicated. B: Insulin-dependent but phospho Tyr-independent, SH2 domain-mediated PSM association with kinase-inactive IR K1018A. CHO cells were transfected as described for (A). Cells were incubated, where indicated, with insulin (+/−) and with cell-permeant peptide mimetic (PSM peptide) either representing the PSM amino terminal Pro-rich sequence (Pro), dimerization domain (DD), SH2 domain, control peptide (C), or no peptide (−). Detergent cell extracts were immunoprecipitated with IR antibody. PSM was subsequently visualized in immunoblots with specific antibody and its position is indicated.

when compared to normal IR. PSM DD and (predictably) Pro-rich domain peptide mimetics did not interfere with the interaction (Fig. 4B). This indicated that PSM via its SH2 domain associated with IR (kinase-inactive or normal) even while PSM dimerization was blocked by DD peptide mimetics. However, DD-mediated PSM dimerization is required for kinase activation of normal IR and the resulting established insulin response as well as for the activation of other Tyr kinases including Src and Jak2 [Nishi et al., 2005; Zhang et al., 2008b].

### OPPOSING REGULATION OF INSULIN-DEPENDENT Akt2 Ser 474 PHOSPHORYLATION BY PSM AND PHLPP1

To begin to address PHLPP1 regulation of the major insulin target Akt2, we evaluated the consequences of cDNA transfection of combinations of PHLPP1 and PSM in response to insulin. Akt2 activity was visualized in immunoblots of detergent cell extracts with Akt phospho-Ser-specific antibody (Fig. 5). We observed the predicted insulin stimulation of Akt2 phosphorylation that was significantly attenuated by PHLPP1 cDNA transfection. In contrast, PSM transfection potentiated Akt2 phosphorylation to a level even observable in the absence of insulin (Fig. 5), which is predicted due

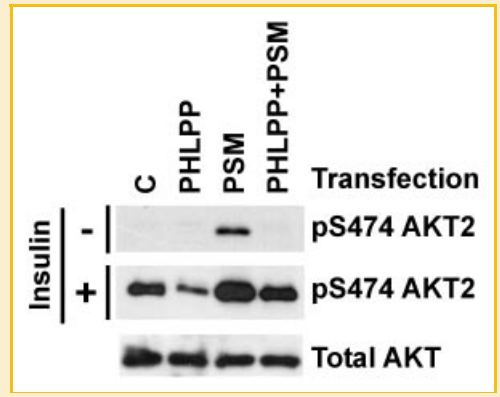


Fig. 5. Opposing regulation of insulin-dependent Akt2 Ser 474 phosphorylation by PSM and PHLPP1. Cells expressing human IR were transfected with control plasmid (C) or combinations of expression plasmids carrying cDNA-encoding PHLPP1 or the alpha variant of PSM as indicated. Cells were incubated, where indicated, with insulin (+/−) and detergent cell extracts were prepared. The presented total Akt was identified in immunoblots with Akt antibody. Akt phosphorylation was analyzed in immunoblots with phospho-specific antibody for Ser 473/474 and the identified band has been labeled as pS474 AKT2.

to the potentiation of IR catalytic activation by PSM even in the absence of insulin [Zhang et al., 2008b]. Co-transfection of PHLPP1 and PSM cDNA approximately canceled the opposing signals out to a basal level of Akt2 phosphorylation as observed with insulin treatment alone (Fig. 5). Since in these experiments the established, catalytic IR-signaling pathway was activated and kinase-independent IR signaling was consequently inactivated due to the presence of normal or increased levels of PSM, negative Akt2 regulation by insulin could not be observed. The presented results are still consistent with the predicted negative regulation of Akt2 by PHLPP1 in insulin action that will be subject to future investigation. The antibodies employed in these experiments cross-react with any of the three Akt isoforms but Akt2 is the major isoform identified in insulin action [Cho et al., 2001; Bae et al., 2003; Katome et al., 2003]. In addition, a clear preference for Akt2 has been reported for the specific isoform PHLPP1 that we employed in our cDNA expression experiments [Brognard et al., 2007]. Since we demonstrate regulation of Akt Ser phosphorylation by insulin and PHLPP1, we have designated the identified target pSer 474 Akt2 but some contribution to the observed signal by pSer 473 phosphorylation of Akt1 cannot be excluded (Fig. 5).

## DISCUSSION

Our findings introduce phosphoprotein phosphatase PHLPP1 as a new component of the insulin-signaling pathway. PHLPP has been described to dephosphorylate Akt on Ser 473 while potentiating apoptosis and suppressing tumor growth [Gao et al., 2005]. In a comparison with a second isoform PHLPP2, both variants differentially attenuate the amplitude and duration of Akt signaling by regulating distinct Akt isoforms and represent the only described protein phosphatases in the human genome that carry a PH domain

[Brognard et al., 2007; Brognard and Newton, 2008]. Their gene product had earlier been identified as suprachiasmatic nucleus circadian oscillatory protein (SCOP) to reflect the oscillation of the mRNA level in the rat hypothalamic suprachiasmatic nucleus in a circadian manner [Shimizu et al., 1999]. We observed that PHLPP1 catalytic activity was stimulated by insulin and that this correlated with PHLPP1 phosphorylation on Tyr (Figs. 1 and 2A). We consider that PHLPP1 Tyr phosphorylation may play a role in its cellular function. Such a role has been reported for protein phosphatase PTP1B phosphorylation by Tyr kinase Fer [Xu et al., 2004], or in the case of PTP alpha phosphorylation on Tyr 789 that is required for proper association with its target complex [Su et al., 1996; den Hertog and Hunter, 1996; Zheng et al., 2000]. For phosphatases such as PTP1B a role in the termination of the catalytic IR signal has been described [Goldstein, 2002]. However, the ligand-dependent stimulation of PHLPP1 Tyr phosphorylation (in response to insulin) appears to represent a new and not yet described type of phosphatase regulation by a hormone signal. This Tyr phosphorylation could possibly play a role in PHLPP1 catalytic activation or alternatively provide an interactive site for association with another signaling protein. This may involve an SH2 domain as in the examples described above for PTP1B or PTP alpha. PHLPP1 Tyr phosphorylation cannot be directly catalyzed by IR since PHLPP1 is activated and Tyr phosphorylated under conditions where the IR kinase is inactive such as in the presence of PSM DD or SH2 domain peptide mimetics [Zhang et al., 2008b] or as mediated by Tyr kinase-inactive IR mutant K1018A (Fig. 3). It should clearly be of interest to identify the Tyr kinase that phosphorylates PHLPP1 in response to insulin in the future (Fig. 6).

The ubiquitously expressed signaling mediator SH2B1/PSM is a critical component of the IR or Src signaling complex and required for the respective Tyr kinase activation [Zhang et al., 2008a,b]. Jak2, IR, or Src have been proposed to be activated by the association of a PSM dimer that can be abolished by PSM siRNA knockdown or by PSM DD or SH2 domain peptide mimetics that disrupt PSM association with the Tyr kinase or PSM dimerization, respectively [Nishi et al., 2005; Maures et al., 2006; Zhang et al., 2008a,b]. The four known PSM variants have been shown to potentiate the IR as well as the Src Tyr kinase in a distinct signature pattern of variant activity [Zhang et al., 2008a,b]. In contrast, PSM regulation of PHLPP1 was observed in an inversely proportional manner. The highest level of PHLPP1 activation was measured under conditions where the IR kinase is least active or vice versa. As a result, PSM dimer association is critical for IR catalytic activation and PSM potentiates the established IR kinase-mediated insulin response via IRS proteins, PI 3-kinase, and Akt2 [Duan et al., 2004; Zhang et al., 2008b] (Fig. 6, left). On the other hand, here we show that disruption of PSM dimer binding to IR exposes an alternative insulin response that activates PHLPP1 and is independent of PI 3-kinase (Figs. 1–3 and 6, right). Specifically, we confirmed in cDNA transfection

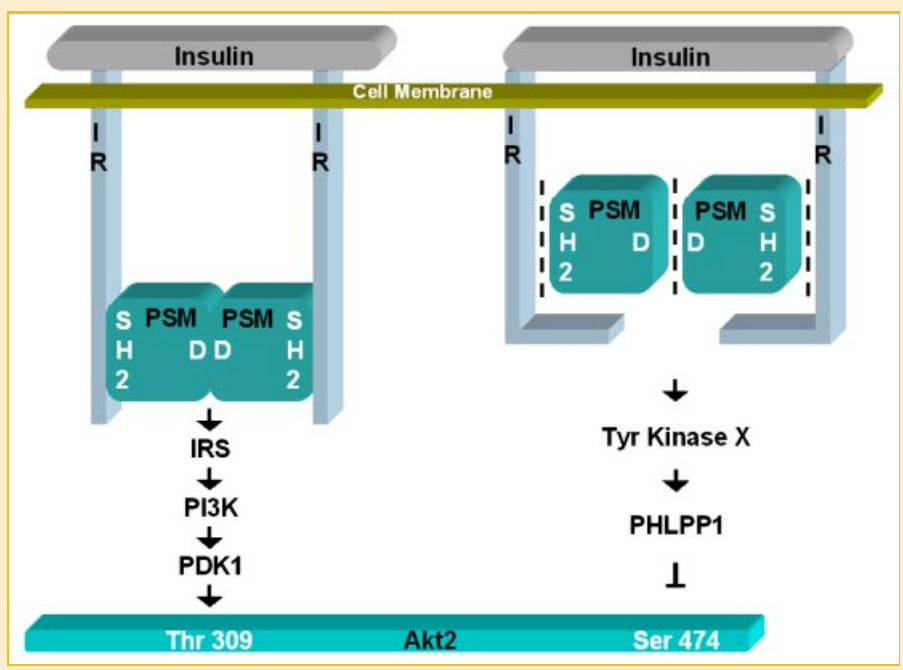


Fig. 6. IR kinase-dependent and -independent insulin-signaling pathways. The established IR-signaling pathway to Akt2 has been presented with associated, dimerized PSM on the left in contrast to the IR kinase-independent signaling pathway on the right that is potentiated by disruption (represented by dashed lines) of PSM dimerization or PSM binding to IR (or by the absence of PSM). The pathways have been extended to the regulation of Akt2, respectively, by phosphorylation of Thr 309 (left) or dephosphorylation of Ser 474 (right). Individual signaling mediators have been identified by specific name in black letters, and insulin, IR, PSM, and Akt2 have been represented by rectangular shapes. Direct contact between objects represents a postulated direct cellular association. Relevant domains or motifs such as PSM dimerization (D) and SH2 domains have been identified by white letters. IR alpha and beta chains have not been distinguished but have been represented by one continuous shape. The pathway leading to phosphorylation of Akt2 Ser 474 by the established catalytic IR-signaling response has been omitted for clarity [Hresko and Mueckler, 2005]. Relative sizes and shapes are arbitrary and have been chosen exclusively to visualize putative interactions in two dimensions.

experiments that increased PHLPP1 attenuated whereas increased PSM potentiated insulin-stimulated Akt2 pSer 474 phosphorylation while both treatments simultaneously canceled out each other (Fig. 5).

While SH2 domain interactions are typically phosho Tyr-dependent [Machida and Mayer, 2005], a number of examples represent phospho Tyr-independent SH2 domain interactions involving signaling mediators Grb2 [Oligino et al., 1997], Grb7 [Pero et al., 2002], SAP/SH2D1A [Li et al., 1999], or receptor Tyr kinase Mer with Vav1 [Mahajan and Earp, 2003]. The observation that a PSM SH2 domain peptide mimetic still potentiated PHLPP1 activation via IR mutant K1018A (Fig. 3) indicated that this SH2 domain still associated with kinase-inactive IR to displace cellular PSM binding. Our observation that insulin-dependent PSM association could also be demonstrated to IR K1018A in co-immunoprecipitation experiments (Fig. 4B) provided a second line of evidence to support that PSM binding to IR is not critically dependent on phospho Tyr. This adds to the growing number of examples of SH2 domain interactions with motifs lacking phospho Tyr. SH2 domain interactions are generally selective [Huang et al., 2008], and the inhibition of the catalytic IR signal by the DD and SH2 domain peptide mimetics was equal and not additive as well as equal to PSM siRNA treatment in our hands [Zhang et al., 2008b]. In particular, the PSM DD peptide mimetic will be selective since it disrupts the interaction of a unique Phe zipper domain that had been newly discovered in SH2B family proteins [Nishi et al., 2005]. The fact that the highly selective PSM siRNA treatment led to the same result as the DD or SH2 domain peptide mimetics also suggests against cross-talk of the SH2 domain peptide. Selectivity of the peptides is also supported by the domain, pathway, and hormone-specific interactions we observed for various peptide mimetics under varying conditions [Wang et al., 1999; Riedel et al., 2000; Deng et al., 2007]. We have earlier shown that siRNA knockdown of the related APS did not interfere with PSM variant regulation of insulin-mediated IR catalytic activation [Zhang et al., 2008b] suggesting that even if cross-interference with APS signaling occurred by the DD or SH2 domain peptides in our experiments, this would not lead to the responses we observe. As a result, we have no evidence to suggest any unspecific action of the employed peptides.

The kinase-inactive IR mutant K1018A has been described to lack any detectable catalytic or physiologic activity in a number of studies [Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987; Russell et al., 1987]. We specifically confirmed in our hands that the IR mutant K1018A and normal IR were expressed at comparable levels in CHO cells (Fig. 4A). Whereas insulin stimulation resulted in a major phospho Tyr signal representing autophosphorylation of normal IR, no change in phosho Tyr was detected in mutant IR K1018A transfected cells when compared to control CHO cells (Fig. 4A) consistent with lack of Tyr phosphorylation for mutant IR K1018A. Yet, this kinase-inactive IR mutant retains full activity to mediate activation of PHLPP1 in response to insulin and its regulation by PSM (Fig. 3). Co-immunoprecipitation (Fig. 4B) demonstrated that PSM associates with IR without any need for PSM dimerization (in the presence of DD peptide mimetic). However, to potentiate IR catalytic activity [Zhang et al., 2008b] or to attenuate insulin-mediated PHLPP1 activation (Figs. 1 and 3) association of dimerized PSM with IR is required. Endogenous PHLPP and PSM protein levels are not easily visible in immunoblots and PHLPP1 or PSM cDNA transfection greatly enhances protein levels (Fig. 2B). As a result it is predicted that PHLPP1 cDNA transfection will substantially enhance the measured PHLPP1 catalytic activity that is further potentiated by PSM SH2 or DD peptide treatment (Figs. 1 and 3). However, the employed phosphatase assay is likely not very sensitive when compared to the physiologic PHLPP1 response and should not be seen to represent low PHLPP1 physiologic activity levels. The signal we observed for endogenous PHLPP1 may well suffice for a relevant physiologic response.

Various earlier reports as summarized at the beginning of this article described IR kinase-independent insulin action. Our findings begin to explain a molecular basis (Fig. 6) for such a mechanism by defining a second, alternative, IR kinase-independent insulin-signaling pathway to Akt2 in addition to the established IR kinase-dependent pathway. Stimulation of an IR kinase-independent pathway appears to be induced and stabilized by specific extracellular IR antibodies or IR mutations [Simpson and Hedo, 1984; Zick et al., 1984; Ponzio et al., 1988; Baron et al., 1989; Soos et al., 1989; Sung et al., 1989; Moller et al., 1991; Sung, 1992a,b; Rolband et al., 1993] or as shown in this study by disruption of PSM dimer binding to IR (Figs. 1 and 3). An initial survey of IR confirmation changes in response to insulin with site-specific IR antibodies distinguished a sequence of two major steps [Baron et al., 1992] that have been represented in the cartoon shown in Figure 6. The first results in conformational changes in the IR extracellular domain as well as at the C-terminus of the two beta chains in the IR complex (Fig. 6, right). This initial insulin response has been shown to be IR kinase-independent and is consequently transmitted by a kinase-deficient IR mutant such as K1018A. Only the subsequent, second step involves ATP binding and IR autophosphorylation which leads to a second conformational change in the major part of the cytoplasmic domain and the resulting established insulin response [Baron et al., 1992] (Fig. 6, left).

We postulate that in response to insulin the initial, IR kinase-independent conformational change at the IR C-terminus transmits a signal that leads to the activation of an unidentified Tyr kinase X that phosphorylates and activates protein phosphatase PHLPP1 (Fig. 6, right). PHLPP1 in turn negatively regulates Akt2 at Ser 474 in response to insulin (Fig. 6, right). Lack of PSM dimer binding to IR enables this mechanism, which we have stimulated by PSM DD or SH2 domain peptide mimetics (Figs. 1–3) or equally by PSM siRNA treatment (not shown). This pathway appears to be critically regulated by events at the IR C-terminus [Gottschalk, 1991] that appear to only play a minimal role in the established IR kinase-dependent insulin response [Myers et al., 1991]. IR sites for PSM binding have been identified at the C-terminus around Y1322 as well as in the juxtamembrane region around Y950 [Wang and Riedel, 1998]. Binding of a PSM dimer to both IR beta chains in these two regions would be predicted to disrupt the IR conformation required for the IR kinase-independent signal at the C-terminus by binding around Y1322 and activate the IR kinase through association around Y950. The IR kinase-independent insulin-signaling pathway that we describe is suppressed by PSM dimer binding to IR. However, this promotes full IR catalytic activation and

the established insulin response via IRS proteins, PI3K, and PDK1 to regulate Akt2 at Thr 309 as a major target (Fig. 6, left) as well as via mTOR-Rictor at Ser 474 [Hresko and Mueckler, 2005].

SH2B1/PSM appears to regulate the established catalytic IR-signaling mechanism when compared to the IR kinase-independent signaling mechanism to PHLPP1 in an opposing and possibly competitive fashion (Figs. 1–3). Insulin activation of PHLPP1 is not just inversely proportional to the total amount of PSM protein in the cell but to the amount of IR that is not available for PSM dimer binding. This functional association also depends on the presence of PSM DD or SH2 domain peptide mimetics. In support of this concept, we observed that simultaneous treatment of cells by increased PSM levels as well as addition of PSM DD or SH2 domain peptide mimetics to disrupt PSM dimer binding to IR did not result in a significant change in PHLPP1 activity likely by both steps canceling each other out (not shown). Since PSM is ubiquitously expressed in essentially all tested tissues [Yousaf et al., 2001; Nishi et al., 2005], in most cell types the relative contribution of either the established catalytic or IR kinase-independent insulin-signaling mechanism should depend on the fraction of IR that is complexed with PSM dimer. This would be defined by the intracellular pools of both molecules that are available to interact. This concept would predict that in addition to IR, the specific regulation of PSM in a given tissue will play an important role in insulin action by defining whether the established catalytic IR-signaling pathway or the IR kinase-independent signaling pathway to PHLPP1 is preferentially induced. How this is regulated—potentially by other hormones—will be the focus of future investigation.

Overall, the data available in lower eukaryotes suggest an IR-related signaling mechanism that may share part of its regulatory network with the mechanism established in mammalian cells with the notable exception of an intrinsic Tyr kinase for the receptor [Kole and Lenard, 1991; Kole et al., 1991; Muller et al., 1998a, 2000]. Its absence implies an alternative mechanism to link the receptor to the reported downstream protein phosphorylation on Ser, Thr, or Tyr. The observations in lower eukaryotes also imply that IR only more recently in evolution captured a Tyr kinase activity by gene fusion that ultimately led to the receptor Tyr kinase protein that has now been well described in *D. melanogaster*, *C. elegans*, and mammals. Prior to this acquisition step, an alternative IR-signaling mechanism is implicated to control the activation of downstream targets. Our data support the concept that an alternative signaling mechanism still co-exists in the mammalian IR today (Fig. 6, right).

At the same time, the importance of the IR Tyr kinase-mediated signaling mechanism is not called into question in this study, which will likely transmit the majority of the modern insulin signal in higher eukaryotes. An analogy with the proposed RNA world may help elucidate this concept [Lamond and Gibson, 1990]. From that proposed early period in evolution, the catalytic function of RNA has been preserved to this date in addition to the well-established structural function of RNA and despite the fact that most catalytic function in the cell is effectively carried out by protein enzymes in the present. Catalytic RNA continues to play a critical role in the ribosome and in mRNA splicing and processing despite the overall dominance of protein enzymes. In analogy, as summarized at the beginning of this article, a considerable number of reports support the concept that IR kinase-independent signaling co-exists today in parallel to the well-established IR kinase-mediated insulin response. Our results show an alternative IR kinase-independent signaling mechanism that provides a second link to the central downstream mediator Akt2 by bypassing PI 3-kinase.

An enigma about Akt2 signaling lies in the question of how it can transmit a specific insulin signal while it plays a prominent role as well in the regulation of cell survival and proliferation including carcinogenesis [Chau and Ashcroft, 2004; Song et al., 2005]. Its level of activity appears highly critical such as that a little too much may cause cancer while just too little may support type II diabetes [Brazil et al., 2004]. The need for precise regulation of Akt2 activity by insulin appears logical but cannot be accomplished by positive regulation via PI 3-kinase alone. The IR kinase-independent insulin-signaling mechanism described in this report adds several new features of control in this context. It transmits a second insulin signal to Akt2 of inverted polarity that will result in a much finer control of total Akt2 activity by providing an additional mechanism to attenuate the insulin signal and a potential feedback mechanism to terminate the established IR kinase-dependent insulin signal. In addition, it provides a mechanism to regulate the level of Akt2 activation at Ser 474 independently and of opposite polarity to the regulation of Thr 309 through the established IR kinase-dependent insulin signal. It appears plausible that only by regulating both Akt2 phosphorylation sites through the combination of both potentiating as well as attenuating signaling mechanisms can Akt2 activity be controlled by insulin with sufficient selectivity and precision. This level of control may be necessary to define a specific insulin signal in the background of interfering Akt signals such as those involved in proliferation and survival.

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