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Synip phosphorylation is required for insulin-stimulated Glut4 translocation

Shuichi Okada [a,*], Kihachi Ohshima [b], Yutaka Uehara [a], Hiroyuki Shimizu [a], Koshi Hashimoto [a], Masanobu Yamada [a], Masatomo Mori [a]

[a] *Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, 3-39-15, Showa-machi, Maebashi, Gunma 371-8511, Japan*
[b] *Health and Science Center, Gunma University, 4-2 Aramaki-machi, Maebashi, Gunma 371-8510, Japan*

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Abstract

Previously we identified an unusual potential dual Akt/protein kinase B consensus phosphorylation motif in the protein Synip ($RxKxRS^{97}xS^{99}$) with serine 99 as a unique Akt2, but not Akt1 or for Akt3, substrate phosphorylation site. Although we have previously reported that serine 99 to phenylalanine (S99F-Synip) resulted in a constitutive inhibition of insulin-stimulated Glut4 translocation, a recent report indicated that Synip serine 99 to alanine mutant (S99A-Synip) failed to inhibit insulin-stimulated Glut4 translocation [H. Sano, S. Kane, E. Sano, G.E. Lienhard, Synip phosphorylation does not regulate insulin-stimulated GLUT4 translocation, Biochem. Biophys. Res. Commun. 332 (2005) 880–884]. To address this apparent discrepancy, we have now examined the S99A-Synip mutant and find that this mutant behaves essentially identical to S99F-Synip in that overexpression inhibits insulin-stimulated Glut4 translocation and is incapable of undergoing insulin-stimulated Syntaxin4 dissociation. These data are consistent with Synip serine 99 phosphorylation required for insulin-stimulated Glut4 translocation.

Keywords: Akt2; Insulin; Glucose transporter 4 (Glut4); Synip; Syntaxin4

Akt/PKB has three isoforms in mammals, each encoded by a different gene (Akt1–3, also known as PKBα, β, and γ). These isoforms share the same general structure that consists of an N-terminal PH domain and C-terminal catalytic domain, both with high degrees of amino acid identity. Nevertheless, recent data that were obtained using knockout mice and siRNA indicate that the isoforms are involved in the regulation of different biological processes. Deletion of Akt1 results in growth retardation and reduced lifespan [1], but no metabolic abnormalities [2]. By contrast, Akt2-deficient mice display insulin resistance and develop diabetes due, at least in part, to the inability of insulin to induce glucose utilization and decrease hepatic glucose output [3]. Consistent with these studies, a mutation in the kinase domain of Akt2 has been found to cause severe insulin resistance and diabetes in humans [4]. Akt3, on the other hand, does not seem to have a role in glucose homeostasis, but has significant effects on neural development [5].

The specificity of action of different Akt/PKB isoforms results from difference in their tissue distribution, subcellular localization and downstream signaling targets. The relative ratios of expression of the three isoforms vary considerably among tissues. Akt3 is predominantly expressed in the nervous system and testis, whereas Akt1 and Akt2 are widely distributed, with Akt2 particularly enriched in insulin-sensitive tissues, such as liver and fat [6]. Studies in adipocytes derived from Akt2 knockout animals have shown that insulin-stimulated glucose uptake is

Abbreviations: VAMP2, vesicle-associated membrane protein 2; Glut4, glucose transporter 4.

* Corresponding author. Fax: +81 27 220 8136.
E-mail address: okadash@showa.gunma-u.ac.jp (S. Okada).

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severely reduced and cannot be compensated for by overexpression of Akt1 [7]. A siRNA-based approach in 3T3L1 adipocyes has also highlighted the primary role of Akt2 on insulin responsiveness in adipocytes [8]. Interestingly, Akt2 has been found to be co-localized with Glut4-containing vesicles, a property not shared by Akt1 and Akt3 [9]. Furthermore, Synip, a protein that regulates the docking and fusion of Glut4-containing vesicles, is phosphorylated by Akt2, but not by Akt1 or Akt3 [10]. Because the main structural differences between Akt isoforms occur at the PH domains [11], it is tempting to speculate that other Akt targets will also show isoform specificity based on the differential binding of their PH domain to various phospholipids or other binding partners.

On the other hand, Sano et al. reported that expression of Synip mutant S99A, which lacks this phosphorylation site, had no effect on insulin-stimulated Glut4 translocation in 3T3L1 adipocytes, and they concluded that phosphorylation of Synip on serine 99 is not required for Glut4 translocation [12]. Since our previous studies were based upon a phenylalanine substitution (S99F-Synip), we therefore, reassessed the hypothesis that serine 99 is a key target of insulin signaling by examining the functional properties of the S99A-Synip mutant. The data presented in this manuscript supports the model that Synip is a key molecule responsible for Akt2 specific action leading to Glut4 translocation in adipocytes.

Material and methods

Reagents. The FLAG M2 monoclonal antibody and Syntaxin4 polyclonal antibody were obtained from Sigma. The Synip rabbit monoclonal antibody was obtained from Epitomics Inc. (CA, USA). ECL and ECL+plus Western Blotting Detection System were obtained from Amersham Biosciences. The anti-mouse and anti-rabbit IgG-HRP were obtained from PIERCE. Cell culture media and reagents were from Invitrogen Life Technologies. β-Gal Assay Kit was purchased from Invitrogen. All of the other chemicals used in this study were purchased from Sigma.

Cell culture. 3T3L1 preadipocytes were cultured in DMEM containing 25 mM glucose, 10% calf serum at 37 °C with 8% CO_2. Confluent cultures were induced to differentiate into adipocytes as previously described [10,13].

Immunoprecipitation and immunoblotting. Scraped frozen cells were rocked for 10 min at 4 °C with NP-40 lysis buffer (25 mM Hepes, pH 7.4, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium phosphate, 137 mM sodium chloride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml leupeptin). Insoluble material was separated from the soluble extract by centrifugation for 10 min at 4 °C, and the total protein amount in the supernatant was determined by BCA method. After the addition of 2 μg antibody to the whole cell lysates, samples (typically 4 mg lysates) were incubated for 2 h at 4 °C. Then 50 μl of protein A/G-agarose was added, and samples were rocked for 1 h at 4 °C. After the incubation, samples were extensively washed three times with the NP-40 lysis buffer. The washed samples were resuspended in SDS sample buffer (125 mM Tris–HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 100 mM dithiothreitol, 0.1% (w/v) bromophenol blue), and heated at 100 °C for 5 min. Samples were separated by SDS polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes. The samples were immunoblotted with monoclonal or polyclonal specific antibody as indicated in the figures and legends.

In order to assess Synip/Syntaxin4 interaction, 90% of samples were applied on SDS–PAGE and subjected to FLAG immunoblotting to detect transfected FLAG-Synip proteins. For the estimation of immunoprecipitated Syntaxin4, the remaining 10% of the immunoprecipitate was applied on a different SDS–PAGE and subjected to Syntaxin4 immunoblotting. The primary monoclonal and polyclonal antibodies for immunoblottings were detected with horseradish peroxidase conjugated anti mouse or anti rabbit IgG antibodies.

Transfection of 3T3L1 adipocytes. 3T3L1 adipocytes were suspended by mild trypsinization and electroporated with various amount of plasmid under low-voltage condition (0.16 kV, 950 μF). The cells were then allowed to adhere to collagen coated tissue culture dishes for 30–48 h, and the adipocytes were then serum-starved for 2 h prior to incubation in the absence or presence of 100 nM insulin at 37 °C for 30 min [10,13].

Quantification of electroporation efficiency by β-galactosidase assay. Electroporation efficacy was quantitated by β-galactosidase activity assay with using β-Gal Assay Kit. 3T3L1 adipocytes were transfected with either 0 μg (replaced with 600 μg of pcDNA3.1 plasmid) or 100 μg (replaced with 500 μg of pcDNA3.1 plasmid) or 600 μg of plasmid expressing lacZ as described above. The cells were then allowed to adhere to collagen coated tissue culture plates for 30–48 h, and the transfected adipocytes were conducted to β-galactosidase assay. Briefly, after cells were washed once with PBS, cells were harvested with trypsin/EDTA solution. Collected cells were resuspended in supplied lysis buffer with homogenizer at 4 °C. After short spin, 30 μl of clear lysate was mixed with 70 μl of ONPG (ortho-nitrophenyl-β-D-galactopyranoside) and 200 μl of supplied cleavage buffer. The reaction mixture was incubated for 60 min at room temperature and reaction was stopped with 500 μl of supplied stop buffer. The absorbance was read at 420 nm against a blank containing ONPG and cleavage buffer [10].

Quantification of insulin-stimulated Glut4 translocation. Quantification of transfected Glut4 translocation was determined using a qualitative colorimetric assay as previously described [10]. Briefly, 3T3L1 adipocytes were co-transfected with 600 μg of eGFP-cMyc-Glut4 plus 400 μg of various other cDNAs as indicated in each figure. Following basal or hormonal stimulation, the cells were cooled to 4 °C and incubated with a myc antibody followed by a HRP-conjugated anti-myc antibody. The specific cell surface bound HRP was then determined by incubation with the *o*-phenylenediamine dihydrochloride peroxidase substrate.

Statistical analysis. All values are expressed as means ± standard error of the mean (SEM). Data were evaluated for statistical significance by analysis of variance and *t* test using the InStat 2 program.

Results

Estimation of electroporation efficiency by β-galactosidase activity assay in 3T3L1 adipocytes

As described earlier [14], cultured adipocytes are highly refractory to most transfection methods for the expression of various cDNAs. To date, electroporation remains the most efficient method to transfect fully 3T3L1 adipocytes with the greatest extent of protein expression [14]. Therefore, we attempted to quantify the relative efficiency of protein expression using two different electroporation conditions of the lacZ (β-galactosidase) reporter activity. As shown in Fig. 1, β-galactosidase activity when transfected with 100 μg of lacZ plasmid DNA under the conditions reported by Sano et al. [12], resulted in 0.207 ± 0.09 nmol of ONPG hydrolyzed, whereas utilizing our previously reported standard conditions [10,13,14] resulted in 22.5 ± 0.3 nmol of ONPG hydrolyzed. These results demonstrate that following electroporation, the adipocytes

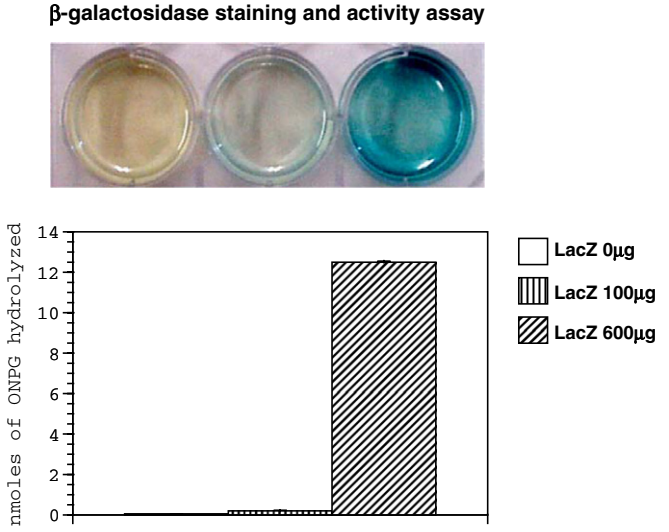

Fig. 1. Estimation of electroporation efficiency by β-galactosidase activity assay in 3T3L1 adipocytes. We attempted to quantify protein expression grade about two different electroporation conditions by β-galactosidase activity assay as described in Materials and methods. β-Galactosidase activity by using 100 μg of lacZ plasmid DNA [12] was 0.207 ± 0.09 nmoles of ONPG hydrolyzed ($n = 3$), whereas β-galactosidase activity was 22.5 ± 0.3 nmoles of ONPG hydrolyzed in the case of 600 μg of lacZ plasmid DNA ($n = 3$) [10,13,14]. β-Galactosidase staining was also performed as described in method to compare electroporation efficiency about two different electroporation conditions.

remain viable, but that the efficiency is markedly different depending on the particular transfection methodology utilized.

Estimation of protein overexpression compared to endogenous protein level

We also confirmed that following electroporation with Synip cDNAs, that proteins (WT-Synip and S99A-Synip) were significantly overexpression by immunoblotting. As shown in Fig. 2, Synip protein expression under our electroporation conditions was approximately 20 times higher than that of the endogenous Synip protein. This level of overexpression is approximately 5 times greater than that reported by Sano et al. [12].

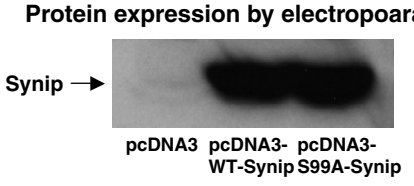

Fig. 2. Estimation of protein overexpression compared to endogenous protein level. We also confirmed Synip protein overexpression condition after electroporation by SDS–PAGE and Western blot as described in Materials and methods. Forty micrograms of whole cell lysate sample per lane was applied. Synip protein expression by electroporation under our condition was 20 times higher than endogenous Synip protein.

Insulin stimulation results in the dissociation of the WT-Synip/Syntaxin4 complex but not the S99A-Synip mutant

Since we have previously reported that insulin stimulation induces the dissociation of Synip from Syntaxin4 [10,13], we tested and determined whether the S99A-Synip mutant, that is no longer an Akt2 substrate, would remain constitutively associated with Syntaxin4. WT-Synip or S99A-Synip was therefore, transfected into 3T3L1 adipocytes under our standard electroporation conditions. As shown in whole cell lysates panel in Fig. 3, WT-Synip and S99A-Synip expression levels were similar and insulin stimulation had no significant effect on the amount of expressed Synip proteins. Syntaxin4 immunoblotting demonstrated equal immunoprecipitation efficiency among each sample (Fig. 3B). Consistent with our previous studies [10,13], WT-Synip was co-immunoprecipitated with Syntaxin 4 and insulin stimulation resulted in the dissociation of WT-Synip (Fig. 3A, lanes 1 and 2). In contrast, S99A-Synip was similarly co-immunoprecipateted with Syntaxin4, but this mutant failed to undergo any insulin-stimulated dissociation (Fig. 3A, lanes 3 and 4). These data are fully consistent with phosphorylation of Synip on serine 99 as a key regulatory mechanism controlling the relative association state of the Synip/Synatxin4 complex.

S99A-Synip inhibits insulin-stimulated Glut4 translocation

Since it was confirmed that phosphorylation of Synip on serine 99 is necessary for Synip dissociation from Syntaxin4, we next examine the functional consequence of S99A-Synip expression on insulin-stimulated myc-Glut4 translocation (Fig. 4). As expected, transfection of 3T3L1 adipocytes with the empty vector had no effect on Glut4 translocation that underwent an approximate 5-fold insulin-stimulated translocation. Similarly, co-transfection of myc-Glut4 with WT-Synip also displayed an approximate 5.0-fold insulin-stimulated translocation. In contrast, overexpression of S99A-Synip resulted in a slightly enhanced (but not statistically significant) basal level of Glut4 at the plasma membrane. More importantly, the amount of insulin-stimulated myc-Glut4 at the plasma membrane was significantly reduced such that the net insulin-stimulated translocation was only ~1.6-fold. Thus, expression of S99A-Synip functioned as a dominant-interfering mutant for insulin-stimulated Glut4 translocation consistent with a required role for serine 99 phosphorylation.

Discussion

It is well recognized that the interaction of Syntaxin4 as t-SNARE and VAMP2 as v-SNARE is a necessary step for insulin-stimulated Glut4 translocation in adipose tissue and skeletal muscle [15]. For examples, overexpression of the Syntaxin4 cytoplasmic domain inhibits insulin-stimulated Glut4 translocation [16]. This inhibition was specific for the VAMP2 binding domain within Syntaxin4, since

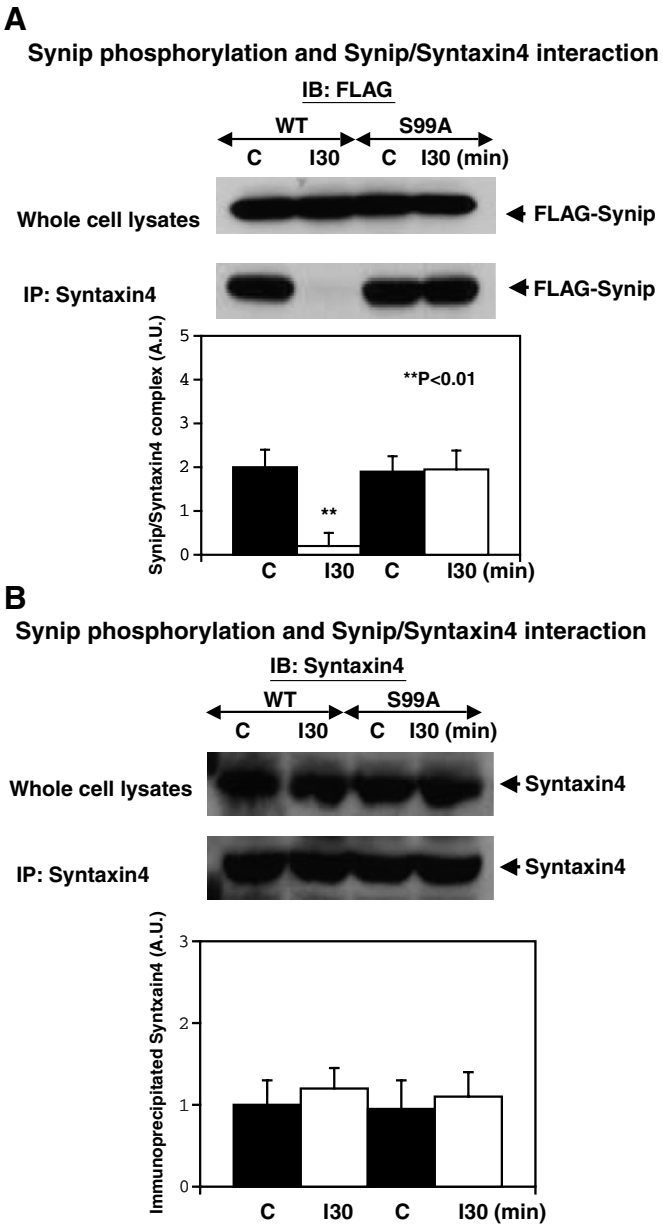

Fig. 3. Insulin stimulation dissociates not S99A-Synip mutant but WT-Synip from Syntaxin4. We expressed either WT-Synip or S99A-Synip in 3T3L1 adipocytes by electroporation with our condition and conducted to Syntaxin4 immunoprecipitation using 4 mg whole cell lysate to assess Synip/Syntaxin4 complex amount with or without insulin (100 nM, 30 min) stimulation. As shown in whole cell lysates panel, WT-Synip and S99A-Synip expression grades were similar and insulin stimulation did not attenuate each expression condition (A). Syntaxin4 immunoblot revealed that immunoprecipitation efficiency was similar among each sample (B).

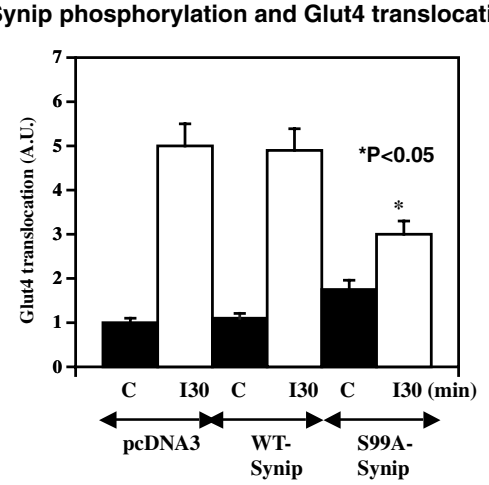

Fig. 4. S99A-Synip inhibits insulin-stimulated Glut4 translocation. Either WT-Synip or S99A-Synip was expressed by electroporation in 3T3L1 adipocytes. After suitable recovery period, insulin-stimulated Glut4 translocation was measured by a quantitative myc-Glut4 colorimetric assay [10]. The overexpressed WT-Synip resulted in a 5.0-fold increase in the plasma membrane insulin-stimulated translocation of Glut4 and it was similar grade with that of pcDNA3 transfected cells. In contrast, overexpressed S99A-Synip resulted in only a 2.8-fold insulin-stimulated Glut4 translocation and the difference was statistically significant compared to either pcDNA3 transfected cells or WT-Synip overexpressed cells ($p < 0.05$).

deletion of this region in Syntaxin4 did not result in inhibition of insulin-stimulated Glut4 translocation [16]. Thus, Syntaxin4 and VAMP2 binding is one of the necessary steps for Glut4 translocation. However, the insulin regulation of the Syntaxin4/VAMP2 interaction and progression into a fusogenic state remains mechanistically unresolved. In this regard, Synip is a Syntaxin4 specific binding protein that regulates the interaction between Syntaxin4 and VAMP2 in an insulin dependent manner [13]. This is based upon the findings that Synip competes for VAMP2 binding to Syntaxin4, and insulin induces the dissociation of the Synip/Syntaxin4 complex in the same time frame as insulin-induced Glut4 vesicle fusion with the plasma [13].

In this regard, we have identified Synip as an Akt2 specific substrate [10]. Akt2 dependent phosphorylation at serine 99 directly modulates the interaction of Synip with Syntaxin4 suggesting a direct mechanism linking Akt2 function with the t-SNARE mediated docking/fusion of Glut4 cargo vesicles [13]. We also demonstrated that insulin-stimulated Akt2 dependent phosphorylation of Synip on serine residue 99 results in reduced binding interactions between Synip and Syntaxin4 [10].

However, in contrast to our findings, Sano et al. reported that Synip serine 99 to alanine mutant (S99A-Synip) failed to inhibit insulin-stimulated Glut4 translocation. It was concluded that the substitution of phenylalanine causes protein conformational and/or functional change compared to alanine mutant [12,13] and therefore, phosphorylation of Synip on serine 99 is not required for Glut4 translocation [12]. In contrast, we have recapitulated these studies using the S99A-Synip mutant and obtained identical results to that using the S99F-Synip mutant.

Although we do not know the basis for this apparent difference between our studies, one likely possibility is the different methodology of adipocyte electorporation to introduce the Synip expression plasmids. We have found that the transfection/expression efficiency in differentiated 3T3L1 adipocytes is markedly dependent upon the amount

of plasmid DNA used during the electroporation. In our case, we obtained a substantial increased in expression compared to the study of Sano et al. [12]. It should also be noted that since we routinely obtain greater than 70% transfection efficiency we were able to use an unbiased colorimetric assay to assess Glut4 translocation in the bulk of the cell population as opposed to single cell analysis [10,13,14].

In any case, our data also demonstrates that insulin stimulation resulted in the dissociation of the Synip/Syntaxin4 complex that is prevented, following mutagenesis of the consensus Akt2 phosphorylation serine 99 to alanine. This mutation also inhibited insulin-stimulated Glut4 translocation consistent with the model that Synip function to repress Glut4 vesicle fusion and insulin-stimulation derepresses Synip function via activation of Akt that induces Synip phosphorylation and dissociation of Synip from Syntaxin4.

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