Ste20-like Protein Kinase SLK (LOSK) Regulates Microtubule Organization by Targeting Dynactin to the Centrosome

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ABSTRACT

A microtubule- and centrosome-associated Ste20-like kinase SLK (LOSK) regulates cytoskeleton organization, cell's polarization and spreading. Its inhibition causes microtubule disorganization and release of centrosomal dynactin. The major function of dynactin is minus-end-directed transport along microtubules in a complex with dynein motor. Also, dynactin is required for maintenance of microtubule radial array in interphase cells, and depletion of its centrosomal pool entails microtubule disorganization. Here we demonstrate that SLK (LOSK) phosphorylates p150Glued subunit of dynactin and thus targets it to the centrosome, where it maintains microtubule radial organization. We show that phosphorylation is required only for centrosomal localization of p150Glued and does not affect its microtubule-organizing properties: artificial targeting of non-phosphorylatable p150Glued to the centrosome restores microtubule radial array in cells with inhibited SLK (LOSK). Phosphorylation site is located in a microtubule-binding region that is variable for two isoforms (1A and 1B) of p150Glued expressed in cultured fibroblast-like cells (1B isoform lacks 20 a.a. in the basic microtubule-binding domain). The fact that SLK (LOSK) phosphorylates only a minor 1A isoform of p150Glued suggests that transport and microtubule-organizing functions of dynactin are distinctly divided between the two
isoforms. Also, we show that dynactin phosphorylation is involved in Golgi reorientation in polarized cells.

INTRODUCTION

Microtubules in interphase cells are organized into a radial array with the minus-ends focused in the centrosome, and plus-ends directed towards cell’s periphery. Such an array maintains polarized transport of molecules and organelles driven by motor proteins. However the molecular mechanisms that regulate radial organization of microtubules still remain unknown. A typical microtubule-organizing center in fibroblast-like cultured cells is represented by the centrosome, where microtubules are nucleated and anchored. Gamma-tubulin ring complexes (gamma-TuRCs) nucleate microtubules and can remain bound to their minus-ends further on (Wiese and Zheng, 2000, 2006; Anders and Sawin, 2011). However gamma-tubulin is not the only anchor of microtubules at the centrosome. Depletion of other centrosomal proteins (ninein (Mogensen et al., 2000, Dammermann and Merdes, 2002; Delgehyr et al., 2005; Ibi et al., 2011), 4.1R (Perez-Ferreiro et al., 2004), Nlp (Casenghi et al., 2005), CAP350 and FOP (Yan et al., 2006), and TACC3/maskin (Albee and Wiese, 2008)) causes randomization of microtubules in cells, although the centrosome retains its nucleating activity, which can be visualized by tracking plus-end binding proteins (EB1 and EB3) at growing ends of microtubules. Inhibition of some dynein-binding proteins such as Bicaudal D and Nudel (Fumoto et al., 2006; Guo et al., 2006), and dynein-dynactin complex does not affect microtubule nucleation at the centrosome (Zhapparova et al., 2007; Burakov et al., 2008a), but disturbs anchoring. Rapid disorganization of microtubules after the inhibition of dynein-dependent transport might result from the compromised delivery of anchoring proteins to the centrosome (Fumoto et al., 2006). In case of dynactin inhibition, the centrosome still contains its major proteins: gamma-tubulin, pericentrin, centrin, ninein, but allows the ability to anchor microtubules (Burakov et al., 2008 a,b). These data indicate that the role of dynactin in microtubule organization is not limited to its transport function.

Dynactin is considered as a multisubunit cofactor of dynein, which ensures cargo binding and processivity of the motor protein (King and Schroer, 2000; Schroer 2004). One of the subunits, p150Glued, contains a MT-binding region (CAP-Gly and basic domains) and can interact with microtubules independently of dynein (Waterman-Storer et al., 1995; Culver-Hanlon et al., 2006; Weisbrich et al., 2007). Therefore, dynactin might contribute to MT organization either by direct or indirect microtubule anchoring at the centrosome, and/or by regulation of dynein activity and dynein-mediated transport of other anchoring proteins to the
centrosome. In either case, depletion of centrosomal dynactin results in inevitable disorganization of microtubules (Quintyne et al., 1999; Kim et al., 2007). Two subunits of the complex (p50 and p150Glued) were shown to interact with centrosomal proteins (Cep135 and PCM1, respectively) (Uetake et al., 2004; Kodani et al., 2010), but the regulation of dynactin localization at the centrosome is far from understanding.

Previously we showed that depletion of centrosomal dynactin and randomization of microtubules were observed after the inhibition of protein kinase LOSK (Long Ste20-like kinase, also called SLK) (Burakov et al., 2008b). SLK (LOSK) is a Ser/Thr-protein kinase associated with the centrosome and microtubules (Zinovkina et al., 1997, 1998; Itoh et al., 1997; Sabourin and Rudnicki, 1999; Yamada et al., 2000; Nadezhdina et al., 2001). Its inhibition results in disorganization of microtubules and disturbed polarization and motility of cells (Burakov et al., 2008b; Roovers et al., 2009). SLK phosphorylates several cytoskeletal proteins: human RhoA (Guilluy et al., 2008), paxillin (Quizi et al., 2012), and, presumably, ezrin (Viswanatha et al., 2012). However, none of them is involved in centrosome-mediated microtubule organization. Regarding the fact that inhibition of SLK resulted in the dramatic decrease of centrosomal dynactin (Burakov et al., 2008b), we suggested that dynactin might mediate the effect of SLK on microtubules.

RESULTS

SLK (LOSK) phosphorylates p150Glued

To test whether SLK could directly phosphorylate dynactin, we purified dynactin from bovine brain, and incubated it with the recombinant kinase domain of SLK (SLKCD) in the presence of 32P-labeled ATP. The label was incorporated into a 150 kDa band (Figure 1A), also recognized by anti-p150Glued antibodies in Western blot (data not shown). We assumed that it might have been a p150Glued subunit of the dynactin complex.

Dynactin phosphorylation was further studied with in vitro kinase assays using recombinant fragments of p150Glued. Two isoforms of p150Glued (1A and 1B) with molecular weight 150 kDa were described previously (Dixit et al., 2008; Zhapparova et al., 2009). They vary in the length of basic microtubule-binding region. We tested GST-fused recombinant fragments of the N-terminal parts of p150Glued-1A with a full-length basic domain (a.a. 1-157) and -1B without the basic domain (a.a. 1-137), and the ΔN-p150Glued fragment (a.a. 151-1281) shared by both isoforms. 32P-phosphate was incorporated only in the N-terminal part of 1A isoform (Figure 1B), indicating that the site of phosphorylation was located somewhere within the variable region. Phosphorylation of the N-terminal part of p150Glued-1A was also confirmed in a kinase assay with cold ATP and anti-phosphothreonine antibodies (Figure 1C).
SLK belongs to the family of serine/threonine protein kinases. The variable region of p150Glued does not contain serine residues, but comprises four threonines: in 141, 145, 146, and 147 positions. We obtained two mutants, where threonines were substituted for alanine: T141A and T145-147A, and tested them in a kinase assay. The label was still incorporated into T141A mutant (data not shown), but not in T145-147A mutant (Figure 1B), indicating that the phosphorylation site was located within threonines 145-147. To define which of three threonines was indeed phosphorylated, we obtained a series of mutants: T145A, T146A and T147A, and performed a kinase assay (Figure 1D). Phosphate incorporated in all three mutants demonstrating that each of three threonines could be phosphorylated (Figure 1D).

Simultaneous phosphorylation of adjacent amino acids is a rare phenomenon, which was described for a few viral proteins (Scheidtmann et al., 1982). It is intriguing, whether SLK phosphorylates all three threonines, or one or two of them, and whether phosphorylation status of dynactin varies under different conditions. We hope to elucidate it in our further research.

Here we studied the effect of dynactin phosphorylation using triple mutants of p150Glued (T145-147A and T145-147E). The identified phosphorylation site in p150Glued (140PTARKT*T*T*RRPKP) has common features with SLK sites in paxillin (Quizi et al., 2012), and RhoA (Guilluy et al., 2008) (Figure 1E), although none of these sites coincides with the consensus site defined by peptide library analysis (X-X-X-Y-X-T-Φ-R/K-X-X-X (Pike et al., 2008), where Φ is aliphatic amino acid) (Figure 1E).

**p150Glued mutants vary in the affinity for microtubules in vitro and in vivo**

Since p150Glued was phosphorylated at the MT-binding domain, we suggested that this phosphorylation might affect the interaction with microtubules. To test it, we performed a copelleting assay. GST-fused N-terminal fragments (1-157 a.a.) of Ala-p150Glued (T145-147A) and Glu-p150Glued (T145-147E) mutants were incubated with taxol-stabilized microtubules, and pelleted through a glycerol cushion (Figure 2A). Glu-mutant exhibited slightly reduced affinity for microtubules compared to Ala-mutant: at 0.8 µM concentration Ala-p150Glued was found only in microtubule pellet, while Glu-p150Glued was also detected in supernatant (Figure 2A). At 2 µM concentration, Ala-p150Glued was found in the pellet and in the supernatant as well, indicating the saturation of microtubule binding. Wild-type p150Glued-1A exhibited similar affinity for microtubules, as Ala-p150Glued: it bound microtubules tightly and was detected mostly in pellet (Figure 2A, Suppl. Table and Zhapparova et al., 2009).

Then we analyzed the interaction of mutants with microtubules in vivo. We expressed GFP-fused full-length p150Glued mutants in Vero cells, and analyzed their distribution along the microtubules (Figure 2B). Consistently with the in vitro results, Ala-mutant demonstrated higher affinity for microtubules and decorated them along the entire length (Figure 2B). Glu-mutant
formed long comets at microtubule plus-ends, but rarely covered the entire microtubules. The localization pattern was independent of the level of recombinant protein expression (data not shown).

The Glu-mutant of p150Glued exhibits higher affinity for the centrosome

The activity of protein kinase SLK and the presence of dynein-dynactin complex at the centrosome are required for the maintenance of microtubule radial array (Quintyne et al., 1999; Burakov et al., 2008b). Considering p150Glued as a substrate of SLK, we studied its interaction with the centrosome regarding phosphorylation status. We expressed GFP-fused full-length Ala- and Glu-p150Glued mutants in Vero cells, and visualized centrosome with anti-gamma-tubulin antibodies (Figure 3A). Strikingly, Ala-p150Glued, which exhibited higher affinity for microtubules, was located at the centrosome only in 36% of cells. At the same time, Glu-p150Glued was concentrated at the centrosome in 64% of cells, indicating that centrosomal localization of p150Glued was regulated by SLK-dependent phosphorylation (Figure 3, A and B).

Glu-mutant of p150Glued restores radial organization of microtubules in cells with inhibited SLK (LOSK)

To reveal the impact of p150Glued phosphorylation on the organization of microtubules, we examined the system of microtubules in cells with inhibited SLK. As we have shown previously, expression of dominant-negative SLK (K63R) disrupts radial organization of microtubules in 80% of cells and entails marked depletion of centrosomal dynactin (Burakov et al., 2008b). Here we coexpressed dsRed-tagged dominant-negative SLK and GFP-fusion either of Ala- or Glu-p150Glued. Glu-p150Glued markedly restored radial organization of microtubules in 53% of Vero cells, while Ala-p150Glued had no effect, and only 22% of Ala-p150Glued cells contained radial array of microtubules (Figure 4, A and B). It should be noted here that the expression of a full-length p150Glued itself induces randomization of microtubules in 58% of cells (Suppl. Figure 1). The effect might be caused by coiled-coil 1 region of p150Glued, which is considered to be responsible for dynein-dynactin interactions (Quintyne et al., 1999). Therefore, the results of coexpression should be compared with corresponding controls of individual Ala- and Glu-p150Glued expression. Notably, Ala-p150Glued itself caused randomization of microtubules in 70% of cells (Figure 4B), indicating that non-phosphorylatable p150Glued could not maintain radial organization of microtubules.

In some cases, microtubule randomization in cells might result from the expression of microtubule-binding proteins, which stabilize microtubules. To test whether such an effect was observed in our system, we expressed GFP-fused wild-type p150Glued-1A and p150Glued-1B. These isoforms vary in the length of microtubule-binding region and have different affinity for microtubules (Zhapparova et al., 2009). 1A isoform, similarly to Ala-p150Glued, had higher
affinity for microtubules, but did not cause such dramatic randomization of microtubules as the mutant protein (Supplementary table and Supplementary figure 1). These data indicate that binding of dynactin to microtubules is not enough for their randomization.

**SLK (LOSK) regulates microtubule organization by targeting p150<sup>Glued</sup> to the centrosome**

The obtained data raise a question about the exact function of dynactin phosphorylation. Phosphorylation might be required only for targeting dynactin to the centrosome (i) or it can affect interactions with microtubules and putative mediator proteins (ii). If the first assumption is true, artificial targeting of non-phosphorylatable p150<sup>Glued</sup> to the centrosome would restore microtubule array in cells with inhibited SLK.

To verify the hypothesis, we fused Ala-p150<sup>Glued</sup> to the PACT domain of AKAP450 protein. PACT-domain is responsible for centrosomal localization of AKAP450 and pericentrin, and its fusion to GFP results in clear centrosomal localization of the latter (Gillingham and Munro, 2000). We expressed full-length GFP-fused Ala-p150<sup>Glued</sup> carrying PACT domain at either N- or C-terminus (Figure 5A). Both proteins accumulated at the centrosome of more than 90% cells (compare to 34% cells in case of Ala-p150<sup>Glued</sup> without PACT domain) (Figure 5A). Then we coexpressed the described constructs and dominant-negative SLK, and examined microtubule organization (Figure 5B). It turned out that PACT-Ala-p150<sup>Glued</sup> restored microtubule array in 47% cells (compare to 50% and 23% in case of Glu-p150<sup>Glued</sup> and Ala-p150<sup>Glued</sup>, respectively) (Figure 4B). Expression of GFP-fused PACT had no effect on microtubule organization (data not shown). The obtained data indicate that phosphorylation itself is required only for dynactin targeting to the centrosome, but not for its interactions with microtubules or microtubule-interacting proteins there.

Unexpected results were obtained with Ala-p150 carrying PACT domain at the C-terminus (Figure 5, A and B). It was unable to restore microtubule array in cells with inhibited SLK, although the protein exhibited clear centrosomal localization (Figure 5A and 4B). Moreover, microtubules were disorganized in 80% cells expressing Ala-p150-PACT chimera indicating either involvement of p150<sup>Glued</sup> C-tail in the interaction with some proteins or importance of dynactin proper orientation in pericentriolar matrix.

To verify the importance of C-terminal interactions for microtubule organization we expressed Glu-p150<sup>Glued</sup> fused to PACT domain at the C-terminus. As we showed in previous experiments, expression of Glu-p150<sup>Glued</sup> successfully restored microtubule array in cells with inhibited SLK. However, expression of Glu-p150<sup>Glued</sup>-PACT caused microtubule randomization and did not restore radial array in cells with inhibited SLK (Figure 5B and 4B).
In cells, p150\textsuperscript{Glued} functions as a part of dynactin complex, and inhibition of SLK results in the release of all dynactin subunits from the centrosome (Burakov et al., 2008). Here we have demonstrated that phosphorylation of p150\textsuperscript{Glued} is required for its centrosomal localization and for the maintenance of microtubule radial array. Next we studied whether other dynactin subunits were implicated in this process. The ability of p150\textsuperscript{Glued} mutants to interact with other dynactin subunits was verified by immunoprecipitation (Suppl. Figure 2). Then cells expressing dominant-negative SLK and Glu-p150\textsuperscript{Glued} were immunostained for dynamitin (p50) subunit. Dynamitin was recruited to the centrosome only in half of cells with centrosomal localization of Glu-p150\textsuperscript{Glued} (data \textit{not shown}). The absence of other dynactin subunits seems to have no effect on microtubule radial organization, once Glu-p150\textsuperscript{Glued} is located at the centrosome.

\textbf{Glu-p150\textsuperscript{Glued} rescues Golgi reorientation in polarized cells with inhibited SLK (LOSK)}

Along with the maintenance of microtubule array, SLK was shown to be required for Golgi reorientation in polarized cells (Burakov \textit{et al.}, 2008b). We examined whether this effect depended on dynactin phosphorylation. Polarized cells were obtained at the edge of experimental wound scratched in a monolayer of Vero cells. Normally, compact Golgi cisternae localize in the perinuclear region, and during polarization they are translocated towards the leading edge. Inhibition of SLK results in Golgi mislocalization in 80\% of cells (Burakov \textit{et al.}, 2008b; Figure 6, A and B). Coexpression of dominant-negative SLK and Glu-p150\textsuperscript{Glued} restored Golgi orientation in 60\% of cells. The observed orientation of Golgi was independent of microtubule organization: only 33\% of cells expressing Ala-p150\textsuperscript{Glued} had radial array of microtubules (Figure 4B), but over 60\% of such cells contained normally oriented Golgi. The p150\textsuperscript{Glued} mutants themselves had almost no effect on Golgi orientation.

Along with reoriented Golgi, polarized migrating fibroblast-like cells usually have their centrosomes translocated towards the leading edge (Euteneuer and Schliwa, 1992). Such localization facilitates microtubule growth towards the leading edge. We examined centrosome positioning in cells coexpressing dominant-negative SLK and one of p150\textsuperscript{Glued} mutants. In all cases centrosome was oriented as in non-transfected control (data \textit{not shown}). The result indicates that Golgi and centrosome, which usually migrate together, are uncoupled in cells with inhibited SLK.

\textbf{DISCUSSION}

Here we have unraveled a new regulatory mechanism of microtubule anchoring at the centrosome. We demonstrated that protein kinase SLK (LOSK), required for the maintenance of microtubule array, phosphorylates p150\textsuperscript{Glued} subunit of the dynactin complex. Phospho-mimicking mutant of p150\textsuperscript{Glued} exhibits high affinity for the centrosome and restores
microtubule array in cells with inhibited SLK. This mutant also restores Golgi polarization in cells with inhibited SLK at the edge of experimental wound.

Phosphorylation status of p150\textsuperscript{Glued} depends on cell signaling pathways (Farshori and Holzbaur, 1997). Although these authors detected only phosphoserine residues in the phosphorylated dynactin, p150\textsuperscript{Glued} phosphorylated at threonines is associated with the centrosome and thus might compose a minor fraction of the whole dynactin pool. Phosphorylation at Ser19 (presumably, by cAMP-dependent protein kinase) was shown to regulate p150\textsuperscript{Glued} interaction with microtubules (Vaughan et al., 2002). In prophase mammalian cells, polo-like kinase Plk1 phosphorylates p150\textsuperscript{Glued} at Ser179 located distally to the basic domain (Li et al., 2010) and thus regulates nuclear envelope breakdown. In mitotic Drosophila cells, dynamics of p150\textsuperscript{Glued} is regulated by Aurora A, which phosphorylates serines in the N-terminal microtubule-binding domain (Rome et al., 2010). It should be noted that Drosophila p150\textsuperscript{Glued} contains a short basic domain, which lacks the variable region with threonines (Zhapparova et al., 2009).

Dynactin can fulfill two possible functions: (1) deliver microtubule-anchoring proteins to the centrosome within dynein-dynactin complex (transport function) and (2) anchor microtubules directly via its MT-binding subunit p150\textsuperscript{Glued} or indirectly via some dynactin-binding proteins or even dynein (anchoring function). SLK does not affect transport function of dynactin, and the major proteins involved in microtubule anchoring retain their centrosomal localization (gamma-tubulin, pericentrin, ninein (Burakov et al., 2008b) and EB1 (data not shown)). However our results indicate that dynactin phosphorylation is definitely required for its microtubule-anchoring function. It seems that the cell contains at least two pools of dynactin with distinct functions. Such functional diversity might be explained by structural differences of p150\textsuperscript{Glued} isoforms that vary in microtubule-binding region (Dixit et al., 2008; Zhapparova et al., 2009). Fibroblasts and other non-neuronal cells express mostly p150\textsuperscript{Glued}-1B, which lacks a 20 a.a. region in basic MT-binding domain. A full-length 1A isoform is expressed in such cells in trace amounts and is located mostly in the centrosomal area, where its functions have remained unknown (Zhapparova et al., 2009). The fact that phosphorylation site is located within the variable region of two isoforms suggests that they might fulfill different functions in cells. The dominant isoform (p150\textsuperscript{Glued}-1B) might be responsible for intracellular transport, while the minor one (1A) might be implicated in microtubule organization.

The precise role of dynactin in microtubule anchoring at the centrosome still remains unknown: dynactin can directly hold microtubules or engage mediator proteins. We demonstrate here that phosphorylation is essential for targeting p150\textsuperscript{Glued} to the centrosome, although it does not affect centrosomal function. It seems that SLK phosphorylates dynactin and recruits it to the centrosome, where the increased amount of dynactin anchors more
microtubules and maintains microtubule radial organization more efficiently. Centrosomal proteins that interact with the phosphorylated basic domain of p150Glued are unknown, and further experiments are required for their identification.

Experiments with overexpression of Glu-p150Glued suggest that centrosomal localization of other dynactin subunits is not essential for microtubule radial organization, when p150Glued is targeted to the centrosome. However in vivo, the integrity of dynactin complex might be required for its transport to the centrosome and for its anchoring there. The mechanism of dyactin retention at the centrosome is still disputable. Until recently, a “shoulder” part of dynactin was considered to be responsible for binding to the centrosome. Dynamitin (p50) was shown to interact with centrosomal protein Cep135 (Uetake et al., 2004), and separation of “shoulder” and “arm” induced by overexpression of p24 subunit resulted in the retention of Arp1 filament and release of p150Glued from the centrosome (Quintyne et al., 1999). Recent data of Kodani et al. (2010) indicate that p150Glued interacts with PCM-1 and thus can bind the centrosome. Alternatively, p150Glued might have some other partners at the centrosome along with PCM-1.

Although p150Glued can directly interact with microtubules, our experiments with masking of C-terminus of p150Glued with PACT domain indicate that dynactin requires some additional proteins to hold a microtubule at the centrosome. C-tail of p150Glued interacts with cargo proteins during vesicular transport: Rab7-interacting lysosomal protein (RILP) (Johansson et al., 2007), transport protein particle TRAPPC9 (Zong et al., 2012), COPII proteins Sec23/Sec24 (Watson et al., 2005). However it is unlikely that these proteins are involved in microtubule organization at the centrosome. C-terminus of p150Glued also might be important for proper positioning of dynactin in a multicomponent and multilayer pericentriolar material (Lawo et al., 2012).

It should be noted that phosphorylation of p150Glued is required not only for microtubule organization, but is also involved in cell’s polarization. In non-neuronal cells 1A isoform of p150Glued is usually accumulated at the centrosome, whereas during cell’s polarization it binds to the microtubules directed towards the leading edge (Zhapparova et al., 2009). Our results demonstrate that SLK-dependent phosphorylation is required for proper orientation of Golgi in polarized cells. The observed phenomenon might be explained by the fact that formation of Golgi and its positioning in polarized cells depend on microtubules that grow from the centrosome (Vinogradova et al., 2012). Cells with inhibited SLK activity contain few such microtubules. Alternatively, the amount of phosphorylated p150Glued might be increased at microtubules directed towards the leading edge, since 1A isoform is actively recruited there during cell’s polarization (Zhapparova et al., 2009). Phosphorylated dynactin might specifically
interact with Golgi proteins and thus ensure reorientation of the organelle. However, we could not detect any differences in the localization of phospho-mimicking p150\textsuperscript{Glued} at microtubules in the leading and rear parts of the cell, which might result from high level of protein expression.

Overall, our data indicate that SLK regulates organization of microtubules and polarization of cell through phosphorylation of p150\textsuperscript{Glued}-1A, which does not affect transport functions of dynactin.

**MATERIALS AND METHODS**

**DNA constructs.** Full-length human cDNA encoding p150\textsuperscript{Glued}-1B and mouse cDNA encoding p150\textsuperscript{Glued}-1A described in (Zhapparova et al., 2009), were cloned into a pEGFP-C1 (Clontech) vector. N-terminal fragments encoding 1-157 a.a. of p150\textsuperscript{Glued}-1A, 1-137 a.a. of p150\textsuperscript{Glued}-1B and ΔN (a.a. 151-1281 a.) fragment of p150\textsuperscript{Glued}-1A were cloned into pGEX-4T3 (GE Healthcare). High-fidelity PCR Enzyme Mix, restriction endonucleases, and other enzymes used for molecular cloning, were from Fermentas (Vilnius, Lithuania). Primers were purchased from Syntol (Moscow, Russia). Site-directed mutagenesis was performed with QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). In GST-fused N-terminal fragments of p150\textsuperscript{Glued}-1A, each threonine in 145-147 positions was substituted for alanine residue to obtain T145A, T146A and T147A mutants; threonine in 141 position was substituted for alanine to obtain T141A mutant; all threonines in 145-147 positions were substituted for alanines to obtain T145-147A mutant. In GFP-fused full-length p150\textsuperscript{Glued}-1A, threonines in 145-147 positions were simultaneously substituted for three alanine or three glutamate residues to obtain Ala-p150\textsuperscript{Glued} and Glu-p150\textsuperscript{Glued} mutants, respectively. GFP-fused catalytic domain of SLK (SLK\textsuperscript{CD}, a.a. 1-342) and a dominant-negative mutant (K63R, a.a. 1-342) were described in Burakov et al. (2008b). Dominant-negative mutant fused to dsRed was obtained as a result of subcloning into a dsRed-C1 vector (Clontech).

For cloning of PACT domain total RNA was isolated from cultured HeLa cells using an RNeasy kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and random hexanucleotide primers (Syntol). PACT domain (a.a. 3702-3789) of AKAP450 (AJ131693.1) was amplified using the following primers: 5’-TATGTTAAATACCTTGCAGGGAACAGG-3’ and 5’ TGACTGATGCCACCGTGAACG-3’. The obtained PACT-domain DNA was amplified with corresponding primers and subcloned into pEGFP-C1 vector at EcoRI/Sall sites (GFP-PACT). Also, PACT-domain DNA was subcloned into pEGFP-C1 vector carrying Ala-p150\textsuperscript{Glued} either upstream of GFP (at AgeI site, PACT-Ala-p150\textsuperscript{Glued}) or downstream of Ala-p150\textsuperscript{Glued} (Ala-p150\textsuperscript{Glued}-PACT). Glu-p150\textsuperscript{Glued}-PACT was obtained as a result of PACT subcloning into pEGFP-C1 vector carrying Glu-p150\textsuperscript{Glued}
downstream of dynactin. All constructs were verified with automated sequencing, and the length of corresponding protein products was confirmed with SDS-PAGE and Western blot.

**Mammalian cell growth, transfection, fixation, immunostaining, and wound healing assay.**

Green monkey kidney Vero epithelium-like cells were cultured in DMEM/F12 (1:1) medium (Paneco, Moscow, Russia) supplemented with 7.5% FBS (Paneco), at 37°C and 5% CO₂. Transfection was performed using TransIT-LT1 reagent (Mirus Bio, Madison WI) or Lipofectamine 2000 (Invitrogen).

For immunostaining, cells were fixed with methanol for 10 minutes at -20°C, followed by 3% PFA for 20 minutes at 4°C. The following antibodies were used: mouse monoclonal antibody to p150Glued (610473, BD Biosciences), p50/dynamitin (611002, BD Biosciences), tubulin DM-1A (Sigma); rat monoclonal anti-tubulin YOL1/34 antibody (Abcam, Cambridge, UK); rabbit polyclonal antibodies to mannosidase II (12277, Abcam), phosphothreonine (71-8200, Invitrogen), and γ-tubulin (kindly provided by Dr. R. Uzbekov, Lomonosov Moscow State University, Russia). Fluorochrome-conjugated (FITC, TRITC, Cy5) secondary antibodies (MultiLabeling type) were obtained from Jackson ImmunoResearch Laboratories (UK). Images of immunostained cells were taken using Carl Zeiss Axiovert 200M microscope, supplied with a 12-bit AxioCamHR camera and Axiovision software (Carl Zeiss). The data were analyzed using ImageJ (NIH) and Excel software.

For wound-healing assay, Vero cells were grown to a monolayer, then scratched with a pipet tip, incubated at 37°C for 2 h, and fixed as described above.

**Tubulin purification and microtubule co-pelleting assay**

Tubulin was isolated from rat brains as described in (Zhapparova et al., 2009). For co-pelleting experiments, 6 mg/ml rat tubulin was incubated in BRB buffer (80 mM PIPES pH 6.8, 1 mM MgCl₂, 1 mM EGTA) with 1 mM GTP for 20 minutes at 37°C, then 2 µM taxol (Sigma) was added. The mixture was incubated at 37°C for 15 minutes, after which the taxol concentration was increased to 20 µM and the mixture was incubated at 37°C for another 15 minutes. 16 µM microtubules, 1 mM GTP, and 15 µM taxol in BRB buffer were mixed with GST-dynactin fragments, incubated at 37°C for 30 minutes and applied over a warm 4 M glycerol cushion with 1 mM GTP and 5 µM taxol in BRB. Microtubules were pelleted in a TLS55 rotor (Beckman) at 50,000 rpm, 25°C for 30 minutes. Supernatants were collected and mixed with 4x sample buffer (SB), cushions were washed three times with BRB and discarded. The pellets (mostly invisible) were resuspended in an equal volume of 2x SB.

**Immunoprecipitation, recombinant protein production, SDS-PAGE and Western blot analysis**
For immunoprecipitation, human embryonic kidney HEK293T cells were transfected and harvested in PHEM buffer (50 mM PIPES, 50 mM HEPES, 1 mM EDTA, 2 mM MgSO₄, pH 7.0) supplemented with 0.5% Nonidet P-40, 0.5% Triton X-100, 0.25% sodium deoxycholate. After centrifugation (TLS55 rotor (Beckman), 32,000 rpm, 4°C, 20 min), supernatant was incubated with protein A-Sepharose (P3391, Sigma) or MabSelect-Sepharose (GE Healthcare) and antibodies for immunoprecipitation against p50/dynamitin (sc-135135, Santa Cruz) or GFP (GMA0311, Protein Synthesis, Russia) for 3 h at 4 °C. The results were analyzed with Western blot using mouse monoclonal anti-p150 Glued (610473, BD Biosciences), anti-dynamitin/p50 (611002, BD Biosciences), anti-GFP (AMA 0311, Protein Synthesis) antibodies; rabbit polyclonal anti-GFP (AB121, Evrogen), Anti-ACTR1B (ab84809, Abcam) antibodies.

Fragments of GST-fused p150 Glued and SLK CD were expressed in E. coli and purified using a glutathione-agarose column (Sigma) according to the manufacturer’s instructions. Purified N-terminal fragments of p150 Glued were used in microtubule co-pelleting and in vitro phosphorylation experiments. SDS-PAGE was performed in 7.5%, 10% or 6-12% gradient gels. Nitrocellulose membranes (Bio-Rad) were blocked in TBS supplemented with 0.05% Tween-20 and 5% fat-free milk for overnight and were subsequently incubated with primary antibodies and goat anti-rabbit or anti-mouse IgG antibodies, conjugated to alkaline phosphatase (KPL, Gaithersburg, MD). The results were visualized with BCIP/NBT solution (KPL).

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In vitro kinase assay

GST-fused SLK CD and N-terminal fragments of p150 Glued were expressed in E. coli and purified on glutathione-agarose. For in vitro kinase assay, 5 μg of kinase and dynactin fragments or 2.5 μg of myelin basic protein (MBP, Sigma) were mixed with γ-32P-ATP (10 μCi, FEI Research Center, Obninsk, Russia) in kinase buffer (50 mM HEPES, pH 7.4, 10mM MgCl₂, 100 mM KCL, 0.5 mM PMSF, 1 mM DTT) and incubated at 25°C for 1h. For the experiments with brain dynactin, 0.2 mM heparin was added to inhibit contaminant endogenous casein kinase activity in dynactin preparation. The results were analyzed with SDS-PAGE and autoradiography. In vitro kinase assay with cold ATP was performed with the same amount of proteins; the reaction was incubated for overnight at 4°C. The results were analyzed with Western blot stained with anti-phosphothreonine antibodies (Invitrogen).

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Figure 1. SLK (LOSK) phosphorylates dynactin in vitro. (A) A catalytic domain of SLK (SLK\textsuperscript{CD}) phosphorylates dynactin preparation from bovine brain. The \textsuperscript{32}P-label is incorporated into a 150 kDa band. Autophosphorylation of the kinase domain is also visible and is likely to cause label incorporation into the lower bands (a result of truncated protein synthesis in E. coli or partial protein degradation). Myelin basic protein is used as positive control. Radioautograph (R/a) and Coomassie staining of the same gel are represented. (B) Identification of the phosphorylation site. SLK\textsuperscript{CD} phosphorylates N-terminal part of p150\textsuperscript{Glued-1A} (a.a. 1-157), but does not phosphorylate N-terminal part of p150\textsuperscript{Glued-1B} (a.a. 1-137), ΔN-p150\textsuperscript{Glued-1A} (a.a. 151-1281), or T145-147A mutant of p150\textsuperscript{Glued-1A} (a.a. 1-157, T145-147A). Asterisks mark positions of recombinant proteins visualized by Ponceau staining prior to radioautography (Ponceau is not shown). (C) In vitro kinase reaction with GST-p150\textsuperscript{Glued-1A} N-terminal fragment in the presence and absence of cold ATP. Western blot is stained with anti-phosphothreonine antibodies. The reaction is inhibited in the absence of phosphatase inhibitors. (D) Threonines in all three positions are phosphorylated by SLK. In vitro kinase assay with the GST-fused N-terminal parts of wild-type p150\textsuperscript{Glued-1A}, mutants (T145-147A, T145A, T146A, and T147A). \textsuperscript{32}P is incorporated in all three threonines (145, 146 and 147)
(lanes 3, 4 and 5). Radioautograph and Ponceau stained membrane are shown. Kinase domain and all fragments of p150Glued shown were synthesized in *E. coli* as GST-fusions and purified on glutathione-agarose. (E) Phosphorylation sites for SLK in human RhoA, paxillin and the identified site in p150Glued-1A. Predicted site is represented according to Pike et al., (2008). Φ corresponds to aliphatic amino acid.
Figure 2. p150\textsuperscript{Glued} mutants vary in the affinity for microtubules \textit{in vitro} and \textit{in vivo}. (A) Co-pelleting of GST-fused N-terminal fragments (a.a. 1-157) of wild type (WT), Ala- and Glu-p150\textsuperscript{Glued} with microtubules. Fragments of p150\textsuperscript{Glued} (0.4, 0.8 and 2 µM) were incubated with microtubules (16 µM) and pelleted through a 4 M glycerol cushion. In control (-MT) fragments of p150\textsuperscript{Glued} were precipitated under the same sedimentation conditions in the absence of microtubules. Protein content in supernatants (S) and pellets (P) was analyzed by Western blot with anti-p150\textsuperscript{Glued} antibodies. Relative content of p150\textsuperscript{Glued} in microtubule pellets is shown. Error bars represent SD. *p < 0.05 for Ala- and Glu-p150\textsuperscript{Glued}. (B) Localization of full-length GFP-fused Ala- and Glu-p150\textsuperscript{Glued} in Vero cells. Microtubules were stained with anti-tubulin antibodies. Ala-p150\textsuperscript{Glued} decorates the entire microtubules, whereas Glu-p150\textsuperscript{Glued} is concentrated mostly at distal ends. Line scans of microtubules indicated with arrowheads are shown. Scale bar 10 µm.
Figure 3. Glu-p150\textsuperscript{Glued} exhibits higher affinity for the centrosome compared to Ala-p150\textsuperscript{Glued}. (A) Expression of full-length GFP-fused Ala- and Glu-p150\textsuperscript{Glued} in Vero cells and immunostaining of fixed cells with anti-gamma-tubulin antibodies to visualize centrosome. Boxed areas are shown at higher magnification. (B) Quantification of cells with centrosomal localization of p150\textsuperscript{Glued} mutants. Ala – a full-length GFP-fused Ala-p150\textsuperscript{Glued} (T145-147A) (n = 349); Glu – a full-length GFP-fused Glu-p150\textsuperscript{Glued} (T145-147E) (n = 420); K63R/Ala – cotransfection of K63R (dsRed-fused dominant-negative mutant of SLK\textsuperscript{CD} (a.a. 1-342)) and Ala-p150\textsuperscript{Glued} (n = 74); K63R/Glu – cotransfection of K63R and Glu-p150\textsuperscript{Glued} (n = 60). Experiments were performed in at least three repeats, n indicate the total number of cells analyzed in all experiments. Error bars represent SD; *p < 0.05 (t-test).
Figure 4. Glu-mutant of p150\textsuperscript{Glued} restores radial organization of microtubules in cells with inhibited SLK. (A) Coexpression of dsRed-fused dominant-negative kinase domain of SLK (K63R-ΔT) and a full-length GFP-fused Ala- or Glu-p150\textsuperscript{Glued} in Vero cells, and immunostaining of fixed cells with anti-tubulin antibodies. Expression of Glu-p150\textsuperscript{Glued} restores radial microtubule array in cells with inhibited SLK, while expression of Ala-p150\textsuperscript{Glued} enhances microtubule randomization in such cells. Microtubule organization was estimated by measurement of fluorescence intensity along lines drawn through the cell’s center. Scans of fluorescence intensity along such lines are represented. (B) Quantification of cells with radial microtubule array. Contr – control non-transfected cells (n = 568); K63R – dsRed-fused dominant-negative mutant of SLK (a.a. 1-342) (n = 948); Ala – a full-length GFP-fused Ala-p150\textsuperscript{Glued} (T145-147A) (n = 389); Glu - a full-length GFP-fused Glu-p150\textsuperscript{Glued} (T145-147E) (n = 225); K63R/Ala – cotransfection of K63R and Ala-p150\textsuperscript{Glued} (n = 400); K63R/Glu – cotransfection of K63R and Glu-p150\textsuperscript{Glued} (n = 862); pact-Ala – PACT-GFP-Ala-p150\textsuperscript{Glued} (n = 104); Ala-pact – GFP-Ala-p150\textsuperscript{Glued}-PACT (n = 77); pact-Glu – PACT-GFP-Glu-p150\textsuperscript{Glued} (n = 68), Glu-pact – GFP-Glu-p150\textsuperscript{Glued}-PACT (n = 273); K63R/pact-Ala cotransfection of K63R and PACT-GFP-Ala-p150\textsuperscript{Glued} (n = 374); K63R/Ala-pact – cotransfection of K63R and GFP-Ala-p150\textsuperscript{Glued}-PACT (n = 179); K63R/Glu-pact – cotransfection of K63R and GFP-Glu-p150\textsuperscript{Glued}-PACT (n = 273). Experiments were performed in at least three repeats, n indicate the total
number of cells analyzed in all experiments. Error bars represent SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (t-test).
Figure 5. SLK (LOSK) regulates microtubule organization by targeting p150\textsuperscript{Glued} to the centrosome. (A) Expression of PACT-fusions in Vero cells and immunostaining of fixed cells with anti-gamma-tubulin antibodies to visualize centrosome. Boxed areas are shown at higher magnification. PACT at either end of Ala-p150\textsuperscript{Glued} ensures centrosomal localization of the protein in more than 90% cells. (B) Coexpression of dsRed-fused dominant-negative kinase domain of SLK (K63R) and p150\textsuperscript{Glued} mutants fused with PACT domain and immunostaining of
fixed cells with anti-tubulin antibodies. PACT at the N-terminus of Ala-p150\textsuperscript{Glued} rescues radial microtubule array in cells with inhibited SLK. PACT at the C-terminus of Ala-p150\textsuperscript{Glued} or Glu-p150\textsuperscript{Glued} does not restore radial microtubule array in cells with inhibited SLK.
Figure 6. Glu-p150^Glued^ rescues Golgi reorientation in polarized cells with inhibited SLK (LOSK). (A) Coexpression of dsRed-fused dominant-negative kinase domain of SLK (K63R) and GFP-fused Ala- or Glu-p150^Glued^ in polarized Vero cells and immunostaining of fixed cells with anti-mannosidase II antibodies to visualize Golgi. Polarized cells were obtained at the edge of experimental wound in a monolayer of Vero cells. Expression of Glu-p150^Glued^ restores normal reorientation of Golgi towards the leading edge of the cell with inhibited SLK. (B) Quantification of cells with polarized Golgi. Contr – control non-transfected cells (n = 157);
K63R – dsRed-fused dominant-negative mutant of SLK (a.a. 1-342) (n = 75); Ala – a full-length GFP-fused Ala-p150Glued (T145-147A) (n = 66); Glu – a full-length GFP-fused Glu-p150Glued (T145-147E) (n = 70); K63R/Ala – cotransfection of K63R and Ala-p150Glued (n = 69); K63R/Glu – cotransfection of K63R and Glu-p150Glued (n = 81). Experiments were performed in at least three repeats, n indicate the total number of cells analyzed in all experiments. Error bars represent SD. ***p < 0.001 (t-test).