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# Tomosyn is a novel Akt substrate mediating insulin-dependent GLUT4 exocytosis

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ABSTRACT

Insulin triggers glucose uptake into skeletal muscle and adipose tissues by gaining the available number of glucose transporter 4 (GLUT4) on the cell surface. GLUT4-loaded vesicles are targeted to plasma membrane from the intracellular reservoir through multiple trafficking and fusion processes that are mainly regulated by Akt. However, it is still largely unknown how GLUT4 expression in the cell surface is promoted by insulin. In the present study, we identified tomosyn at Ser-783 as a possible Akt-substrate motif and examined whether the phosphorylation at Ser-783 is involved in the regulation of GLUT4 expression. Both Akt1 and Akt2 phosphorylated the wild-type tomosyn, but not the mutant tomosyn in which Ser-783 was replaced with Ala. Phosphorylation of tomosyn at Ser-783 was also observed in the intact cells by insulin stimulation, which was blocked by PI3K inhibitor, LY294002. *In vitro* pull-down assay showed that phosphorylation of tomosyn at Ser-783 by Akt inhibited the interaction with syntaxin 4. Insulin stimulation increased GLUT4 in the cell surface of CHO-K1 cells to promote glucose uptake, however exogenous expression of the mutant tomosyn attenuated the increase by insulin. These results suggest that Ser-783 of tomosyn is a target of Akt and is implicated in the interaction with syntaxin 4.


## 1. Introduction

Blood glucose level is strictly maintained by various hormones to prevent hyperglycemia and its complications. Insulin plays the major role in blood glucose homeostasis by stimulating glucose transport in skeletal muscle and adipose tissues, and impairment of its action leads to type 2 diabetes (Saltiel, 2001). Insulin triggers exocytosis of glucose transporter 4 (GLUT4)-loaded vesicles localized at intracellular reservoir compartment to the plasma membrane through multiple trafficking steps involving being released from its storage site, targeting to the cell periphery, docking/priming/fusion (Bryant et al., 2002; Ramm and James, 2005; Watson et al., 2004; Welsh et al., 2005). The final step of exocytosis, membrane fusion, is mediated by hetero-trimeric complexes of SNARE [soluble NSF (*N*-ethylmaleimide-sensitive factor)-attachment protein receptors] proteins (Malsam et al., 2008; Sollner et al., 1993; Weber et al., 1998) consisting of members of the VAMP (vesicle-associated membrane protein, also called synaptobrevin) family on the vesicular membrane (v-SNARE), and syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) families on the target plasma membrane (t-SNARE). α-Helical SNARE motifs from VAMP and syntaxin and two from SNAP-25 form parallel coiled-coil bundles believed to promote fusion of vesicular and target membranes (Bonifacino and Glick, 2004; Sutton et al., 1998). A number of accessory proteins regulating SNARE-mediated membrane fusion has been shown to interact directly with each SNARE protein and/or with assembled SNARE protein complexes (McNew et al., 2000). Primary SNARE complex consisting of syntaxin 1, SNAP-25 and VAMP2, which is involved in synaptic vesicle exocytosis, has been extensively studied (Linial, 1997). In GLUT4 translocation, syntaxin 4 and SNAP-23 in the plasma membrane and VAMP2 on the GLUT4-loaded vesicles have been shown to be involved (Bryant et al., 2002), analogously to insulin secretion.

*Abbreviations:* GLUT4, glucose transporter 4; EGFP, enhanced green fluorescence protein; GST, glutathione S-transferase; PKA, protein kinase A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNARE, soluble NSF-attachment protein receptors; SNAP-25, synaptosome-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; WT, wild type; SUMO, small ubiquitin-related modifier.

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Activation of the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway plays the central role in overall insulin-regulated GLUT4 trafficking. Indeed, Akt2, the primary isoform in insulin-responsive tissues of mice, has been reported to associate with GLUT4-loaded vesicles upon insulin stimulation of adipocytes (Calera et al., 1998) and to phosphorylate their component proteins in response to insulin (Kupriyanova and Kandror, 1999). Furthermore, specific activation of Akt without activating any upstream signaling was sufficient to drive GLUT4-loaded vesicles to fuse with the plasma membrane in 3T3-L1 adipocytes to a similar extent as insulin stimulation (Ng et al., 2008). Identification of Akt substrate of 160 kDa (AS160, also known as TBC1 domain family member 4; TBC1D4) was the first example to uncover the direct link between Akt signaling and GLUT4 exocytosis (Eguez et al., 2005; Kane et al., 2002; Sano et al., 2003); AS160 is a membrane-associated Rab family GTPase-activating protein (GAP), maintaining Rab in an inactive GDP-bound form, but the phosphorylation triggered by Akt in response to insulin suppresses its GAP activity, thereby allowing Rab to be a GTP-bound active form, leading to the docking of the GLUT4-loaded vesicles at the plasma membrane (Eguez et al., 2005; Sano et al., 2003). However, it is also suggested that there is AS160-independent regulation (Bai et al., 2007), and other Akt targets involved in various steps of GLUT4 translocation have also been identified, including PIKfyve (Berwick et al., 2004) and Synip (Okada et al., 2007; Yamada et al., 2005). Furthermore, several lines of evidences suggest that the processes regulated by AS160 phosphorylation might be a step prior to membrane fusion of GLUT4-loaded vesicle, such as docking (Bai et al., 2007; Fujita et al., 2010; Randhawa et al., 2008; Zeigerer et al., 2004). Thus, it is still unclear if there is a mechanism by which Akt directly regulate priming/fusion of GLUT4-loaded vesicles after the docking step. One possible molecule involved in these steps is Synip (Min et al., 1999), a syntaxin 4-binding protein, whose interaction is regulated by direct phosphorylation by Akt2 (Okada et al., 2007; Yamada et al., 2005).

Tomosyn was first identified as a syntaxin 1 binding protein with a molecular mass of 130 kDa, which consists of N-terminal WD40 repeats, a tail domain and C-terminal VAMP-like domain (Fujita et al., 1998). Tomosyn inhibits ternary SNARE complex formation by competing with VAMP2 *via* VAMP-like domain for syntaxin, and thereby inhibits SNARE-mediated neurotransmitter release (Ashery et al., 2009; Hatsuzawa et al., 2003; Pobbati et al., 2004; Sakisaka et al., 2008; Yamamoto et al., 2009). There are two mammalian genes encoding tomosyn-1 and tomosyn-2, and the alternative splicing leads to express three and four different variants with distinct distribution patterns (Groffen et al., 2005; Yokoyama et al., 1999). The s- and m-tomosyn isoforms are expressed primarily in brain and probably implicated in synaptic transmission, while b-tomosyn is ubiquitously expressed (Yokoyama et al., 1999) and thus plays a role in regulated exocytosis in non-neuronal cells. Interestingly, several recent genetic studies have shown that tomosyn-2 is implicated in type 2 diabetes (Bhatnagar et al., 2011; Joost and Schurmann, 2014). Indeed, b-tomosyn has been shown to regulate insulin-stimulated GLUT4 exocytosis (Widberg et al., 2003) and b-tomosyn-2 has been shown to be a negative regulator of insulin secretion (Bhatnagar et al., 2011, 2014). Molecular mechanism how tomosyn regulate SNARE-mediated membrane fusion is still unknown, however, there are growing evidence that phosphorylation of tomosyn plays important role. The PKA (cyclic AMP-dependent protein kinase)-mediated phosphorylation on Ser-724 of rat m-tomosyn-1 was well characterized for attenuating inhibitory effect of tomosyn on synaptic vesicle exocytosis (Baba et al., 2005). Bhatnagar et al. (2014) demonstrated that several serine-residues of tomosyn-2 were phosphorylated in INS1 cells stimulated by either glucose, membrane-permeable analogs of cyclic adenosine monophosphate (8-Br-cAMP) and phorbol esters, and mutation of 11 residues in tomosin-2 to aspartate to mimic phospho-residues resulted in accelerated turnover of tomosyn-2 by increased ubiquitination by Hrd-1 E3-ubuiquitin ligase. However, roles of each phosphorylated residue are uncovered yet.

In this study, we showed that b-tomosyn-1 (simply tomosyn hereafter) was directly phosphorylated by Akt on Ser-783 in response to insulin. Insulin-induced phosphorylation of tomosyn triggered the dissociation from syntaxin 4, possibly allowing v-SNARE protein on GLUT4-loaded vesicles to assemble with t-SNARE complex (syntaxin 4 and SNAP-23) on the target plasma membrane, and therefore resulted in the promotion of membrane fusion. The results suggested that tomosyn is a candidate for yet unidentified Akt substrate that is involved in GLUT4 exocytosis.

## 2. Materials and methods

### 2.1. Reagents and antibodies

[γ-$^{32}$P]ATP, [$^{32}$P]orthophosphate and [$^{3}$H]2-deoxyglucose were from Perkin-Elmer (Boston, MA). GST-Akt1 and GST-Akt2 were from CARNA BIOSCIENCES (Natick, MA). ATP and His-Akt2 was from Sigma–Aldrich (St. Louis, MO). Insulin and forskolin were from Wako Pure Chemical, Ltd. (Osaka, Japan). PKA was from Promega (Madison, WI). LY294002 was from Calbiochem/Merck (Darmstadt, Germany). 2-Deoxyglucose and cytochalasin B were from Sigma–Aldrich. Antibodies used were as follows; tomosyn (Santa Cruz Biotechnology, Santa Cruz, CA), GST (glutathione *S*-transferase) (MBL, Nagoya, Japan), phospho-Akt (Ser-473) (Cell Signaling Technology, Danvers, MA), FLAG (Sigma–Aldrich), syntaxin 4 (Synaptic systems, Göttingen, Germany). Normal rabbit and mouse globulins were from Jackson ImmunoReserch Laboratories (West Grove, PA).

### 2.2. DNA constructs

To generate the construct to express FLAG-tagged rat tomosyn, the open reading frame of tomosyn was PCR-amplified using the reverse transcript of rat cerebral total RNA as the template with the primers 5′-TAGGATCCACCATGAGGAAATTCAACATCAG-3′ (forward) and 5′-TCGAATTCAGAACTGGTACCACTTCTTATCTTTG-3′ (reverse). The BamHI/EcoRI fragment of the PCR product was inserted into BamHI/EcoRI digested pcDNA3-FLAG2 plasmid (Takeuchi et al., 2010). The short version of tomosyn (698-793) was PCR-amplified from the full-length tomosyn construct using the primers 5′-TAGGATCCTGTGATATTACCGAAGGAACTGTCG-3′ and 5′-TCGAATTCCCGGGACTCTTTGTCAATG-3′ to created the expression plasmid. The other short version of tomosyn (655-1156) was also PCR-amplified using the primers 5′-TAGGATCCTGTGATATTACCGAAGGAACTGTCG-3′ (forward) and the same reverse primer used for the full-length construct. The BamHI/EcoRI fragment of the PCR product was inserted into BamHI/EcoRI-site of pCold-TF expression vector (Takara Bio, Shiga, Japan). Site directed mutagenesis of tomosyn (S782A, S783D) were performed using Quik Change kit (Stratagene/Agilent Technologies) using the primers 5′-GCAGATCTCGGAGTTCAGCTGTGACCAGCATTGACAA-3′ and 5′-TTGTCAA TGCTGGTCACTCGTGAACTCCGAGATCTGC-3′ for S783A, and 5′-GCAGA TCTCCGGAGTTCAGATGTGACCAGCATTGACAA-3′ and 5′-TTGTCAATGCTGGTCACATCTGAACTCCGAGATCTGC-3′ for S783D. SNARE constructs and human GLUT4 cDNA containing a HA epitope tag in the first exofacial loop were kindly provided by Profs. Tom Martin and Sun Sik Bae, respectively. HA-GLUT4 construct was PCR-amplified using the primers 5′-TAAAGCTTCACCATGCCGTCGGGCTTC-3′ and



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