Isolation of Full-Length Wild-Type LGN Protein

By Adam Howard, Ryan Elnicki, Autumn Smith, Justin Galardi, Mikalia Thomas-Ibitoye, Kyle Cohen, Laurie B. Cook, Ph.D, and Brandy Sreeilayam, Ph.D

Abstract

LGN, named for the many repeats of the amino acids leucine (L), glycine (G), and asparagine (N), is crucial for mammalian cellular division. Specifically, LGN plays a significant role in cell polarity and alignment of the mitotic spindles and, in its absence, the organism ceases to develop. LGN has been shown to be upregulated in multiple clinical breast cancer cells and several breast cancer cell lines, and mutations in LGN have resulted in Chudley-McCullough syndrome and non-syndromic hearing loss DFNB82. The goal of this project was to overexpress and isolate full-length, wild-type LGN protein so that it could be purified to 95% and protein crystals could be grown to result in a 3-D structure of LGN. To date, full-length LGN has only been isolated in the presence of $G\alpha_i$ and the end product was not 95% pure. Furthermore, several crystal structures of LGN have been solved, but none are of the full-length LGN. It was discovered that overexpression of LGN in E. coli cells resulted in significant degradation of the protein, so a mammalian system was utilized for expression. Wild-type LGN has been overexpressed transiently in baby hamster kidney (BHK)-570 tissue culture cells using a pCMV-LGN plasmid and LipoD 293 reagent. Sonication was used to break open the BHK-570 cells to preserve the native structure of LGN, and then LGN was isolated by performing a modified immunoprecipitation in which a rabbit anti-LGN polyclonal antibody and Protein A/G Plus-Agarose beads captured the LGN protein and a synthetic LGN peptide released the LGN protein. It was estimated that about one-third of the total LGN protein was isolated using the current protocol, so optimization is necessary before scaling up the process. Ultimately, solving the crystal structure of wild-type LGN may facilitate the process of novel treatments for breast cancer, Chudley-McCullough syndrome and non-syndromic hearing loss DFNB82.

Introduction

LGN protein is named due to the presence of ten leucine (L), glycine (G) and asparagine (N) repeats in the N-terminal half of the protein; its official name as approved by HUGO Gene Nomenclature Committee is G-Protein Signaling Modulator 2 (GPSM2).[1] It has a molecular mass of 76 kDa, a basal pI of 6.49, and is expressed in a variety of tissue including: brain, heart, lung, liver, pancreas, spleen, kidney, testis, ovary, and skeletal muscle.[1,2,3] It has been shown that LGN is critical in the receptor-independent G-protein signaling pathway that regulates mitotic spindle orientation in cellular division.[4,5,6]

Due to the important role LGN plays in cellular division, it is not surprising that LGN is involved in multiple medical conditions. Clinical breast cancer cells have shown upregulation of LGN in comparison with normal mammary epithelial cells.[7] In cancerous MCF7, MDA-MB-231, and T47D tissue culture cells, the localization of LGN mimicked the localization observed in noncancerous culture cells; LGN was in the cytoplasm during interphase and moved to the spindle poles during metaphase and anaphase.[7] It was determined that the T450 residue of LGN was phosphorylated by the serine/threonine kinase PBK/TOPK in the breast cancer cell lines, and knocking down the activity of LGN using siRNAs resulted in significant growth reduction in T47D tissue culture cells.[7] Overexpression of a T450A mutant of LGN also suppressed cell growth in non-cancerous COS-7 culture cells.[7] Another medical condition in which LGN is altered is Chudley-McCullough syndrome (CMCS), an autosomal-recessive disorder characterized by early and severe onset of sensorineural deafness and various abnormalities in the brain.[8,9,10] Thus far, it has been determined that several different pathological mutations have led to truncations of the LGN protein, causing CMCS.[8,9,10] Finally, two different nonsense mutations in LGN have been discovered in patients with autosomal recessive non-syndromic hearing loss DFNB82.[11,12] Nonsyndromic hearing loss is a partial or total loss of hearing that is not associated with other signs and symptoms affecting other parts of the body, and the effects patients experience vary greatly depending on which gene contains the mutation.[11,12]

LGN was initially classified as a mosaic protein with two distinct domains.[1] The N-terminal domain of LGN consists of eight tetratricopeptide repeat (TPR) motifs which can bind to a wide range of protein targets, including nuclear mitotic apparatus protein (NuMA), an F-actin binding protein Afadin, and the multidomain protein Frmpd4 (also known as Preso1).[13,14,15,16,17] Specifically, TPR5-8 interact with NuMA and TPR2-6 interact with Frmpd4.[17] The C-terminal domain contains 4 GoLoco (GL) domains, also known as G-protein regulatory (GPR) domains, which bind to $G\alpha_i$-GDP subunits independently of each other and with high affinity.[1,18] Within the GL domains, there is a highly conserved D/EQR triad called an arginine finger, which binds to the Ras-like and all-helical domains of $G\alpha_i$-GDP.[19] Interestingly, GL1-2 and GL3-4 domains bind to TPR0-3 and TPR4-7 motifs respectively, resulting in a closed, auto-inhibited conformation of LGN.[4] A truncated LGN crystal structure also shows that GL3-4 domains form a pair of parallel α helices and bind to the concave surface of the TPR4-7 motifs, thus preventing LGN from binding to other proteins.[4] When $G\alpha_i$-GDP binds to the GL domains, the TPR motifs are released from the GL domains, thus allowing the TPR motifs to interact with their binding partner proteins NuMA, Afadin, or Frmpd4.[17] The crystal structure of the GL3-4 domains bound to TPR4-7 motifs reveals a slightly different conformation of the GL domains compared to the crystal structures of GL3 or GL4 domains bound to $G\alpha_i$-GDP.[4,20] Furthermore, there is less than 50% overlap in the amino acids of the GL3-4 motifs that interact with $G\alpha_i$-GDP and TPR4-7 domains.[4] To date, there are no

protein structures of the full-length LGN protein and, thus, there is no information about the region of LGN that connects the N-terminal TPR motifs and the C-terminal GoLoco domains.

The long-term goal of this project is to purify full-length, wild-type LGN protein to 95% purity and grow protein crystals for protein X-ray crystallography. This paper focuses on the short-term goal of isolating LGN from baby hamster kidney (BHK)-570 tissue culture cells. Overexpression of full-length LGN protein was attempted in the lab in *E. coli* cells, but there was significant protein aggregation, so a mammalian system was used. The purification of full-length LGN had been reported using *Sf9* cells, but it was in complex with $G\alpha_i$-GDP and the purity was less than 95%.[21] In order to isolate the full-length LGN from transfected BHK-570 tissue culture cells, cell membranes were broken using sonication, then a modified immunoprecipitation reaction was used in which the LGN protein is captured using a rabbit anti-LGN antibody and Protein A/G Plus-Agarose beads. The LGN protein is then released by adding the synthetic LGN peptide that was used to generate the LGN antibody sequence. The presence of LGN in the supernatant fractions of a Western blot indicated that the LGN protein has successfully been competed off the antibody by the peptide.

Methods

LGN Expression in BHK-570 Tissue Culture Cells

The full-length, wild-type pCMV-LGN plasmid was purchased from OriGene and transformed in DH5α cells. A 1 mg/mL stock of pCMV-LGN was isolated using a maxi-prep and stored at -20 °C until needed. BHK-570 tissue culture cells (obtained from ATCC) were grown in DMEM growth media containing 10% FBS at 37 °C and 5% CO_2. For LGN isolation experiments, the BHK-570 tissue culture cells were grown to 80% confluency in 10 cm dishes before the transfection protocol was performed using LipoD293 DNA In Vitro Transfection Reagent, per the manufacturer's, SignaGen Laboratories, protocol. Cells were allowed to express LGN protein for 24 hours for the isolation experiments.

Lysis of the BHK-570 Tissue Culture Cells

BHK-570 tissue culture cells were either lysed with a buffer or by sonication. For the lysis buffer procedure, BHK-570 tissue culture cells were rinsed with ice cold PBS three times in the 10 cm dishes. One mL of lysis buffer (50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1:1000 protease & phosphatase inhibitors (purchased from Sigma-Aldrich) was added to each dish on ice for 5 minutes. The cells were scraped off the dishes and the lysates were transferred to chilled microfuge tubes, incubated on ice for 20 min, and centrifuged at 13,200 rpm for 10 minutes at 4 °C. The supernatants were transferred into new chilled microfuge tubes and stored at 4 °C until ready for use. For the sonication procedure, BHK-570 tissue culture cells were rinsed with ice cold PBS three times in the 10 cm dishes. One mL of sonication buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1:1000 protease & phosphatase inhibitors (purchased from Sigma-Aldrich)) was added to a 10 cm dish. Cells were scraped off the dishes and the lysates were transferred to chilled microfuge tubes and centrifuged at 13,200 rpm for 5 min at 4 °C. The supernatants were discarded and 500 µL of sonication buffer was added to the pellets. Microfuge tubes were sonicated for a total of 10 seconds in pulse mode (5 seconds on followed by 30 seconds off). Microfuge tubes were centrifuged at 13,200 rpm for 5 min at 4 °C and the supernatants were transferred into new chilled microfuge tubes and stored at 4 °C until ready for use.

Isolation of LGN

One µL of rabbit anti-LGN polyclonal primary antibody (1 mg/mL; purchased from Aviva Systems Biology) was added to the supernatant microfuge tubes, and the mixtures were gently rocked overnight at 4 °C. Sixty µL of protein A/G Plus-Agarose beads (purchased from Santa Cruz) were added and gently rocked at 4 °C for one hour. The microfuge tubes were centrifuged at 13,200 rpm at 4 °C for 2 minutes. The supernatant fraction was removed and labeled S*. The pellets were washed with either 500 µL of lysis buffer or sonication buffer three times. The pellet was resuspended in 300 µL of lysis buffer or sonication buffer and the lysate was equally distributed to 3 new microfuge tubes. Various amounts of LGN peptide (1 mg/mL; purchased from Aviva Systems Biology) and additional NaCl (0-350 mM) were added to the tubes and incubated overnight at 4 °C with gentle rocking. The microfuge tubes were centrifuged at 13,200 rpm at 4 °C for 5 minutes and 90 µL of the supernatant was transferred into 3 new chilled microfuge tubes. The supernatant microfuge tubes were centrifuged at 13,200 rpm at 4 °C for 5 minutes and 80 µL of the supernatant was transferred into 3 new chilled microfuge tubes (S1-S3). Pellets were washed with 200 µL of lysis buffer or sonication buffer 3 times, discarding the supernatant after each wash (P1-P3). The presence of LGN was determined by SDS-PAGE (10% gels) and Western blot analysis using PVDF membranes, rabbit anti-LGN polyclonal primary antibody (1:1000; purchased from Aviva Systems Biology) in 5% milk, and goat anti-rabbit IgG-HRP secondary antibody (1:5000; purchased from Fisher Scientific) in 5% milk. Detection of LGN bands was determined by film or computer imaging software (LI-COR C-Digit Blot Scanner).

Results

LGN protein was first attempted to be overexpressed and purified in *E. coli* using pET15b-LGN and pET28a-LGN vectors, but the SDS-PAGE and Western results indicated significant degradation of the protein (data not shown). The mammalian cell line of baby hamster kidney (BHK)-570 tissue culture cells was then utilized. The isolation of LGN was first attempted using

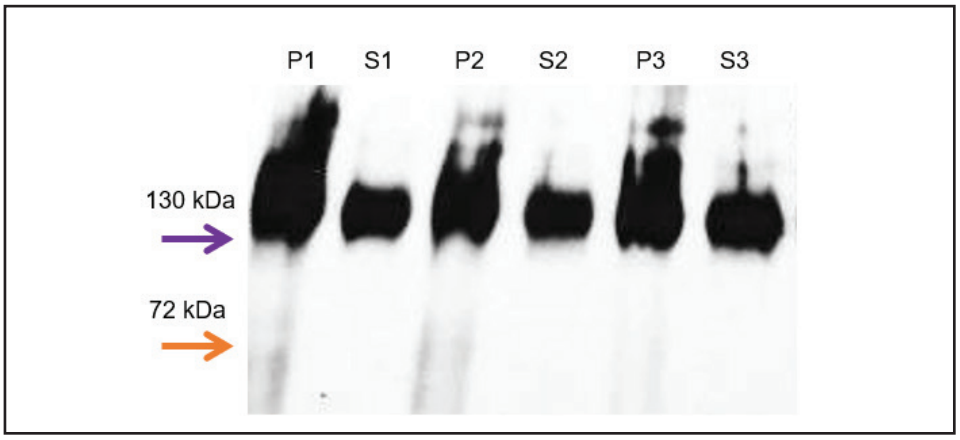

Figure 1: Isolation of LGN after lysing the BHK-570 tissue culture cells with lysis buffer. LGN protein was released from the antibody in the presence of 2 µg of peptide and various amounts of NaCl (100-500 mM). Lane contents from left to right: P1, S1, P2, S2, P3, S3. Orange arrow – 72 kDa; Purple arrow – 130 kDa.

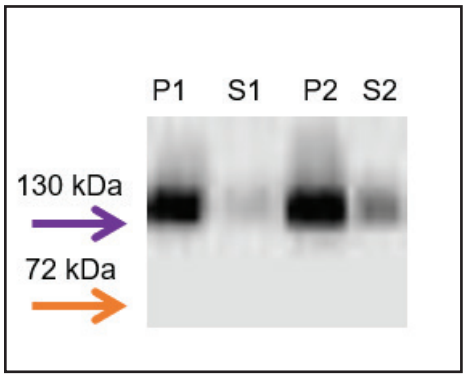

Figure 2: Isolation of LGN after sonication of the BHK-570 tissue culture cells. LGN protein was released from the antibody in the presence of 1 (left 2 lanes) or 2 µg (right 2 lanes) of peptide. Lane contents from left to right: P1, S1, P2, S2. Orange arrow – 72 kDa; Purple arrow – 130 kDa.

lysis buffer to break open the BHK-570 tissue culture cells. The cytosolic LGN protein that was expressed by the cells was captured in the pellet fraction using the anti-LGN primary antibody and protein A/G agarose beads. In order to compete LGN into the supernatant fraction, 2 µg of the peptide that was used to generate the anti-LGN antibody was added to each pellet fraction. Furthermore, an additional 100 mM, 250 mM or 500 mM NaCl was added to the P1, P2, and P3 fractions respectively. The presence of bands in lanes 2, 4, and 6, which correspond to S1, S2, and S3 respectively, indicate that LGN was successfully competed off the LGN antibody (Figure 1). Interestingly, the LGN protein detected by Western analysis appears to be around 150 kDa instead of the 76 kDa that corresponds to monomeric LGN.

Next, sonication was attempted to break open the BHK-570 tissue culture cells followed by the modified immunoprecipitation method the lab developed. After several sonication conditions were tested, it was determined that 40% amplitude and pulsing for 5 seconds on followed by 30 seconds off for a total duration of 10 seconds on resulted in the least harsh conditions to produce maximal amounts of protein in the cytosolic fraction (data not shown). When performing the modified immunoprecipitation procedure after lysing the cells using sonication in the presence of either 1 µg (left 2 lanes) or 2 µg (right 2 lanes) of peptide, it was evident that LGN protein was being competed off the LGN antibody into the supernatant fractions, lanes labeled as S1 and S2 (Figure 2). Furthermore, the sample that had 2 µg of peptide added competed about twice as much LGN into the supernatant fraction compared to the 1 µg of peptide being added (Figure 3, S2 and S1 respectively). Finally, the immunoprecipitation reaction was performed in which the presence of additional NaCl was added to the sonication buffer in BHK-570 tissue culture cells that were transfected in the presence (Figure 3 left image) or absence (Figure 3 right image) of pCMV-LGN. Interestingly, it appeared as though the BHK-570 tissue culture cells that were transfected with the pCMV-LGN plasmid resulted in more LGN being competed off the antibody, as the bands are thicker on the left Western compared to the right Western for lanes labeled S1, S2, and S3 (Figure 3). In contrast to the results obtained when using the lysis buffer to break open the cells, the amount of LGN protein competed off the antibody in the presence of 2 µg of peptide and additional 150 mM, 350 mM or 0 mM NaCl to the sonication buffer, represented by lanes labeled S1, S3, and S5 respectively, did not appear to compete off any more LGN protein (Figure 3). It is estimated that about one-third of the total LGN captured on the LGN primary antibody is being competed off into the supernatant fraction (Figure 3). The large band in the S* lane may indicate that not enough primary antibody is being added to the supernatant of the lysed cells and we are only capturing about half of the LGN being expressed from the BHK-570 tissue culture cells (Figure 3).

Discussion

LGN is known to be a crucial protein involved in cell division and mutations in LGN have resulted in several medical conditions. Although several crystal structures of the TPR motifs and GoLoco domains of LGN have been solved, there is no full-length LGN protein structure. Given that the first step of protein crystallization is to obtain 95% pure protein, it is important to be able to express and isolate functional protein before attempting to set up conditions to grow protein crystals. After it was determined that *E. coli* could not be used to overexpress LGN, BHK-570 tissue culture cells were selected based on their natural expression of LGN, suggesting that overexpression of LGN would not damage the cells or interfere with their normal function. Although it appears that the same amount of LGN is present in BHK-570 tissue culture cells transfected with and without pCMV-LGN, the amount of LGN competed off the LGN antibody using the modified immunoprecipitation procedure is higher in the BHK-570 tissue culture cells in which pCMV-LGN was added during the transfection (Figure 3). This could be due to the endogenous LGN protein having certain post-translational modifications that may not be present on the LGN expressed from the plasmid, such as phosphorylation of the T450 residue that is observed in breast cancer cell lines.[7]

Due to the potential misfolding of the LGN protein induced by the lysis buffer's detergent, sonication was utilized to allow LGN to maintain its native conformation. Even though more LGN peptide was added during the modified immunoprecipitation procedure, the fraction of LGN present in the supernatant fractions appeared to be less than when lysis buffer was used (Figures 3 and 1, respectively). This is likely due to the detergent helping to compete LGN off the antibody.

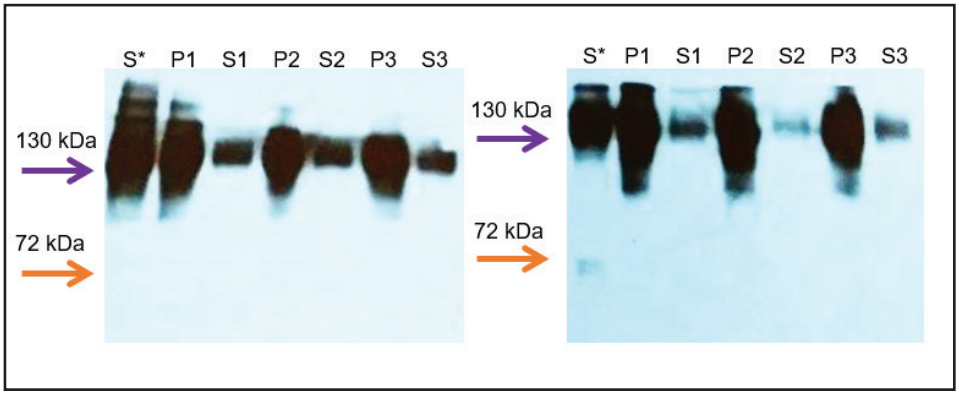

Figure 3: Isolation of LGN after sonication of the BHK-570 tissue culture cells using peptide and additional NaCl. LGN protein was released from the antibody in the presence of 2 µg of peptide and 150, 350 or 0 mM NaCl. BHK-570 tissue culture cells were transfected in the presence (left image) or absence (right image) of pCMV-LGN. Lane contents from left to right: S*, P1, S1, P2, S2, P3, S3. Orange arrow – 72 kDa; Purple arrow – 130 kDa.

Regardless of the way the BHK-570 tissue culture cells were broken open, the LGN bands from the Western analyses all have a significant band near 150 kDa instead of the expected 76 kDa that would correspond to monomeric LGN. Given that the TPR motifs and GoLoco domains of LGN bind, it is conceivable that dimers of LGN are forming where the TPR motifs of one protein are binding to the GoLoco domains of anther LGN protein and vice versa. Another possibility is that LGN is bound to one of its binding partners instead of dimerizing. However, this is highly unlikely because a denaturing gel was run, indicating that the interaction between the binding partners would be covalent, which is not consistent with the literature.[1,13,14,15,16,17,18,19] Given that each experimental condition was only run once, this data is preliminary and more experiments need to be performed to be confident that LGN is primarily at the 150 kDa band. Additionally, using an anti-LGN primary antibody that recognizes a different site than the primary antibody being used to sequester the LGN during the modified immunoprecipitation experiment and performing a functional assay to ensure that LGN can still bind to either $G\alpha_i$-GDP and/or NuMA would strengthen the possibility that LGN is being isolated as a dimer.

Given that only about one-third of the LGN is located in the supernatant fraction using the conditions tested thus far (Figure 3), the modified immunoprecipitation procedure needs to be optimized before scaling it up to obtain the amount of LGN protein necessary for screening crystallization conditions to grow protein crystals. Furthermore, the purity of the isolated LGN needs to be determined by SDS-PAGE and Coomassie blue staining. It is expected that size exclusion chromatography will be able to be utilized to obtain 95% pure LGN protein and ensure no LGN peptide is present in the final LGN fractions. Upon solving the crystal structure of the full-length, wild-type LGN, it may aid in the design of selective treatments to lessen or alleviate the symptoms experienced by patients with breast cancer, Chudley-McCullough syndrome, and/or non-syndromic hearing loss DFNB82.

Conclusion

Based on the preliminary data collected thus far, it is concluded that (i) LGN protein can be expressed in BHK-570 tissue culture cells and be isolated using a modified immunoprecipitation procedure, and (ii) more LGN protein is competed off during the modified immunoprecipitation procedure when the BHK-570 tissue culture cells are transfected in the presence of pCMV-LGN. Given that all experiments and Western data presented are representative of only one experiment, each experiment needs to be repeated at least two more times to draw firm conclusions about the data.

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