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Regulation of the formation and trafficking of vesicles from Golgi by PCH family proteins during chemotaxis

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ABSTRACT

Previous study demonstrated that WASP localizes on vesicles during *Dictyostelium* chemotaxis and these vesicles appear to be preferentially distributed at the leading and trailing edge of migrating cells. In this study, we have examined the role of PCH family proteins, Nwk/Bzz1p-like protein (NLP) and Syndapin-like protein (SLP), in the regulation of the formation and trafficking of WASP-vesicles during chemotaxis. NLP and SLP appear to be functionally redundant and deletion of both *nlp* and *slp* genes causes the loss of polarized F-actin organization and significant defects in chemotaxis. WASP and NLP are colocalized on vesicles and interactions between two molecules via the SH3 domain of NLP/SLP and the proline-rich repeats of WASP are required for vesicle formation from Golgi. Microtubules are required for polarized trafficking of these vesicles as vesicles showing high directed mobility are absent in cells treated with nocodazole. Our results suggest that interaction of WASP with NLP/SLP is required for the formation and trafficking of vesicles from Golgi to the membrane, which might play a central role in the establishment of cell polarity during chemotaxis.

1. Introduction

Chemotaxis, directed movement toward a chemoattractant agent, is a fundamental process of many cell types and is involved in diverse biological responses, including: migration of leukocytes and macrophages to inflammation sites, metastasis of tumor cells, and aggregation leading to the formation of the multicellular organism in *Dictyostelium* [1–4]. During these processes, the actin cytoskeleton is dynamically changed, a process that involves F-actin polymerization and depolymerization and the reorganization of existing filament networks. The first step of chemotactic movement is a chemoattractant-mediated increase in F-actin polymerization at the leading edge of the cell, which provides the motive force for pseudopod extension and cell movement. The Wiskott–Aldrich Syndrome protein (WASP) and related proteins (N-WASP and SCAR/WAVE) have emerged as key downstream components converging on multiple signaling pathways to F-actin polymerization. The proline-rich segment of WASP interacts with a number of proteins containing SH3 domains, many of which are involved in the regulation of cytoskeletal structure [5,6]. One of the SH3 proteins known to interact with WASP is Cdc42-interacting Protein 4 (CIP4), one of the pombe Cdc15 homology (PCH) family proteins.

The PCH family proteins are characterized by the presence of an evolutionarily conserved FER-CIP4 homology (FCH) domain and coiled-coil (CC) region, and they induce tubular membrane invagination *in vivo* and deform liposomes into tubules *in vitro* [7–9]. They are also known as F-BAR-domain-containing proteins (F-BAR proteins) since the FCH and coiled-coil domains are structurally similar to Bin/amphiphysin/RVS (BAR) domains [10,11]. These two domains together were also called as the extended FC (EFC) domain [12]. Some PCH family members contain homology region 1 (HR1) domain, which interact with the Rho GTPases Cdc42, TC10, and Rnd2 [13]. Most PCH proteins have one or more Src homology 3 (SH3) domains at the COOH terminus, binding to various target molecules, including dynamins, N-WASP, and formin [8,9,12,14,15]. Different members of the PCH family appear to regulate various aspects of actin organization. CIP4 binds to activated Cdc42 and, when overexpressed, decreases the number of stress fibers in fibroblasts [7]. Overexpression of rat synaptic, dynamin-associated protein I (Syndapin I) or Syndapin II, caused reorganization of cortical F-actin and formation of filopodia in HeLa cells [16]. PSTPIP2 is an actin bundling protein that stimulates formation of filopodia, inhibits ruffling and increases the motility of macrophages [17].

In a previous study, we demonstrated that actin cytoskeleton is highly polarized in chemotaxing *Dictyostelium* cells and that WASP, a major regulator of F-actin assembly, localizes on vesicles, and these vesicles appear to be preferentially distributed at the leading edge and uropod of chemotaxing cells [18]. In this study, we have examined the role of two PCH family proteins, NLP and SLP, in the regulation of the formation and trafficking of vesicles that are associated with WASP. NLP and SLP appear to be functionally redundant and the deletion of both

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NLP and SLP causes a loss of polarized F-actin organization and severe defects in chemotaxis. WASP and NLP/SLP are colocalized on vesicles, and interactions between SH3 domains of NLP/SLP and the proline-rich region of WASP are required for the formation and trafficking of these vesicles. Our results demonstrate that NLP/SLP play an essential role in controlling the formation and trafficking of WASP-associated vesicles.

2. Materials and methods

2.1. Molecular biology

The full coding sequence of the *nlp* and *slp* cDNAs was generated by PCR using a series of primers based on a sequence in The *Dictyostelium* Genome Project database (http://www.dictybase.org). PCR products were subcloned into the EcoRI–XhoI site of pSP72 and sequenced. An *nlp* knockout construct was made by inserting the blasticidin resistance cassette into a BglII site. An *slp* knockout construct was made by inserting hygromycin resistance cassette into a BamHI site created at nucleotide 465 of the *slp* cDNA. These constructs were used for a gene replacement in the KAx-3 parental strain. Randomly selected clones were screened for a gene disruption by PCR, which was then confirmed by Southern blot analysis and RT-PCR. To YFP- or GST-fusion protein expression, *nlp* and *slp* cDNAs were subcloned into EXP-4(+) or pGEX plasmids, respectively.

2.2. Immunostaining and F-actin staining

Immunostaining was performed as described in the previous study [19]. For colocalization studies, a series of images with varying vertical focus (typically 10 images with 0.2 μm distance) was taken and reconstituted with deconvolution using Metamorph software and analyzed with colocalization measurement application of the Metamorph. Both source images were thresholded prior to performing the colocalization measurement and the area of overlap between the two probes was measured as the percentage of whole image.

For phalloidin staining, cells were starved in 12 mM sodium phosphate buffer (pH 6.2) for more than 5 h and fixed with 3.7% formaldehyde for 5 min. Cells were permeabilized with 0.5% Triton X-100, washed, and incubated with FITC or TRITC-labeled phalloidin (Sigma) in PBS containing 0.5% BSA and 0.05% Tween-20 for 1 h. Cells were washed in PBS containing 0.5% Tween-20. Images were captured with Roper Coolsnap camera and Metamorph software.

For labeling barbed ends, aggregation-competent cells were permeabilized with 100 mM PIPES pH 6.9, 1% Triton X-100, 4% PEG, 1 mM EGTA, 1 mM MgCl2, 3 μM phalloidin for 3 min and 0.4 μM Rhodamine-labeled actin in 1 μM ATP solution was added. After 5 min staining, cells were washed 3 times with PIPES buffer and fixed with 3.7% paraformaldehyde.

2.3. Chemotaxis assay

Cells competent to chemotax toward cAMP (aggregation-competent cells) were obtained by pulsing cells in suspension for 5 h with 30 nM cAMP and plated on glass-bottomed microwell dishes. A micropipette filled with 100 μM cAMP was positioned to stimulate cells by using a micromanipulator and DIC images of migrating cells were taken in 6 s intervals for 15 min and analyzed with Metamorph software (Universal Imaging Corp., Downingtown, PA). Chemotaxis index is defined as $CI\ (t) = \cos(\theta\ (t))$, where θ = the frame by frame angle of turn for each cell in our sample and the index presented in the paper is the average value of indices over the experiment.

2.4. In vivo actin polymerization assay

F-actin was quantified from TRITC phalloidin staining of *Dictyostelium* cells as described in the previous study [20]. Cells were pulsed with 30 nM cAMP at 6 min intervals for 5 h. Cells were diluted to 1×10^7 cells/ml and shaken at 200 rpm with 2 mM caffeine for 20 min to synchronize the signaling of the cells. Cells were spun and resuspended with phosphate buffer (10 mM PO_4 buffer, pH 6.1, and 2 mM $MgSO_4$) at 5×10^7 cells/ml and stimulated with 100 μM cAMP. 500 μl of cells were taken at 5, 10, 20, 30, 50, and 80 second time points and mixed with actin buffer (20 mM KH_2PO_4, 10 mM PIPES, pH 6.8, 5 mM EGTA, 2 mM $MgCl_2$) containing 6% formaldehyde, 0.15% Triton X-100, 1 μM TRITC phalloidin. Cells were fixed and stained for 1 h and spun down at 14,000 rpm for 5 min in the microfuge. Pelleted cells were extracted with 1 ml of 100% methanol and fluorescence was measured (540 ex/575 em). To determine nonsaturable binding, 100 μM unlabeled phalloidin was included.

2.5. Vesicle formation reconstitution assay

Vesicle budding assay was done as described in previous studies [24,25].

2.5.1. Preparation of cytosol

Dictyostelium cells were pulsed with 50 nM cAMP at 6-min intervals for 5 h. Cells were then pelleted at 800 g for 5 min, and washed once in homogenization buffer containing 125 mM KCl, 25 mM Hepes, pH 7.4. The cells were resuspended in homogenization buffer followed by homogenization using a stainless steel ball bearing homogenizer. The homogenate was centrifuged for 30 min at 214,000 *g* to get the cytosol fraction.

2.5.2. Preparation of permeabilized Dictyostelium cells

Approximately 2×10^7 cells were plated on 100 mm Petri-dish. Cells were washed 3 times with ice-cold swelling buffer containing 10 mM Hepes, pH 7.2 and 15 mM KCl. After a 10 min incubation on ice, the swelling buffer was aspirated and replaced with 1 ml breaking buffer (10 mM Hepes, 90 mM KCl) after which the cells were broken by scraping with a rubber policeman and 1 stroke of homogenization. The cells were centrifuged at 800 *g* for 5 min, washed in 1 ml of breaking buffer, and resuspended in 400 ml of breaking buffer.

2.5.3. Reconstitution assay

Reconstitution assays for vesicle budding were done in a final volume of 600 ml containing: 240 ml permeabilized cells, 240 ml cytosolic extract (360 mg protein), 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 110 mM KCl, 1 mM ATP, 0.02 mM GTP, 10 mM creatine phosphate, 80 mg/ml creatine phosphate kinase, and protease inhibitors. Incubations were at RT for 1 h and then centrifuged at 800 *g* for 5 min. After centrifugation, supernatant was removed and centrifuged for 30 min at 214,000 *g*. Supernatant and pellet were collected and run on SDS-PAGE gel to determine the amount of comitin by Western blot.

3. Results

3.1. WASP is associated with vesicles derived from Golgi

Our previous study demonstrated that GFP-WASP is associated with vesicles enriched with $PI(4,5)P_2$ and the basic (B) domain plays a major role for the interaction with $PI(4,5)P_2$ [18], compared to uniform distribution of GFP (Fig. 1A). This vesicular localization of GFP-WASP can be recapitulated with YFP-tagged basic (B) and GTPase-binding (GBD) domains of WASP (YFP-B-GBD). To identify the origin of the these WASP-vesicles, we first examined the possibility of WASP- or YFP-B-GBD-associated vesicles being endosomes by staining cells with FM 4–64, a membrane-impermient lipophilic dye that fluoresces only when associated with membranes, and the internalization of the dye was monitored by fluorescence microscopy after washing out the dye. Plasma membranes become fully stained within seconds, and fluorescence then gradually