Direct Binding of Lgl2 to LGN during Mitosis and Its Requirement for Normal Cell Division*

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The Drosophila tumor suppressor protein lethal (2) giant larvae (l(2)gl) is involved in asymmetric cell division during development and epithelial cell polarity through interaction with the aPKC-Par-6 complex. We showed here that Lgl2, a mammalian homolog of l(2)gl, directly bound to LGN, a mammalian homolog of Partner of inscuteable (HEK293 cells. The C-terminal tail of Lgl2 bound to LGN with a Kd value of about 56 nM. Endogenous Lgl2 formed a complex with aPKC, Par-6, and LGN. This complex formation was enhanced in metaphase of the synchronized cells by treatment with thymidine and nocodazole. Immunofluorescence staining of the complex was the strongest at the cell periphery of the metaphase cells. Overexpression of the C-terminal tail of Lgl2 induced mis-localization of the nuclear mitotic apparatus protein NuMA and disorganization of the mitotic spindle during mitosis, eventually causing formation of multiple micronuclei. Knockdown of endogenous Lgl (Lgl1 and Lgl2) also induced disorganization of the mitotic spindle, thereby causing formation of multiple micronuclei. The binding between Lgl2 and LGN played a role in the mitotic spindle organization through regulating formation of the LGN/NuMA complex. These results indicate that Lgl2 forms a Lgl2-Par-6-aPKC-LGN complex, which responds to mitotic signaling to establish normal cell division.

The gene product of Drosophila lethal (2) giant larvae (D-Lgl) is essential for development of polarized epithelia and for cell polarity associated with asymmetric cell division of neuroblasts during fly development (1–5). D-Lgl is in the same genetic pathway as Discs-large (Dlg) and Scribble, which are necessary for establishing and maintaining the basolateral membrane domain and basal protein targeting (3, 5, 6), and functions competitively with Crumbs/Stardust and Par-3-Par-6-aPKC protein complexes that are necessary for the apical membrane domain (7–9).

Mammals have two Lgl homologs, Lgl1 (Mlgl, Hugl) and Lgl2. Lgl1 and Lgl2 directly interact with the Par-6-aPKC protein complex (7, 10). Loss of Lgl1 in mice results in formation of neuroepithelial rosette-like structures, similar to the neuroblastic rosettes in human primitive neuroectodermal tumors. The newborn Lgl1 knock-out mice develop severe hydrocephalus and die neonatally. Due to loss of mitotic spindle orientation, a large proportion of neural progenitor cells fail to exit the cell cycle and differentiate, and instead, continue to proliferate (11). These results have revealed a critical role for mammalian Lgl1 in the regulation of mitotic spindle orientation, proliferation, differentiation, and tissue organization of neuroepithelial cells. Although there is growing information on the protein partners of Lgl, the molecular details of the roles of Lgl in mitotic spindle orientation and cell cycle, however, remain obscure.

In Drosophila neuroblasts, the rotation of the spindle requires the activities of Par-3, Inscuteable (Insc), and Partner of inscuteable (Pins) (12). Although no mammalian gene similar to Insc is known, mammalian Par-3 participates in the establishment of epithelial cell polarity and several mammalian proteins related to Pins have been identified (13, 14). The mammalian Pins homolog, LGN, binds the nuclear mitotic apparatus protein NuMA and plays a key role in spindle pole organization during mitosis in mammalian cells (15, 16). NuMA is a large coiled-coil and microtubule (MT)-binding protein that organizes the spindle poles orientation (17, 18). The binding of NuMA to LGN is necessary for the binding of LGN to the α subunits of heterotrimetric G-protein (Go) at the cell cortex (19). The ternary NuMA-LGN-Gα complex regulates the interaction of aster MTs with the cell cortex, thereby causing the chromosome segregation during mitosis (19).

In this study, we demonstrated that Lgl2 bound LCN directly. Lgl2 formed a complex with aPKC, Par-6, and LGN favorably in mitotic cells. The inhibition of the binding between Lgl2 and LGN caused the mitotic spindle disorganization and the chromosome mis-segregation through affecting the formation of the LGN-NuMA complex.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—One bait vector, pGBD Lgl2 (full-length, aa 1–1015), was constructed by subcloning the insert encoding the amino acid residue of Lgl2 into pGBD-C1 (20). Yeast two-hybrid screening using the yeast strain PJ69-4A (MATa trp1–901 leu2–3, 112 ura3–52 his3–200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ) was performed as described (20). Standard procedures for yeast manipulations were performed as described (21).
Constructions of Expression Vectors—The cDNA encoding full-length human Lgl2 was obtained as described previously (10). The cDNA encoding full-length human LGN (IMAGE: T12445) was obtained from ATCC (Rockville, MD). Expression vectors were constructed in pCMV-HA (22), pGEX-4T-1 (Amersham Biosciences), pMAL-C2 (New England Biolabs Inc.), and pFastBac1-maltose-binding protein (MBP) using standard molecular biology methods. The pFastBac1-MBP was constructed with a baculovirus transfer vector, pFastBac1 (Invitrogen), to express a fusion protein with N-terminal MBP. Constructs of human Lgl2 and LGN contained the following aa: pCMV-HA-Lgl2-Full, aa 1–1015; pCMV-HA-Lgl2-N, aa 1–640; pCMV-HA-Lgl2-C, aa 636–1015; and glutathione S-transferase (GST)-LGN-Full, aa 1–677; GST-LGN-N, aa 1–365; GST-LGN-C, aa 360–677.

Knockdown of Lgl and LGN by the RNA Interference Method—The mammalian expression vector, pBS-H1 (23), was used for expression of small interfering RNA in HEK293 cells. The following inserts were used: for Lgl2, a human Lgl2 gene-specific insert was a 19-nucleotide (nt) sequence corresponding to nts 124–142 (GCTGCAGTTCAAGTTGGAA) of human Lgl2 cDNA; human LGN gene-specific insert was a 19-nucleotide nt sequence corresponding to nts 605–623 (ACCAGATCCTGATCGGCTA) of human LGN cDNA; and a control (Scramble) insert was a 19-nt sequence (TCTAACAGTGTCCGAGCCA) with no significant homology to any mammalian gene sequence, all of which were separated by a 9 nt noncomplementary spacer (TCTAAGAGA) from the reverse complement of the same 19-nt sequence.

Antibodies—The GST fusion fragment of LGN (aa 360–677) was produced in Escherichia coli, purified, and used as antigen to raise a polyclonal antibody (pAb) in rabbit. The rabbit anti-LGN pAb was affinity-purified using MBP-LGN-C (aa 360–677) immobilized on Amine-link agarose beads (Pierce). A mouse anti-HA monoclonal Ab (mAb) was purchased from Babco. A rat anti-HA mAb was purchased from Roche Applied Science. A mouse anti-aPKCa mAb was purchased from Transduction Laboratories. A goat anti-Par-6 pAb was purchased from Santa Cruz Biotechnology. A mouse anti-NeuMA mAb was purchased from Calbiochem. A mouse anti-α-tubulin mAb was purchased from Sigma. A mouse anti-setin mAb was purchased from Chemicon. Rabbit anti-LG1 and Lgl2 pAbs were made as described previously (10).

Pull-down Assay—To determine the binding site of Lgl2 to LGN, HEK293 cells were transfected with pCMV-HA-Lgl2-Full, -N, and -C. After 48-h incubation, the cells were harvested and suspended in Buffer A (20 mM Tris-HCl at pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 1% Triton X-100, 10 μM α-phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The cell extract was obtained by centrifugation at 100,000 × g for 15 min. The extract was applied onto the full-length or truncated form of GST-LGN (500 pmol) immobilized on glutathione-Sepharose beads (Amersham Biosciences) (100 μl wet volume). After the beads were extensively washed with Buffer A, the bound proteins were eluted by boiling in the SDS sample buffer (60 mM Tris-HCl at pH 6.7, 3% SDS, 2% 2-mercaptoethanol, and 5% glycerol) for 10 min. The samples were then subjected to SDS-PAGE, followed by Western blotting with the anti-HA mAb.

Direct Binding of Lgl2 to LGN—MBP-Lgl2-C or MBP-Lgl2-Full was incubated with GST-LGN-Full or GST alone (20 pmol each) immobilized on 40 μl (wet volume) of glutathione-Sepharose beads in 400 μl of Buffer B (20 mM Tris-HCl at pH 8.0, 27.5 mM NaCl, 25 mM KCl, 0.1% Triton X-100) at 25 °C for 2 h. After the beads were extensively washed with Buffer B, the bound proteins were eluted by boiling in the SDS sample buffer. The samples were then subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue. The amount of bound MBP-Lgl2 was determined by comparing the intensity of its band with that of various amounts of bovine serum albumin using a densitometer (FluorChem™ Alpha; Innotech Corp.). The Kd value was calculated by ScatChard analysis.

Cell Culture, Cell Cycle Synchronization, and Transfection—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum. To synchronize the cells, they were seeded in Dulbecco’s modified Eagle’s medium containing 5% thymidine and nocodazole treatment was performed as described (24). Briefly, HEK293 cells were cultured in DMEM containing 5 mM thymidine (Sigma) for 16 h and incubated in fresh DMEM containing 10% fetal calf serum for 8 h. After the incubation, nocodazole (Sigma) was added at a final concentration of 0.33 μM, and the incubation was continued for another 4 h. HEK293 cells were transfected with the full-length or truncated form of pCMV-HA-Lgl2 using the Lipofectamine 2000 (Invitrogen). After 48-h incubation, the cells were seeded at a density of 2.5 × 105 cells per dish onto 35-mm collagen type I-coated dish. At 24 h after the seeding, the cells were fixed and stained with DAPI (Nacalai Tesque) and the rat anti-HA mAb.

Co-immunoprecipitation Experiments—HEK293 cells treated with or without the thymidine and nocodazole treatment were extracted by the addition of Buffer C (25 mM Tris-HCl at pH 8.0, 27.5 mM NaCl, 25 mM KCl, 10% sucrose, 0.1% Nonidet P-40, 50 mM NaF, 2 mM Na3VO4, 10 mM EDTA, 100 μM α-phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The cell extract was obtained by centrifugation at 100,000 × g for 60 min. The extract was incubated with the anti-LGN pAb (5 μg) or the anti-Lg2 pAb (5 μg) at 4 °C for 18 h. Immunocomplexes were then precipitated with protein A-Sepharose CL-4B beads (Amersham Biosciences). After the beads were extensively washed with Buffer C, the bound proteins were eluted by boiling in the SDS sample...
buffer. The samples were then subjected to SDS-PAGE, followed by Western blotting with the anti-Lgl2, anti-aPKC/H9261, anti-Par-6, or anti-LGN Abs.

Other Methods—Immunofluorescence microscopy of cultured cells was done as described (25). Protein concentrations were determined with bovine serum albumin as a reference protein (26). SDS-PAGE was done as described by Laemmli (27).

RESULTS AND DISCUSSION

To identify a Lgl2-binding protein(s), we performed the yeast two-hybrid screening using a human brain library with full-length Lgl2 as a bait. Purified MBP-Lgl2-C and MBP-Lgl2-Full were incubated with either GST or GST-LGN-Full immobilized on glutathione-Sepharose beads. After the beads were extensively washed, the beads were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue. The asterisks indicate the degraded proteins of GST-LGN-Full. The indicated concentrations of MBP-Lgl2-C were incubated with GST-LGN-Full coated beads. Panel a, the bound proteins were analyzed by protein staining with Coomassie Brilliant Blue. The arrowheads indicate MBP-Lgl2-C. The arrowhead with an asterisk indicates the degraded proteins of MBP-Lgl2-C. Panel b, quantification of the bound proteins. Panel c, the $K_d$ value was calculated by Scatchard analysis. Each point represents the mean ± S.D. of triplicate experiments.

with bovine serum albumin as a reference protein (26). SDS-PAGE was done as described by Laemmli (27).

FIG. 2. Direct binding of Lgl2 to LGN. A, direct binding of the full-length and C-terminal region of Lgl2 to LGN. Purified MBP-Lgl2-C and MBP-Lgl2-Full were incubated with either GST or GST-LGN-Full immobilized on glutathione-Sepharose beads. After the beads were extensively washed, the beads were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue. The asterisks indicate the degraded proteins of GST-LGN-Full. The indicated concentrations of MBP-Lgl2-C and GST-LGN-Full. The indicated concentrations of MBP-Lgl2-C were incubated with GST-LGN-Full coated beads. Panel a, the bound proteins were analyzed by protein staining with Coomassie Brilliant Blue. The arrowheads indicate MBP-Lgl2-C. The arrowhead with an asterisk indicates the degraded proteins of MBP-Lgl2-C. Panel b, quantification of the bound proteins. Panel c, the $K_d$ value was calculated by Scatchard analysis. Each point represents the mean ± S.D. of triplicate experiments.

with bovine serum albumin as a reference protein (26). SDS-PAGE was done as described by Laemmli (27).

FIG. 3. Cell cycle-dependent binding of Lgl2 to LGN. A, enhanced formation of the Lgl2-aPKC/Par-6-LGN complex during mitosis. HEK293 cells were synchronized by use of thymidine and nocodazole (Thy + Noco). The extract of HEK293 cells was incubated with the control IgG, the anti-Lgl2 pAb, or the anti-LGN pAb. The immunoprecipitates were analyzed by Western blotting with the anti-Lgl2 pAb, the anti-LGN pAb, the anti-Par-6 pAb, and the anti-aPKC mAb. The quantification of immunoblot is shown on the bottom. B, co-localization of LGN and Lgl2 during mitosis. HEK293 cells were triple-stained with the anti-Lgl2 pAb, the anti-aPKC mAb, and DAPI in the interphase (without thymidine and nocodazole treatment) and in the metaphase (with thymidine and nocodazole treatment). HEK293 cells were triple-stained with the anti-LGN pAb, the anti-aPKC mAb, and DAPI in the interphase and in the metaphase. The arrowheads indicate the co-localization of Lgl2 or LGN with aPKC at the cell periphery. Bars, 10 μm. The results shown are representative of three independent experiments.
regulatory domain for the interaction between the GPR domain and G/H9251 (28) or may play an important role in the localization of LGN (28). The GPR domain interacts with G/H9251 and stabilizes the GDP-bound form of G/H9251 (29, 30).

We next confirmed the binding of Lgl2 to LGN and determined their binding regions by a pull-down assay. HA-tagged full-length Lgl2 expressed in HEK293 cells bound the N-terminal fragment of LGN (LGN-N, aa 1–365) but not the C-terminal fragment (LGN-C, aa 360–677) (Fig. 1B). HA-tagged C-terminal fragment of Lgl2 (Lgl2-C, aa 636–1015), but not N-terminal fragment of Lgl2 (Lgl2-N, aa 1–640), bound the N-terminal fragment of LGN (Fig. 1C). These results indicate that the C-terminal region of Lgl2 binds the N-terminal region of LGN.

To confirm the direct binding of Lgl2 to LGN, we performed affinity chromatography using the pure recombinant proteins of Lgl2 and LGN. MBP-Lgl2-C bound to immobilized GST-LGN-full-length but not the C-terminal fragment (LGN-C, aa 360–677) (Fig. 1B). HA-tagged C-terminal fragment of Lgl2 (Lgl2-C, aa 636–1015), but not N-terminal fragment of Lgl2 (Lgl2-N, aa 1–640), bound the N-terminal fragment of LGN (Fig. 1C). These results indicate that the C-terminal region of Lgl2 binds the N-terminal region of LGN.

To examine the in vivo binding of Lgl2 to LGN, a co-immunoprecipitation assay was performed using the HEK293 cell lysate. When Lgl2 was immunoprecipitated with the anti-Lgl2 pAb, LGN was co-immunoprecipitated with the anti-Lgl2 pAb. In addition to LGN, Par-6 and aPKC were co-immunoprecipitated with Lgl2, consistent with the earlier observations that Lgl2 forms a complex with Par-6 and aPKC (7, 10). Thus, Lgl2 forms a novel complex with Par-6, aPKC, and LGN.

LGN has been shown to play an important role in spindle pole orientation and to localize at cell periphery during mitosis (29). Therefore, we next examined by a co-immunoprecipitation assay whether Lgl2 binds to LGN and whether the binding of Lgl2 to LGN is affected by the expression of Lgl2-C and knockdown of Lgl2. HEK293 cells were transfected with an empty control vector (HA) or various fragments of HA-Lgl2 or co-transfected with DsRED as a morphological marker along with scramble RNAi (control), LGN RNAi, or the combination of Lgl1 and Lgl2 RNAi. The percentages of the cells with multiple micronuclei are shown on the right. B, mislocalization of NuMA and MTs of mitotic spindle by expression of Lgl2-C and knockdown of LGN. HEK293 cells were transfected with an empty control vector (HA) or various fragments of HA-Lgl2, or co-transfected with DsRED as a morphological marker along with scramble RNAi (control), LGN RNAi, or the combination of Lgl1 and Lgl2 RNAi. The transfected cells were identified by the expression of HA or DsRED (arrowheads), and their nuclear structures were examined by various combinations of DAPI (blue) and immunostaining with the anti-a-tubulin mAb (green), the anti-Lg1 pAb (green), or the anti-aPKC mAb (green). Bars, 10 μm. C, decreased expression levels of LGN and Lgl by the RNAi method. HEK293 cells were transfected with scramble RNAi (control), LGN RNAi, or the combination of Lgl1 and Lgl2 RNAi, and cultured for 48h. The whole cell lysates (50% transfection efficiency) were subjected to SDS-PAGE, followed by Western blotting with the anti-LGN pAb, the anti-Lg1 pAb, the anti-Lgl2 pAb, and the anti-aPKC mAb. D, enhancement of the LGN-NuMA complex formation by binding of HA-Lgl2-C to endogenous LGN. HEK293 cells were transfected with an empty control vector (HA) and various fragments of HA-Lgl2. The extract of HEK293 cells was incubated with the anti-LGN pAb. The immunoprecipitates were analyzed by Western blotting with the anti-HA mAb, the anti-NuMA mAb, and the anti-LGN pAb.
assay whether the amount of the Lgl2-Par-6-aPKC-LGN complex is changed during mitosis. To produce large numbers of mitotic cells, HEK293 cells were treated with thymidine and nocodazole. Over 80% of the cells showed mitotic cell phenotype (data not shown). The Lgl2-Par-6-aPKC-LGN complex was more predominantly formed in the mitotic cells than in the control cells (Fig. 3A). We furthermore examined by immunostaining the localization of these proteins in the interphase and the metaphase of HEK293 cells. As shown previously (29), LGN localized in the cytoplasm in the interphase and at the cell periphery in the metaphase (Fig. 3B). aPKC co-localized with LGN at the cell periphery in the metaphase. Lgl2 and aPKC co-localized in the perinuclear structure and faintly at the cell-cell contact sites in the interphase. In the metaphase, Lgl2 and aPKC as well as LGN localized at the cell periphery. These immunostaining data are consistent with the biochemical data shown in Fig. 2A and suggest that Lgl2 forms a complex with LGN, aPKC, and Par-6 during mitosis.

Dysfunction of LGN has been shown to have the phenotype of multiple micronuclei due to chromosomal mis-segregation and defect in cell division through mis-localization of mitotic spindle regulator NuMA, a LGN binding partner (15, 16). Therefore, to investigate the functional significance of the binding between Lgl2 and LGN, we examined the effect of overexpression of Lgl2-C, the LGN-binding fragment, on cell division.

Formation of the Lgl2-LGN complex was analyzed by western blotting. To overexpress HA-LGN and Lgl2, we used HEK293 cells which were stably transfected with HA-LGN expression vector. Lgl2 and a PKC were overexpressed, and the Lgl-LGN complex was detected in the cytoplasm. To further confirm this result, we performed a loss-of-function analysis by use of the RNA interference (RNAi) method. We knocked down endogenous Lgl (Lgl1 and Lgl2) and LGN by the RNAi method (Fig. 4). The Lgl-knockdown cells had apparently a similar multinucleate phenotype as that of the LGN-knockdown cells (Fig. 4A). The percentage of the Lgl-knockdown cells showing the multinucleate phenotype was 6% and slightly less than that of the LGN-knockdown cells. Both NuMA and MTs of mitotic spindle were mis-localized in the Lgl-knockdown cells similarly to those of the LGN-knockdown cells. In addition, LGN, but not aPKC and Par-6, was not localized at the cell cortex during mitosis in the Lgl-knockdown cells. These results suggest that Lgl plays a role in chromosome segregation through regulating the LGN and NuMA localization during mitosis. In addition, to examine the Lgl-C effect on binding between endogenous LGN and NuMA in the cells, we examined the amount of NuMA bound to endogenous LGN by a co-immunoprecipitation assay using the anti-LGN pAb. The LGN-NuMA complex was more predominantly formed in the cells overexpressing Lgl-C than in the control cells (Fig. 4D). Lgl2-C indeed bound to LGN more predominantly than Lgl2-full-length. These results indicate that the binding of Lgl2 to LGN is important for the formation of the LGN-NuMA complex to regulate chromosome segregation.

Previous studies have shown that Lgl2 is largely expressed at the cell-cell contact sites in epithelial cells (10). However, there have been no analysis of potential change in the pattern of Lgl2 complex formation during mitosis. We have first presented here that Lgl2 forms a novel complex with LGN, Par-6, and aPKC and that this complex formation is enhanced during mitosis. This complex shows cortical localization in mitotic cells. The functional significance of the cycle-dependent cortical localization of this complex is not yet clear, but the localization of LGN at the cell cortex during mitosis is somewhat similar to what is described for Drosophila Pins. In Drosophila, Pins is normally found in the lateral cortex of epithelial cells and only become asymmetricaly localized upon the expression of inscuteable in neuroblasts, for which a mammalian homolog has not been found so far (31). Pins is also dependent on heterotrimeric G-protein activity for its localization (30). Furthermore, Pins and LGN have been shown to bind Gα through their C-terminal GPR domain (29, 30). They behave as GDP dissociation inhibitors of the Gα. For the efficient binding of LGN to Go at the cell cortex, the binding of LGN to NuMA seems to be prerequisite (19). LGN normally folds back onto itself and shows a closed conformation. The binding to NuMA triggers a partially open state of LGN for further interaction with Gα. The formation of ternary NuMA-LGN-Gα complex regulates the interaction of aster MTs with the cell cortex, thereby causing the chromosome segregation during mitosis.

The binding of Lgl2 to LGN enhances the formation of NuMA-LGN complex. Lgl2 seems to induce the full activation of LGN in cooperation with NuMA at the cell cortex during mitosis. Formation of the Lgl2-Par-6-aPKC-LGN complex might activate the activity of LGN during mitosis. Therefore, the Lgl2-Par-6-aPKC-LGN complex may play a role in coupling the Par signaling pathway to LGN and the heterotrimeric G-proteins, to ensure that bipolar spindles are correctly organized and oriented for normal cell division.

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