Biochemical and Biophysical Research Communications 381 (2009) 345–349

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The role of the Ser/Thr cluster in the phosphorylation of PPPSP motifs in Wnt coreceptors

Soohwan Yum [a], Su-Jin Lee [b], Shunfu Piao [a], Yongbin Xu [a], Jiyoung Jung [a,1], Yunjin Jung [a], Sangtaek Oh [c], Jaewon Lee [a], Bum-Joon Park [b], Nam-Chul Ha [a,*]

[a] College of Pharmacy and Research Institute for Drug Development, Pusan National University, Busan 609-735, Republic of Korea
[b] Department of Molecular Biology, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea
[c] PharmacoGenomics Research Center, Inje University, Busan 614-735, Republic of Korea

ARTICLE INFO

Article history:
Received 9 February 2009
Available online 23 February 2009

Keywords:
Wnt signaling pathway
LRP6
GSK3β
CK1
Biochemical role
Phosphorylation
β-Catenin

ABSTRACT

Wnt/β-catenin signaling controls a variety of cellular processes, including cell growth, oncogenesis, and development. Upon Wnt stimulation, the intracellular region of the coreceptor, LRP6 or 5, is phosphorylated by the membrane-recruited GSK3β and CK1. The cytoplasmic domain of LRP6/5 contains one Ser/Thr cluster and the PPPSP motifs, both of which are essential for propagation of the signal. While the phosphorylated PPPSP motifs are known to directly inhibit GSK3β, the biochemical role of the phosphorylated Ser/Thr cluster remains to be elucidated. Herein, we reveal that the Ser/Thr cluster plays an important role in the phosphorylation of the PPPSP motif. Interestingly, we observe that GSK3β activity on the PPPSP motif requires a high ATP concentration, close to that of the physiological condition. Taken together, these data suggest that the phosphorylated Ser/Thr cluster serves as a docking site for GSK3β to promote the phosphorylation of the PPPSP motif. Our results provide insight into the molecular mechanism for the initial events of the Wnt/β-catenin signaling.

Introduction

GSK3β and CK1 are protein Ser/Thr kinases and are involved in a wide variety of cellular signal transduction pathways. In particular, both kinases are key components in the Wnt/β-catenin signaling pathway, which is linked to a number of cancers and other diseases [1]. This signaling pathway functions by regulating the phosphorylation and degradation of the transcriptional co-activator, β-catenin [2,3]. Interestingly, two enzymes play key roles in both the down-regulation and the activation of the signaling pathway. In the absence of the extracellular ligand, Wnt, β-catenin is sequentially phosphorylated by CK1 and GSK3β in the Axin complex, consisting of the scaffold protein Axin, CK1, GSK3β, and β-catenin [3].

Wnt stimulation shuts off β-catenin degradation by the specific inhibition of GSK3β in the Axin complex [4]; this action is mediated by the phosphorylated cytoplasmic domain of the Wnt coreceptors LRP6 and 5 (LRP6/5) [5–8]. The cytoplasmic domain of LRP6/5 contains one Ser/Thr cluster and five iterative PPPSP motifs, all of which phosphorylated by CK1 and GSK3β in response to Wnt stimulation [5,7]. The Ser/Thr cluster and the PPPSP motifs are essential elements for the signal transduction [5,7]. Our recent study revealed that the Ser/Thr cluster has an intrinsic affinity for GSK3β, and that affinity is increased when the cluster is phosphorylated [8]. We provided biochemical evidence that the recruitment of the Axin complex to LRP6 is mediated by GSK3β during the activation of Wnt/β-catenin signaling, during which the affinity of the Ser/Thr cluster to GSK3β may be important [8]. Moreover, the phosphorylated PPPSP motif can directly inhibit GSK3β; this inhibition occurs predominantly when the first Ser residue of the PPPSP motif is phosphorylated by GSK3β. However, the relationship between the Ser/Thr cluster and the PPPSP motif was not tested.

GSK3β and CK1 are "phosphate-directed" protein kinases, able to phosphorylate with high efficiency. They target Ser/Thr residues specified by a pre-phosphorylated (or primed) Ser/Thr at position $n+4$ or $n-3$ as a canonical sequence, respectively [9–11]. However, GSK3β and CK1 can also phosphorylate substrates that are not pre-phosphorylated (or non-primed) substrates *in vivo*; however, it is not easy to predict these non-canonical target sites. Recent studies have revealed that the transient binding through the docking site provides CK1 selective binding to cognate substrate molecules. This docking site-mediated interaction dramatically increases the efficacy of CK1 toward non-canonical sequences of β-catenin and NFAT1 by reducing K_M values close to a level compatible with physiological conditions [12,13].

* Corresponding author.
E-mail address: hnc@pusan.ac.kr (N.-C. Ha).
[1] Pusan National University Hospital, 305 Gudeok-Ro, Busan 602-739, Republic of Korea.

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.02.044

In this study, we show that the Ser/Thr cluster of LRP6/5 is an indispensable element for the phosphorylation of the PPPSP motif by GSK3β, and that the Ser/Thr cluster of LRP6/5 functions as the docking site for GSK3β.

Materials and methods

Plasmids for transfection. Mammalian expression vectors, pEGFP-miniCL and pEGFP-miniCLMT, encoding GFP-fused LRP6 miniCL and LRP6 miniCLMT proteins, were described previously [8]. DNA fragment encoding LRP6 PPPSP (residues 1485–1497) were obtained from pEGFP-miniCL by PCR and inserted into the pEGFP-C1 vector (Clontech) using HindIII and BamHI sites to generate a GFP-tagged protein, resulting in pEGFP-PPPSP.

Antibodies. Antibodies were used according to the manufacturer's instructions. Antibodies used include mouse monoclonal anti-β-catenin (Santa Cruz), anti-GFP (Santa Cruz), rabbit polyclonal anti-GSK3β (Cell signaling), anti-phospho LRP6 (Ser1490) (Cell signaling), and goat polyclonal anti-actin (Santa Cruz).

Transfection and protein analysis. Vectors pEGFP-miniCL, pEGFP-miniCLMT, and pEGFP-PPPSP were transfected into 293 cells using jetPEI according to the manufacturer's protocol (Polyplus transfection). In brief, 2 μg of DNA and jetPEI mixtures were added to 293 cells and incubated for 3 h under serum-free conditions; then, equal volumes of DMEM containing 20% serum were added. After 24 h of incubation, cells were harvested using RIPA (containing protease cocktail). Subsequently, 20 μg of protein lysate was applied to SDS–PAGE. After transferring the proteins to a PVDF membrane, samples were incubated with the appropriate antibodies, according to standard western blot protocol.

Expression and purification of recombinant proteins. The catalytic domains of mouse GSK3β (residues 27–393) and CK1ε (residues 1–319) were expressed and purified as previously described [14]. The N-terminal region (residues 1–133) of β-catenin was expressed in *Escherichia coli* as a GST-fusion protein with a TEV protease cleavage site and then purified using Glutathione-agarose. Subsequently, the GST tag was cleaved using recombinant TEV protease and was removed by repeated HiTrapQ anion-exchange chromatography. To obtain the primed β-catenin (residues 1–133) by CK1, the purified β-catenin N-terminal segment (1–133) was phosphorylated by incubation with the catalytic domain of CK1ε (0.28 mg/ml) at 37 °C for 20 min in 50 mM Tris buffer (pH 8.0) containing 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 1 mM ATP. The phosphorylated β-catenin fragment was then further purified using HiTrapQ anion-exchange chromatography to remove the CK1ε enzyme. The mouse Axin GBD (residues 512–530), a human Frat1 fragment (residues 166–279), and variants of human LRP6 (miniCL, residues 1470–1510; miniCLMT, residues 1470–1510 with substitution at Ser1490 with Ala; LRP6 PPPSP, residues 1485–1497) were expressed in *E. coli* as GST-fusion proteins and purified using Glutathione-agarose and a HiTrapQ anion-exchange chromatographic column. The final buffer for the GST-fusion proteins was changed to 20 mM Tris buffer (pH 8.0) using Centriprep (10 kDa cutoff; Millipore).

Phosphorylation of GST-fused LRP6 fragments. To phosphorylate GST-fused LRP6 fragments, 15 μg of each GST-fusion protein was incubated for 4 h at 37 °C with 0.2 μg of recombinant GSK3β and/or 0.4 μg of recombinant CK1ε in 10 μl of 50 mM Tris (pH 8.0) buffer containing 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and a given concentration of ATP. The phosphorylation at Ser1490 in the LRP6 fragments was measured by western blot analysis using anti-phospho LRP6 (Ser1490) antibody. The CK1 activity toward GST-miniCLMT was measured by band shift on the Coomassie-stained SDS–acrylamide gel. The correlation between the band shift and CK1 activity on the LRP6 intracellular domain was previously characterized [5].

Phosphorylation of β-catenin N-terminal segment (residues 1–133). To measure the GSK3β activity under varying concentration of ATP, the reaction mixture (7.5 μl) contained 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 300 ng of recombinant CK1ε catalytic domain, and/or 3 ng of recombinant GSK3β catalytic domain, 7 μM of the primed β-catenin N-terminal fragment, and varying concentrations of ATP. The reaction mixtures were incubated at 37 °C for 30 min. After the reaction was stopped via the addition of SDS-loading buffer and immediate boiling, the resulting mixture was applied to a 15% SDS–polyacrylamide gel for analysis. The CK1 activity at β-catenin Ser45 and the GSK3β activity at β-catenin Thr41/Ser37/Ser33 were measured on Coomassie-stained SDS–polyacrylamide gels, as described previously [8].

Results

The LRP6 Ser/Thr cluster is required for the phosphorylation of the PPPSP motif

Using a short peptide containing the first PPPSP motif of LRP6 (residues 1485–1497; Fig. 1A) is injected into *Xenopus* embryo, we previously reported that only the phosphorylated PPPSP motif of LRP6 can promote the activation of Wnt/β-catenin signaling [8]. However, Cselenyi et al. reported that a larger fragment (residues 1397–1614; Fig. 1A) of the LRP6 intracellular domain, which contains the Ser/Thr cluster and the five PPPSP motifs, strongly activated the Wnt/β-catenin signaling in the embryo even when the injected fragment was not pre-phosphorylated [15]. Likewise, when the LRP6 miniCL region (residues 1470–1510; Fig. 1A), which contains the Ser/Thr cluster and the first PPPSP motif, was cytosolically overexpressed in a cell, the Wnt/β-catenin signaling was activated without exogenous Wnt stimulation [8]. However, when the residues in the Ser/Thr cluster are mutated to alanines, the Wnt/β-catenin signaling was not activated, regardless of its cellular location [5,15]. These observations suggest that the Ser/Thr cluster may promote the phosphorylation of the PPPSP motif by endogenous kinases in the cell or embryo, since pre-phosphorylation of the PPPSP motif was required for the Wnt/β-catenin signaling only in the absence of the Ser/Thr cluster in the introduced LRP6 fragments.

To elucidate the role of the Ser/Thr cluster in the phosphorylation of the PPPSP motif, we measured phosphorylation of the Ser1490 residue in the first PPPSP motif. Mammalian expression vectors encoding GFP-fused miniCL and GFP-fused PPPSP motif (Fig. 1A) were transfected into 293 cells, and the phosphorylation of the PPPSP motif was determined by using the phospho-specific antibody for detection of the phosphorylated Ser1490 residue of LRP6. The phosphorylation of Ser1490 was observed only in the miniCL construct and not in the PPPSP construct, and was in accordance with the level of β-catenin in the cell (Fig. 1B). These observations suggest a new role of the Ser/Thr cluster of the LRP6 intracellular domain as a requisite for the phosphorylation of the PPPSP motif.

To confirm the importance of the Ser/Thr cluster in the phosphorylation of the PPPSP motif in a purified system, we incubated recombinant GST-fused miniCL and GST-fused PPPSP proteins (as substrates) with the purified proteins of the catalytic domains of GSK3β and CK1 enzymes, in a reaction buffer containing 1 mM ATP, which is close to physiological ATP concentrations (5–10 mM) [16]. As shown in Fig. 1C, LRP6 Ser1490 was phosphorylated only on the miniCL fragment and not on the PPPSP fragment. This result shows that the Ser/Thr cluster is a crucial element for the phosphorylation of Ser1490 and indicates that the role of the Ser/Thr cluster in the activation of the Wnt/β-catenin is associated with the phosphorylation of the PPPSP motif.