Guanylate kinase domains of the MAGUK family scaffold proteins as specific phospho-protein-binding modules

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Membrane-associated guanylate kinases (MAGUKs) are a large family of scaffold proteins that play essential roles in tissue developments, cell–cell communications, cell polarity control, and cellular signal transductions. Despite extensive studies over the past two decades, the functions of the signature guanylate kinase domain (GK) of MAGUKs are poorly understood. Here we show that the GK domain of DLG1/SAP97 binds to asymmetric cell division regulatory protein LGN in a phosphorylation-dependent manner. The structure of the DLG1 SH3-GK tandem in complex with a phospho-LGN peptide reveals that the GMP-binding site of GK has evolved into a specific pSer/pThr-binding pocket. Residues both N- and C-terminal to the pSer are also critical for the specific binding of the phospho-LGN peptide to GK. We further demonstrate that the previously reported GK domain-mediated interactions of DLGs with other targets, such as GKAP/DLGAP1/SAPAP1 and SPAR, are also phosphorylation dependent. Finally, we provide evidence that other MAGUK GKs also function as phospho-peptide-binding modules. The discovery of the phosphorylation-dependent MAGUK GK/target interactions indicates that MAGUK scaffold-mediated signalling complex organizations are dynamically regulated.

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Introduction

MAGUKs originally referred to a group of cell junction proteins composed of synaptic scaffold protein PSD-95 from mammals, DLG tumour suppressor from *Drosophila*, and tight

junction protein ZO-1 from mammalian epithelia (Cho *et al*, 1992; Willott *et al*, 1993; Woods and Bryant, 1993). The family has since grown to encompass a large number of scaffold proteins that play critical roles in diverse cellular processes including inter-cellular connections, cell polarity development and maintenance, synaptic plasticity, and cell survival in multicellular eukaryotes (Funke *et al*, 2005; Velthuis *et al*, 2007; Mendoza *et al*, 2010). Despite of large differences in their lengths and amino acid sequences, every member of the MAGUK family proteins contains a catalytically inactive GK-like domain. All MAGUKs, with the exception of MAGI, share a common structural core consisting of an SH3 domain followed by a GK domain (termed as the SH3-GK tandem). Extensive structural and functional studies of a number of MAGUKs have revealed that the SH3 domain and the GK domain in a SH3-GK tandem interact with each other forming an integral structural unit (also referred to as the SH3-GK supramodule), which often has functions distinct from those of the isolated or simple summation of the SH3 and GK domains (Shin *et al*, 2000; McGee *et al*, 2001, 2004; Tavares *et al*, 2001; Chen *et al*, 2004; Takahashi *et al*, 2004; Lye *et al*, 2010).

Extensive studies in the past have provided numerous evidences regarding the critical cellular functions of the MAGUK GK domains. For example, *Drosophila* DLG is a tumour suppressor; mutations leading to truncations of a part or the entire GK domain of DLG lead to the tumour growth of fly imaginal discs and the eventual death of animals (Woods *et al*, 1996). A DLG truncation mutant lacking the C-terminal 43 amino acid residues of the GK domain (*DLG*^{1P20}) is defective in the neuro-precursor asymmetric cell divisions (Bellaïche *et al*, 2001), most likely due to the mutation-induced disruption of the DLG/Pins interaction (Sans *et al*, 2005; Johnston *et al*, 2009). Mice homozygous for a *DLG1/SAP97* mutation, which lacks the SH3-GK tandem due to the truncation caused by the mutation, die perinatally with severe and multi-faceted developmental defects such as cleft palate (Caruana and Bernstein, 2001). The GK domain of the voltage-gated calcium channel β -subunit binds to a small peptide fragment from the $\alpha 1$ -subunit, and the GK-mediated interaction with the $\alpha 1$ -subunit plays an essential role in regulating the surface expression and gating of the channel (Chen *et al*, 2004; McGee *et al*, 2004; Takahashi *et al*, 2004; Buraei and Yang, 2010).

Despite of their well-established functional roles, little is known about the molecular basis governing the functions of MAGUK GK domains. It is puzzling that little progress has been made in understanding how the GK domains of DLG family MAGUKs function mechanistically, although the structure of the DLG4/PSD-95 SH3-GK tandem has been solved for ~10 years (McGee *et al*, 2001; Tavares *et al*, 2001) and many GK domain-binding proteins have been identified since the discovery of the DLG MAGUKs (see Kim *et al*, 1997; Brenman *et al*, 1998; Deguchi *et al*, 1998; Hanada *et al*, 2000; Pak *et al*,

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2001 for examples). The structures of the currently solved MAGUK GK domains have convincingly demonstrated that MAGUK GKs cannot catalyze the conversion of GMP to GDP due to the lack of critical catalytic residues and the loss or dramatically weakening of GMP binding (McGee *et al*, 2001; Tavares *et al*, 2001; Chen *et al*, 2004; Lye *et al*, 2010). Instead, the MAGUK GK domains have evolved into protein interaction modules. Except for the GK domain of the calcium channel β -subunit (Chen *et al*, 2004; McGee *et al*, 2004; Takahashi *et al*, 2004), which can be viewed as an atypical member of the MAGUK family, the biochemical and structural basis underlying the MAGUK GK/target interactions are poorly understood. These GK/target interactions include the bindings of various isoforms of DLG GKs to the mitotic spindle regulatory protein LGN (or Pins in fly; Sans *et al*, 2005; Johnston *et al*, 2009), major post-synaptic scaffold DLGAP1 (also known as GKAP/SAPAP1; Kim *et al*, 1997), microtubule-associated protein MAP1a (Brenman *et al*, 1998), kinesin-like motor protein GAKIN (Hanada *et al*, 2000), synaptic scaffold protein BEGAIN (Deguchi *et al*, 1998), and Rap-specific GTPase-activating protein SPAR (Pak *et al*, 2001).

Here we show that the bindings of the DLG1 and 4 (SAP97 and PSD95) GK domains with LGN require the phosphorylation of a specific Ser residue located in the linker region between the N-terminal TPR repeats and the C-terminal GoLoco motifs of LGN. The 3D structure of the DLG1 SH3-GK tandem in complex with a synthetic phospho-peptide comprising the GK-binding domain of LGN reveals two important properties of this highly specific DLG/LGN interaction. First, the GMP-binding pockets of DLG GK domains have evolved into specific phospho-Ser/phospho-Thr (pSer/pThr)-binding domains. Second, a number of amino acid residues both N- and C-terminal to the pSer are also critical for the specific interaction between DLG GK and LGN. We further demonstrate that the bindings of DLG GK domains to DLGAP1 and SPAR also require the phosphorylation of specific Ser residues in these two DLG target proteins. Finally, we present evidence showing that other MAGUK GK domains, such as those from CASK and MPPs, are also phospho-peptide-binding modules. The discovery of the specific MAGUK GK/phospho-target interactions strongly suggests that MAGUK-mediated organizations of large protein complexes functioning in diverse cellular processes are subjected to dynamic regulations by various protein kinases.

Results

The phosphorylation-dependent interaction between SAP97 SH3-GK and LGN

To understand the molecular basis underlying the bindings of the SH3-GK tandems of DLG4/PSD-95 and DLG2/SAP102 with the linker domain of LGN/mPins reported earlier (Bellalche *et al*, 2001; Sans *et al*, 2005), we performed a series of biochemical binding experiments, including analytical gel filtration chromatography and pull-down assays, using bacterially expressed, highly purified SH3-GK tandems of PSD-95 and SAP97 and the linker region of LGN (residues 374–479). To our disappointment, we could not detect any bindings between the SH3-GK tandems and the LGN linker in these *in vitro* assays (data not shown). However, in HEK293T

cells co-expressing GFP-tagged full-length mouse LGN and Myc-tagged full-length rat SAP97, LGN could be coimmunoprecipitated with SAP97 as reported earlier (Bellalche *et al*, 2001; Sans *et al*, 2005). Interestingly, addition of a cocktail of phosphatase inhibitors (100 mM sodium fluoride, 10 mM β -glycerophosphate, 20 mM PNPP, and 50 μ M sodium vanadate) into the cell lysate led to a two-fold enhancement of the amount of LGN precipitated by SAP97 (Figure 1A), suggesting that the interaction between LGN and SAP97 might be regulated by phosphorylation. This finding is encouraging, as recent studies have shown that Ser401 in the linker region of LGN is phosphorylated by aPKC during mitosis in polarized epithelial cells (Hao *et al*, 2010), and that Ser436 of *Drosophila* Pins (which is the equivalent of Ser401 in LGN) is phosphorylated by Aurora-A kinase in S2 cells (Johnston *et al*, 2009). Elimination of the phosphorylation of LGN Ser401 (or Pins Ser436) led to mitotic spindle orientation defects (Johnston *et al*, 2009; Hao *et al*, 2010). To test whether Ser401 of LGN could be phosphorylated by aPKC directly, we performed an *in vitro* phosphorylation assay on the purified LGN linker (aa 374–479) by GFP-aPKC. Indeed, the LGN linker was robustly phosphorylated by aPKC. Substitution of Ser401 with Ala largely eliminated the aPKC-mediated phosphorylation of the LGN linker (Figure 1B), indicating that Ser401 is the major aPKC phosphorylation site of the LGN linker. We next tested whether the previously reported bindings of DLG SH3-GK tandems to the LGN linker (Sans *et al*, 2005) might be directly regulated by the aPKC-mediated phosphorylation of Ser401. As shown in Figure 1C, the interaction between SAP97 SH3-GK and the LGN linker could only be observed when the LGN linker was treated with aPKC. Additionally, the S401A mutant of the LGN linker could hardly bind to SAP97 SH3-GK even in the presence of aPKC, confirming our hypothesis that the SAP97 SH3-GK/LGN interaction requires the phosphorylation of Ser401 in the LGN linker. Amino acid sequence analysis of the linker region of LGN from different species revealed a highly conserved, short peptide fragment (aa 395–414) surrounding the aPKC phosphorylation site Ser401 (Figure 1D). We asked whether this short peptide fragment of LGN is sufficient to bind to SAP97 SH3-GK upon phosphorylation. To test this hypothesis, we assayed the binding between SAP97 SH3-GK and an 18-residue synthetic phospho-LGN peptide ('GRRHpSMENLELMKLTPEK', referred to as p-LGN18) using fluorescence spectroscopy, and found that p-LGN18 binds to SAP97 SH3-GK with a high affinity ($K_d \sim 0.22 \mu$ M; Figure 1E). Importantly, the unphosphorylated LGN18 peptide showed a ~ 500 -fold weaker binding ($K_d \sim 102 \mu$ M) to SAP97 SH3-GK compared to the p-LGN18 peptide (Figure 1E). Taken together, the above biochemical results demonstrate that the direct interaction between the SAP97 SH3-GK tandem and the LGN linker requires the phosphorylation of Ser401, an event possibly mediated by aPKC (Hao *et al*, 2010; and this study), or other kinases such as Aurora-A kinase (Johnston *et al*, 2009) and PKA or PKB/AKT as predicted by Scansite (<http://scansite.mit.edu/>).

Crystal structure of the SAP97 SH3-GK/p-LGN complex

To elucidate the molecular mechanism underlying the SAP97 SH3-GK/p-LGN18 interaction, we solved the crystal structure of the SAP97 SH3-GK/p-LGN18 complex at 2.7 Å resolution (Table I). The SAP97 SH3-GK tandem is composed

Figure 1 Discovery of the phosphorylation-dependent interaction between SAP97 SH3-GK and LGN. (A) SAP97/LGN association is enhanced with the treatment of HEK293T cell lysates with a cocktail of phosphatase inhibitors composed of 100 mM sodium fluoride, 10 mM β-glycerophosphate, 20 mM PNPP, and 50 μM sodium vanadate). The ratio of the GFP-LGN band intensities with and without the treatment of the phosphatase inhibitors was quantified. Values are mean ± s.d. from three independent experiments. (B) Purified aPKC specifically phosphorylates LGN on Ser401 in the linker region. Substitution of Ser401 with Ala largely eliminated aPKC phosphorylation of the LGN linker. (C) aPKC promotes the binding of the LGN linker to SAP97 SH3-GK. No interaction between SAP97 SH3-GK and the LGN linker could be detected in the absence of aPKC. aPKC-treated S401A-LGN linker showed a background level binding to SAP97. In this experiment, highly purified His-tagged LGN linker or S401A mutant were first treated with affinity purified GFP-aPKC and then mixed with GST-tagged SAP97 SH3-GK (see ‘Materials and methods’ for details). The SAP97 SH3-GK-bound LGN linker proteins were detected by western blot using anti-His antibody. (D) Sequence alignment showing the conserved core within the linker region of LGN from different species. For comparison, the AGS3 linker sequences are also included. In this alignment, the absolutely conserved amino acids are highlighted in red, and the highly conserved residues are in green. The pSer site is highlighted with cyan box and referred to as the ‘0’ position of the peptide sequence at the bottom of the alignment. Residues involved in direct contact with the GK domain are marked with orange triangles. The residues numbers corresponding to the mouse LGN sequence are labelled at the top of the alignment. (E) Fluorescence polarization-based measurement of the binding affinity of SAP97 SH3-GK to an 18-residue phospho-LGN peptide referred to as ‘p-LGN18’. The nonphosphorylated counterpart peptide serves as the control. The insert shows the expanded view of the binding curve of p-LGN18 to SAP97 SH3-GK.

of a split SH3 domain and a GK domain (Figure 2). The SH3 domain is split by an elongated HOOK/hinge-region composed of an α-helix (αA) and a ~50 residue unstructured hinge sequence (Figure 2A). The βE strand after the HOOK region and the βF strand at the very C-terminal following the GK domain form an antiparallel sheet, which completes the SH3 domain fold (Figure 2A). The overall structure of SAP97 SH3-GK is highly similar to that of the PSD-95 SH3-GK structures solved earlier (Figure 2C; McGee *et al*, 2001; Tavares *et al*, 2001), which is not surprising as the amino acid sequences of the SH3-GK tandems of the two DLGs (except for the unstructured HOOK regions) are highly similar (Supplementary Figure 1). Beside the N-terminal first residue and the C-terminal a few residues, the electron densities of the rest of the p-LGN18 peptide are clearly defined in the complex (Figure 2B). The p-LGN18 peptide occupies the elongated concave groove formed by the NMP-binding domain and the helical core sub-domain of GK (Figure 2A and B; Supplementary Figure 2), although the vast majority of the p-LGN18 contacting sites are limited to the residues in

the NMP-binding sub-domain of GK (Figure 3A and B). The centre of the p-LGN18 peptide forms a one-turn α-helix and followed by a 4 residue β-strand conformation in the complex. There is no direct contact between the p-LGN18 peptide and the SH3-Hinge domains, implying that the GK domain is sufficient for the p-LGN18 binding (Figure 2A). Indeed, the isolated GK domain from either SAP97 or PSD-95 binds to p-LGN18 with indistinguishable affinities compared to their SH3-GK counterparts (Figure 3D). We also show that truncation of the last 14 residues of SAP97 SH3-GK (‘SAP97 SH3-GKΔ14C’ in Figure 3D, the equivalent of the fly DLG^{SW} mutant, (Woods *et al*, 1996)) has no impact on the p-LGN18 binding, further demonstrating that the SH3 domain is not required for the DLG/LGN interaction. Structural analysis further showed that the structure of SAP97 SH3-GK in the SAP97 SH3-GK/p-LGN18 complex is slightly closer to the PSD-95 SH3-GK structure crystallized in the GMP-bound form (RMSD = 0.625 Å) than that crystallized in the GMP-free form (RMSD = 0.704 Å; Figure 2C). However, this difference is minor.

Table 1 Crystallographic Data and Refinement Statistics

Data collection	
Data sets	SAP97 SH3-GK/p-LGN18
Source	SSRF-BL17U
Space group	P21
Unit cell parameters (Å, deg)	$a = 47.6$, $b = 66.2$, $c = 55.2$, $\beta = 102.4$
Resolution range (Å)	50.00–2.70 (2.75–2.70)
No. of unique reflections	9281 (464)
Redundancy	4.7 (4.8)
I/σ	20.6 (3.5)
Completeness (%)	99.8 (99.8)
R_{merge} (%) ^a	8.5 (42.7)
Structure refinement	
Resolution (Å)	46.5–2.70 (3.09–2.70)
$R_{\text{cryst}}/R_{\text{free}}$ (%) ^b	18.7 (23.9)/24.2 (32.1)
RMSD bonds (Å)/angles (deg)	0.003/0.8
No. of reflections	
Working set	8826
Test set	442
Protein/peptide/other atoms	2171/125/36
Average ADP of Protein/peptide ^c	54.8/60.32
Ramachandran plot	
Most favored regions (%)	96.9
Additionally allowed (%)	3.1
Outliers (%)	0

Numbers in parentheses represent the value for the highest resolution shell.

^a $R_{\text{merge}} = \sum |I_i - I_m| / \sum I_i$, where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry related reflections.

^b $R_{\text{cryst}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$, where F_{obs} and F_{calc} are observed and calculated structure factors. $R_{\text{free}} = \sum_T ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_T |F_{\text{obs}}|$, where T is a test data set of about 5% of the total reflections randomly chosen and set aside before refinement.

^cRefinement is processed with Phenix.refinement (Adams *et al*, 2010), and ADP values are extracted by the Phenix validation tools.

The SAP97 GK/p-LGN18 interface

The binding of p-LGN18 to the GK domain is mainly mediated by extensive polar (charge–charge and hydrogen bonding) and hydrophobic interactions (Figure 3A and B; Supplementary Figure 3). The GK/p-LGN18 interface can be divided into two sites: the phospho-Ser-binding site (Phospho-site) and the Specificity-site (Figure 3C). At the Phospho-site, the phosphate group of pSer at the 0 position from the p-LGN18 peptide forms an extensive charge–charge and hydrogen bonding interaction network with Arg755, Arg758, and Glu761 from the $\beta 3/\beta 4$ -loop, Tyr767 from $\beta 4$, and Tyr796 from $\beta 6$ of the GK domain. The side chain of Arg at the -3 position (R-3) of p-LGN18 forms salt bridges with Glu761 and Asp766 from the $\beta 3/\beta 4$ -loop of GK and a hydrogen bond with the phosphate group from pSer(0) (Figure 3A and Supplementary Figure 3). The extensive interactions between p-LGN18 and the GK domain in the Phospho-site provide a clear mechanistic explanation as to why Ser(0) needs to be phosphorylated for LGN to bind to DLG1 SH3-GK. Consistent with these structural data, substitution of two Arg residues of GK domain in the Phospho-site (Arg755&Arg758 in SAP97, Arg568&Arg571 in PSD-95) with Gly led to a >70-fold decrease in p-LGN18 binding (Figure 3D). A quadruple substitution mutant (R755,758G/Y767,796A) of SAP97 SH3-GK had no detectable binding to p-LGN18 (Figure 3D).

Replacement of Arg(-3) with an Ala caused a 5-fold decrease in p-LGN18's binding to PSD-95 SH3-GK. Finally, although substitution of Ser(0) of LGN18 with Glu led to a ~14-fold affinity decrease between the DLG SH3-GK/LGN complex formation, the S(0)E-LGN18 mutant peptide still displays a respectable binding affinity to the SH3-GK tandem (Kd ~4.6 μ M, Figure 3D). This finding points to the possibility that some MAGUK SH3-GK tandems may also recognize target proteins in a phosphorylation-independent manner if the residue corresponding to the Ser(0) of LGN18 in such targets is a negatively charged amino acid.

The interactions at the Specificity-site are mainly hydrophobic. The side chains of Met(+1) and Leu(+4) from p-LGN18 project into a pocket formed by the side chains of Cys749, Pro751, Tyr767, Tyr796, and Thr798 from GK domain. Met(+7) and Leu(+9) from p-LGN18 make hydrophobic contacts with the side chains of Ile780, Ala788, and Leu795 from the GK domain (Figures 3A and B; Supplementary Figure 3). Again, entirely consistent with the above structural findings, removal of the C-terminal four residues from the p-LGN18 peptide, none of which form any direct contact with the GK domain, did not alter its binding to the SH3-GK tandems of both SAP97 and PSD-95 (Figure 3D, 'p-LGN14'). Further truncation of the p-LGN14 peptide by removing the hydrophobic Met(+7) and Leu(+9) led to a >50-fold decrease in its binding to SH3-GK (Figure 3D, 'p-LGN9'). Thus, the residues C-terminal to pSer(0) play critical roles in establishing the specificity of the interaction between LGN and DLG SH3-GK. In agreement with the above analysis, an 16-residue synthetic peptide corresponding to residues 405–420 of mouse AGS3, equivalent to p-LGN18 in LGN (Figure 1D), displayed a very weak binding to DLG SH3-GK (Kd ~63 μ M, Figure 3D). This much weaker binding of the p-AGS3 peptide to SH3-GK can be explained by the differences of several critical residues between p-LGN18 and p-AGS3. These differences are: the -3 residue is a Gln instead of Arg, the +1 position is a small Ala instead of a more bulky Met, and the +4 position is a very bulky Trp instead of a Leu in AGS3 (Figure 1D). Consistent with the above analysis, substitution of Gly789 in the shallow hydrophobic pocket accommodating Met(+1) and Leu(+4) of p-LGN18 with a bulky Tyr decreased SAP97 SH3-GK's binding to p-LGN18 by ~100-fold (Figure 3B and D). In agreement with the above structural and biochemical analysis, it has been shown that AGS3 cannot replace LGN in regulating mitotic spindle orientations during asymmetric cell divisions (Williams *et al*, 2011) and DLG/LGN complex-regulated NMDA receptor trafficking (Sans *et al*, 2005).

The amino acid sequence conservation map of GK domains of the DLG-, CASK-, MPP-, and MAGI-sub-family MAGUKs showed that the residues forming the phosphopeptide-binding groove (the Phospho-site in particular) are highly conserved (Figure 3C), suggesting that the GK domains from these MAGUKs share similar phosphopeptide-binding properties.

The NMP-binding pockets of DLG GK domains have evolved to be specific pSer/pThr-binding pockets

Although the GK domain of PSD-95 displays a very weak GMP binding (Olsen and Bredt, 2003), the PSD-95 SH3-GK tandem could be crystallized with a bound GMP in the presence of a very high concentration (5 mM) of GMP in

Figure 2 The overall structure of the SAP97 SH3-GK/p-LGN18 complex in stereo-view. (A) Ribbon diagram representation of the SAP97 SH3-GK/p-LGN18 complex in stereo-view. The SAP97 SH3 domain is shown in pale orange, Hinge domain in grey, and GK domain in green. The p-LGN18 peptide is shown in purple with the phosphate group in the stick-and-ball model. (B) A close-up view of the SAP97 SH3-GK/p-LGN18 complex. The SAP97 SH3-GK (grey) is shown in ribbon and the p-LGN18 peptide (yellow) is drawn in the stick-and-ball model. The 2Fo-Fc density map for the p-LGN18 peptide is shown in blue and contoured at 1.0 σ . (C) Superposition analysis of the backbone structures of the SAP97 SH3-GK/p-LGN18 complex (green), the PSD-95 SH3-GK/GMP (cyan, PDB ID: 1JXM) complex, and the apo PSD-95 SH3-GK (orange, PDB ID: 1KJW).

the crystallization buffer (Tavares *et al*, 2001). A comparison of the structures of the SAP97 SH3-GK/p-LGN18 complex, the PSD-95 SH3-GK/GMP complex, and the yeast GK/GMP complex illustrates how the DLG GK domains have evolved into specific pSer/pThr-binding modules (Figure 4). All of the residues involved in binding to the phosphate group of GMP in yeast GK (Arg39, Arg42, Glu45, Tyr51, and Tyr79) are retained in DLG GKs (Arg 568, Arg571, Glu574, Tyr580, and Tyr609 in PSD-95 GK). The structure of GMP-bound SH3-GK illustrates that the mechanisms of GMP and peptide phosphate group recognitions by DLG GKs are essentially the same. Interestingly, the GMP-bound PSD-95 SH3-GK structure contains a guanidine group from the crystallization buffer (Figure 4B). This guanidine group occupies the same position as the Arg(-3) side chain guanidine in the SAP97 SH3-GK/p-LGN18 structure (Figure 4A), illustrating the importance of Arg(-3) of p-LGN18 in binding to DLG GKs. The three critical

residues responsible for the guanine ring binding in yeast GK (Ser35, Glu70, and Asp101) are not present in DLG GKs (Figure 4C), explaining why the GMP binding affinities of DLG GKs are so low (Olsen and Bredt, 2003). Instead, a number of conserved, hydrophobic residues from the NMP-binding domain of DLG GKs function to recognize several hydrophobic residues C-terminal to the pSer in p-LGN18 (Figure 3A and Supplementary Figure 3). Taken together, the above structural analysis indicates that the GK domains of DLG have evolved from GMP-binding domains into specific pSer/pThr peptide-binding modules.

The bindings of PSD-95 SH3-GK to DLGAP1 and SPAR are also phosphorylation-dependent

Among previously identified DLG GK-binding proteins, we have been particularly interested in DLGAP1 (Kim *et al*, 1997), as DLGAP1 is a major post-synaptic scaffold protein

Figure 3 The interaction interface between SAP97 GK and p-LGN18. (A) Stereo-view plot showing the interaction details between SAP97 GK domain (green) and p-LGN18 peptide (purple). In this presentation, the side chains of the residues involved in the inter-domain interactions are drawn in the stick model. Charge-charge and hydrogen bonding interactions are highlighted by dashed lines in yellow. (B) Surface representation showing the GK/p-LGN18 interface. In this drawing, the hydrophobic residues are in yellow, the positively charged residues are in blue, the negatively charged residues are in red, and the rest of the amino acids are in grey. The side chains of the residues from p-LGN18 peptide (purple) involved in binding to GK are drawn in the stick model. The orientation of the structure is the same as in (A). (C) The amino acid sequence conservation map of the residues from DLG GKs involved in the p-LGN18 binding. The conservation map is calculated based on the sequence alignment of DLG, CASK, MPP, and MAGI GK domains shown in Supplementary Figure 1 using the Scorecons Server (Valdar, 2002). In this drawing, the highly conserved residues (with score >0.8) are drawn in green, the conserved residues (with score >0.5) in light green, and the rest in grey. We arbitrarily divide the p-LGN18-binding site on SAP97 GK into the 'Phospho-site' and the 'Specificity-site' as indicated by dashed circles in red. (D) Summary of the quantitative binding constants between various SAP97/PSD-95 SH3-GK fragments and different LGN/AGS3 peptides. All quantitative binding data were derived from fluorescence-based titration assays following the method described for Figure 1E.

with an abundance similar to that of PSD-95 and each accounts for $>5\%$ of total PSD proteins (Walikonis *et al*, 2000; Peng *et al*, 2004; Sheng and Hoogenraad, 2007; Feng and Zhang, 2009). Understanding of the molecular basis governing the PSD-95/DLGAP1 interaction is important for understanding the functional roles of DLGAP1 and its isoforms in synaptic plasticities, the potential involvement of DLGAP3 in obsessive-compulsive disorder caused by the deletion of DLGAP3 (Welch *et al*, 2007), and DLGAP-mediated neuronal polarity development (Manneville *et al*, 2010). However, biochemical evidence supporting the direct

binding between DLGAP1 and PSD-95 SH3-GK has eluded us in the past, although the PSD-95 GK-binding domain of DLGAP1 has been narrowed to the N-terminal five 14 aa-repeats (Kim *et al*, 1997). With the discovery of the specific phosphorylation-dependent interaction of LGN to DLG SH3-GK tandems, we revisited the DLGAP1/PSD-95 SH3-GK interaction. Amino acid sequence alignment analysis immediately revealed many similarities between the 5 N-terminal 14 aa-repeats and LGN18 (Figure 5A). Specifically, each DLGAP1 14 aa-repeat contains absolutely conserved Ser and Arg residues, which are aligned perfectly with

Figure 4 Comparison of the SAP97 SH3-GK/p-LGN18 (A), PSD-95 SH3-GK/GMP (B), and the yeast GK/GMP (C) interaction interfaces. Amino acid residues, which are involved in direct contact with the phosphate groups in the complexes, are drawn in the stick model. Charge-charge and hydrogen bonding interactions are highlighted by dashed lines in yellow. The PDB access code for the yeast GK/GMP complex is 1EX7 (Błaszczuk *et al*, 2001).

Ser(0) and Arg(-3) of LGN18. Additionally, each DLGAP1 14 aa-repeat also contains two hydrophobic residues immediately following Ser(0). These two hydrophobic residues can align well with Met(+1) and Leu(+4) of the LGN18 peptide if we assume that the corresponding sequences in 14 aa-repeats adopt extended structures instead of an α -helix, as in p-LGN18 (Figure 5A). The above analysis suggests that the DLGAP1 14 aa-repeats are likely to bind to PSD-95 SH3-GK in a phosphorylation-dependent manner. We chose two repeats (DLGAP1-R2 and 3) to test this prediction. Exactly as we predicted, both the p-DLGAP1-R2 and p-DLGAP1-R3 peptides bind to PSD-95 SH3-GK with affinities comparable to that of p-LGN18 (Figure 5B). In sharp contrast, the non-phosphorylated DLGAP1-R2 peptide bound very weakly to PSD-95 SH3-GK ($K_d \sim 143 \mu\text{M}$). On the basis of the structure of the SAP97 SH3-GK/p-LGN18 complex, we built a structure model of the SAP97 SH3-GK/p-DLGAP1-R2 complex (Figure 5C). In this model, pSer(0) of p-DLGAP1-R2 occupies the GMP-binding pocket of SAP97 GK as pSer(0) of p-LGN18 does. Residues immediately C-terminal to pSer(0) adopts an extended structure, allowing Tyr(+1) and Leu(+2) of p-DLGAP1-R2 to occupy positions in the shallow hydrophobic pocket of GK analogous to Met(+1) and Leu(+4) of p-LGN18 (Figure 5C). Thr(+5) and Pro(+7) of p-DLGAP1-R2 make additional hydrophobic contacts with GK, just as Met(+7) and Leu(+9) of p-LGN18 do (Figure 5C). To validate the structural model of the SAP97 SH3-GK/p-DLGAP1-R2 complex, we showed that the changes of the residues at the Phospho-site (the 'R755,758G/Y767,796A' mutant) and the shallow hydrophobic pocket (the 'P751K,G789Y' mutant) of SH3-GK eliminated its binding to p-DLGAP1-R2 (Figure 5C and D). We have tested the role of the hydrophobic residues in the +2 and +5 positions of the DLGAP1-R2/3 in binding to PSD-95 SH3-GK by substituting the Leu(+2) and Val(+5) of DLGAP1-R3 with Gln. The mutant p-DLGAP1-R3 peptide binds to PSD-95 SH3-GK with a ~ 10 -fold lower affinity than the WT peptide does (Supplementary Figure 5). Finally, amino acid sequence analysis reveals that all members of the DLGAP family contain highly similar DLG SH3-GK-binding sequence motifs in their N-termini (Figure 5E), suggesting that the phosphorylation-dependent binding to DLGs is a property common to all isoforms of DLGAPs.

With our successful prediction of DLGAP1 as a phosphorylation-dependent binder of DLG, we wondered whether we might be able to extend this prediction to other previously identified DLG-interacting proteins. We carefully analyzed amino acid sequences of two additional DLG-binding proteins, namely a Rap-specific GTPase-activating protein called SPAR (Pak *et al*, 2001) and a synaptic scaffold protein BEGAIN (Deguchi *et al*, 1998). To our delight, we found that both of these proteins contain a highly similar DLG GK-binding sequence motif (Figure 5A). Importantly, all of these predicted DLG GK-binding sequences are highly conserved in vertebrates (data not shown). We chose the DLG GK-binding motif of SPAR to test our prediction. Indeed, a phospho-SPAR peptide ('PLRRPpSYTLGMKSL') binds to PSD-95 SH3-GK with a K_d of $\sim 15 \mu\text{M}$ (Figure 5F). The slightly weaker affinity of the phospho-SPAR peptide for PSD-95 SH3-GK is likely due to a Pro at the -1 position, as this Pro would introduce an unfavourable backbone torsion angle in restricting Arg(-3) to stabilize the phosphate group of pSer(0) as shown in Figure 4A. With the above findings, we conclude that the majority of previously identified DLG GK/target interactions probably occur via the GK-binding sequence motif identified in this study in a phosphorylation-dependent manner.

Other MAGUK GK domains

MAGUKs can be divided into 11 sub-families (Velthuis *et al*, 2007; Mendoza *et al*, 2010). Every member of the MAGUK family proteins contains one GK domain (Figure 6A). On the basis of the structural and biochemical data obtained from the DLG GK/target interactions described above, we analyzed possible target binding properties of the GK domains from other MAGUKs. We performed a detailed structure-based amino acid sequence alignment analysis of all MAGUK family GK domains (Supplementary Figure 1), and selected the whole DLG family and one representative member from each of the other MAGUK subfamilies for detailed comparison in Figure 6B. In this sequence alignment analysis, the residues forming the pSer/pThr-recognition Phospho-site of the GK domains are boxed and shaded in yellow, and the residues forming the target binding Specificity-site are boxed and highlighted in sky-blue (Figure 6B). All of the DLG GKs contain absolutely conserved amino acid residues in the both

Figure 5 Phosphorylation-dependent bindings of PSD-95 SH3-GK to DLGAP1 and SPAR. (A) Sequence alignment of the GK-binding motif of LGN with various fragments identified in previously identified DLG GK-binding proteins including DLGAP1, SPAR, and BEGIN. All five human DLGAP1 14 aa-repeat sequences are included in the alignment. In this alignment, the absolutely conserved amino acids are highlighted in red, and the highly conserved residues are in green. Residues from LGN involved in direct contact with the GK domain are marked with orange triangles. The corresponding positions are labelled at the top of the sequences. (B) Fluorescence polarization-based measurement of the binding affinities of PSD-95 SH3-GK to the phosphorylated- or nonphosphorylated DLGAP1-R2 and -R3 peptides. The insert shows the expanded view of the binding curves of p-DLGAP1-R2, p-DLGAP1-R3, and a p-DLGAP1-R3 peptide with pSer(0) replace with pThr to PSD-95 SH3-GK. (C) The SAP97 SH3-GK/p-DLGAP1-R2 complex structural model derived from docking-based model building. In this drawing, the GK domain is shown in pink ribbon, and the p-DLGAP1-R2 is drawn with the stick model in blue. (D) Comparison of the bindings of the two SAP97 SH3-GK mutants to p-DLGAP1-R2 with that of the wild type SH3-GK to p-DLGAP1-R2. (E) Amino acid sequence alignment of DLG GK binding DLGAP N-terminal repeats from the different isoforms of DLGAPs from human and zebrafish. For simplicity, we chose to include DLGAPs from human only in the alignment as all DLGAPs from mammals are highly conserved. The colouring scheme of the alignment is the same as in (A). (F) Fluorescence polarization-based measurement of the binding affinity of PSD-95 SH3-GK to a 14-residue p-SPAR peptide with sequence derived from the analysis shown in (A).

Phospho- and Specificity-sites (Figure 6), and thus the DLG GKs should share common phospho-target binding sequence motif shown in Figure 5A. As we have demonstrated that the

the residues forming the Phospho-site are absolutely required for DLG GK/target interactions, the sequence alignment shown in Figure 6B immediately reveals that the ZO-,

Figure 6 GK domains from other MAGUKs are likely to function as phosphor-protein-binding modules. (A) Domain organization of the 11 sub-families of MAGUK scaffolds. (B) Structural-based sequence alignment of the pSer/pThr-binding site and Specificity-site of MAGUK GK domains. In this alignment, the absolutely conserved amino acids are highlighted in red, and the highly conserved residues are in green. Corresponding residues forming the pSer/pThr-site in SAP97 GK domain are highlighted with yellow boxes, and corresponding residues forming the Specificity-site are shaded with cyan boxes. The secondary structures and the residues numbers matching the SAP97 GK domain are labelled at the top of the alignment. An expanded version of the structure-based sequence alignment analysis of MAGUK GK domains can be found in Supplementary Figure 1.

DLG5-, CARMA-, and β -subunit calcium-sub-families of MAGUK GKs are not likely to bind to phospho-target proteins, due to the lack of critical phosphate recognition residues in their Phospho-site. The CASK-, MPP-, and MAGI-sub-family MAGUKs all contain complete pSer-binding pockets, and thus these MAGUK GKs are likely to bind to their targets in a phosphorylation-dependent manner. It is further noted that the residues in the Specificity-site of the CASK, MPP, and MAGI GK domains are distinctly different from those of DLG GKs, indicating that CASK, MPP, and MAGI GKs are likely to recognize very different amino acid residues surrounding the pSer. On the basis of the above analysis, we predicted a possible CASK SH3-GK-binding peptide sequence of whirlin, which is a reported binding target of CASK (Mburu *et al*, 2006). Consistent with this prediction, this whirlin peptide ('GQTL(pS)EDSGVDAGEA') binds to CASK SH3-GK only when the Ser at the position 0 is phosphorylated (Supplementary Figure 6). It is further noted that the amino acid sequence of the p-whirlin peptide is quite different from that of p-LGN18.

Discussion

We discover in this study that the GK domains of SAP97 and PSD-95 function as specific phospho-Ser-binding modules.

Analysis of the structure of the SAP97 SH3-GK/p-LGN18 peptide complex (Figure 3A) and the structural model of the PSD-95 SH3-GK/p-DLGAP-R2 complex (Figure 5C) indicate that the substitution of pSer(0) with pThr will not introduce obvious steric hindrance in the Phospho-site of DLG GKs. To confirm this analysis, we measured the PSD-95 SH3-GK-binding affinity of a pThr-substituted p-DLGAP1-R3 peptide. The pThr-containing DLGAP1-R3 peptide binds to PSD-95 SH3-GK with a K_d of $\sim 0.8 \mu\text{M}$ (Supplementary Figure 5). Thus, we conclude that the DLG GK domains are capable of specifically binding to either pSer- or pThr-containing peptides. We further show that the specific pSer/pThr binding of MAGUK GK domains are likely evolved from the GMP-binding pocket of their ancestor, the GMP-GDP conversion enzyme GK. Our structure-based amino acid analysis also suggests that binding to phosphorylated protein targets is likely to be a property common to the most MAGUK family scaffold proteins. The finding that MAGUK GK domains function as specific phospho-peptide-binding modules expands the repertoire of the phospho-peptide-binding modules (Yaffe and Elia, 2001).

Our discovery of the phosphorylation-dependent interaction between DLG1 and LGN, together with an earlier finding of the aPKC-mediated phosphorylation of Ser401 in the LGN linker (Hao *et al*, 2010), suggests a direct functional

connection between the Par-3/Par-6/aPKC complex and the Gai/LGN/Numa complex in the regulations of cell polarities as well as asymmetric cell divisions. For example, in 3D MDCK culture cells and chick neuroepithelia, both Gai-mediated targeting of LGN to cell cortex and cortical aPKC activities are required for proper mitotic spindle orientations and planar cell divisions (Zheng *et al*, 2010; Peyre *et al*, 2011). The planar cell divisions in these two epithelial systems require a lateral belt of LGN to guide the mitotic spindles. Since DLG is well-known to be laterally localized (Bilder *et al*, 2000; Hanada *et al*, 2003), it is highly likely that the formation of the lateral LGN belt is mediated by the interaction between the laterally localized DLG and aPKC-phosphorylated LGN. In *Drosophila* neuroblasts, the apically localized DLG is required to couple mitotic spindle pole to the apical Par3/Par6/aPKC and Gai/Pins complexes via the DLG GK/Pins interaction (Siegrist and Doe, 2005; Johnston *et al*, 2009). Again, the aPKC-mediated phosphorylation of Pins at Ser436 likely serves as the regulatory switch for the assembly of the DLG/Pins/Gai complex at the apical cortex, which in return couples with the apical spindle pole via the Pins/Mud interaction (Siegrist and Doe, 2005; Izumi *et al*, 2006; Zhu *et al*, 2011).

The discovery of the phosphorylation-dependent interaction between DLGs and DLGAPs has major implications for understanding of synaptic protein complex organizations. Both DLGs and DLGAPs are highly abundant, multi-modular scaffold proteins in excitatory synapses (Sheng and Hoogenraad, 2007; Feng and Zhang, 2009). The phosphorylation-dependent formation of DLG/DLGAP1 complexes provides a mechanistic switch for the activity-regulated, dynamic assembly and disassembly of protein complexes in synapses, which is likely connected with the molecular basis of synaptic plasticity in animal brains. The phosphorylation-dependent, regulated DLG/DLGAP1 interaction may also shed light on the mechanisms by which deletion of DLGAP3/SAPAP3 cause obsessive-compulsive disorder-like disease in the mouse model (Welch *et al*, 2007). It should be pointed out that our work reveals that, from a biochemical standpoint, the interaction between DLGs and DLGAP1 is phosphorylation-dependent. It is critically important to establish whether this interaction is indeed regulated by phosphorylation in animal brains, and if so, which protein kinases participate in this regulation. By scanning the DLG-binding DLGAP1 repeat sequences using Scansite (<http://scansite.mit.edu/>), protein kinase A, aPKC, PKB, and AKT came up as possible candidate kinases. Given the high amino acid sequence identity and structure similarity between the DLGAP1 N-terminal repeats and LGN18 and the fact that LGN can be robustly phosphorylated by aPKC, we predict that DLGAP1 may also be phosphorylated by aPKC as well. Further work is required to experimentally test the above predictions.

Materials and methods

Antibodies

Anti-GFP antibody was purchased from Santa Cruz; anti-Myc antibody was from DSHB, and anti-His-tag antibody was purchased from Sigma.

Protein expression and purification

Rat SAP97 SH3-GK (aa 578–911), SAP97 GK (aa 719–911); human PSD-95 SH3-GK (aa 426–724), PSD-95 GK (aa 531–724); and the

LGN linker (aa 374–479) were individually cloned into the pET-15b vector. All the mutations used in this study were created using the standard PCR-based mutagenesis method and confirmed by DNA sequencing. Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) host cells at 16°C, and purified by using a Ni²⁺-NTA agarose affinity chromatography followed by size-exclusion chromatography. For *in vitro* biochemical analysis, SAP97 SH3-GK and PSD-95 SH3-GK were expressed as the GST-fused proteins and purified by GSH-Sepharose affinity chromatography.

Co-immunoprecipitations

For immunoprecipitation of overexpressed proteins, HEK293T cells were co-transfected with the GFP-tagged full-length LGN and the Myc-tagged full-length SAP97. Cells were harvested 24 h post-transfection and divided equally into two tubes. Cells were lysed in a lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EGTA, and 1% Triton) with and without a cocktail of phosphatase inhibitors (100 mM sodium fluoride, 10 mM β -glycerophosphate, 20 mM PNPP, and 50 μ M sodium vanadate) for 30 min at 4°C. Each cell lysate was incubated with 50 μ l anti-Myc antibody-coupled Protein G beads (50% slurry). After incubation for 2 h at 4°C, the beads were washed with the lysis buffer extensively. The captured proteins were then eluted by the SDS-PAGE sample buffer and immune-detected with specific antibodies.

GST pull-down assay

For the GST pull-down assays, GST-tagged DLG SH3-GKs (50 μ l from 1 mg/ml stock solutions) were first loaded to 30 μ l GSH-sepharose 4B slurry beads in an assay buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA). The GST fusion protein loaded beads were then incubated with GFP-aPKC-treated or -untreated potential binding partners for 2 h at 4°C. After three times washing, the proteins captured were eluted by boiling, resolved by 15% SDS-PAGE, and detected by western blot.

In vitro kinase assay

About 1 mg/ml purified His-tagged WT mouse LGN linker (aa 374–479) and S401A mutant were dialyzed in the kinase buffer containing 20 mM MOPS (pH 7.4), 15 mM MgCl₂, and 2 mM DTT. GFP-tagged aPKC was transfected in HEK293T cells and harvested 24 h after transfection. Cell lysate was incubated with GFP antibody-coupled Protein G beads. Beads were washed with the lysis buffer and the kinase assay buffer, respectively. GFP-tagged aPKC was eluted with purified GFP protein. In each kinase assay, 10 μ l of each protein sample was mixed with 10 μ l of affinity purified GFP-tagged aPKC. The mixture was incubated at 25°C for 1 h in the kinase assay buffer containing 2 μ Ci [γ -³²P]-ATP (PerkinElmer) and 0.1 mM nonradioactive ATP. [³²P] phosphate labelling of bound proteins was detected by SDS-PAGE electrophoresis followed by autoradiography.

Fluorescence assay

Fluorescence assays were performed on a PerkinElmer LS-55 fluorimeter equipped with an automated polarizer at 25°C. In a typical assay, a FITC (Molecular Probes)-labeled peptide (~1 μ M) was titrated with each binding partner in a 50 mM Tris pH 8.0 buffer containing 100 mM NaCl, 1 mM DTT, and 1 mM EDTA. The K_d values were obtained by fitting the titration curves with the classical one-site binding model.

Crystallization, data collection, and processing

Crystals of SAP97 SH3-GK tandem in complex with the p-LGN18 peptide were obtained by the hanging drop vapour diffusion method at 16°C. Freshly purified SAP97 SH3-GK was concentrated to 10 mg/ml before a saturating amount of p-LGN18 was added. The SAP97 SH3-GK/p-LGN18 complex was grown in 0.2 M ammonium nitrate, 20% w/v polyethylene glycol 3350. Glycerol (25%) was added as the cryo-protectant. A 2.7 Å resolution X-ray data set was collected at the beam-line BL17U1 of the Shanghai Synchrotron Radiation Facility. The diffraction data were processed and scaled by HKL2000 (Otwinowski and Minor, 1997).

Using the structure of the PSD-95 SH3-GK tandem (PDB code: 1KJW) as the search model, the initial structural model was obtained using the molecular replacement method in PHASER (McCoy *et al*, 2007). The model was then refined by the phenix.refinement (Adams *et al*, 2010). COOT (Emsley and Cowtan, 2004) was used for the peptide modelling and model adjustments.

TLS refinement was applied at the final refinement stage. The final structure was validated by the phenix.model_vs_data (Adams *et al*, 2010) validation tools. The final refinement statistics are listed in Table I. The structure figures were prepared using the program PyMOL (<http://www.pymol.org/>).

The SAP97 SH3-GK/p-DLGAP1-R2 complex model building

To build the SAP97 SH3-GK/p-DLGAP1-R2 complex model, the residues in the p-DLGAP1-R2 were first aligned with the corresponding residues of p-LGN18 in the SAP97 SH3-GK/p-LGN18 structure. After changing the residues of the p-LGN18 peptide into those of the p-DLGAP1-R2 peptide using COOT, FlexPepDocking from Rosetta (Raveh *et al*, 2010) was used to calculate 200 refined docking models. These 200 structures of the SAP97 SH3-GK/p-DLGAP1-R2 complex model converged well (Supplementary Figure 4), and the top 20 structure models with the lowest energy functions were selected for analyses.

Coordinates

The atomic coordinates of SAP97 SH3-GK/p-LGN18 complex have been deposited to the Protein Data Bank under the accession codes 3UAT.

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Supplementary data

Supplementary data are available at *The EMBO Journal* online (<http://www.embojournal.org>).

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Author contributions: JZ, YS and CX performed experiments; JZ, YS, W Wang, W Wen and MZ analyzed data; JZ, YS, W Wen and MZ designed experiments and wrote the paper; and MZ coordinated the project.

Conflict of interest

The authors declare that they have no conflict of interest.

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