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Regulation of Actin Cytoskeleton by WasC and Electric Field-Directed Cell Migration by G-Proteins in *Dictyostelium*

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딕티오스텔리움에서 WasC에 의한 액틴 세포 골격 및 G-단백질에 의한 전기 자극 매개 세포 이동 조절

2020 년 2 월 25 일

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ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
GBD	GTPase binding domain
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPCRs	G-protein coupled receptors
GTP	Guanosine triphosphate
LatB	Latrunculin B
PBS	Phosphate-buggered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PPR	Poly proline-rich region
SD	Standard deviation
SEM	Standard error of measurement
TRITC	Tetramethylrhodamine
VCA	Verprolin homology, central, acidic region
WASP	Wiskott-aldrich syndrome protein

ABSTRACT

Regulation of Actin Cytoskeleton by WasC and Electric Field-Directed Cell Migration by G-Proteins in *Dictyostelium*

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The actin cytoskeleton is involved in the regulation of cell morphology and cell migration. For the cells to migrate, first the actin cytoskeleton is highly polarized in chemotaxing cells. Wiskott-Aldrich Syndrome Protein (WASP) plays an important role in controlling actin polymerization by activating Arp 2/3 complex. The WASP family proteins are key regulators that link multiple signaling pathways to regulate the actin cytoskeleton. I investigated the function and regulation of WasC, one of 3 WASP family proteins in *Dictyostelium* in cell migration and development. To investigate the functions of WasC, I prepared *wasC* null cells by homologous recombination. *wasC* null cells showed spread morphology and strong cell adhesion compared to wild-type cells. Loss of WasC exhibited 1.8-fold increase in the level of F-actin polymerization compared to wild-type cells in response to chemoattractant stimulation. I investigated the roles of WasC in phagocytosis and cell migration of *Dictyostelium* cells and found that *wasC* null cells exhibited a negative effect on phagocytosis and chemoattractant-mediated cell migration. These phenotypes of *wasC* null cells were rescued by overexpressing GFP-WasC. The localization assay showed that WasC localized at the cell cortex dependently of F-actin and translocated in response to chemoattractant stimulation. In addition, WasC

localized at the leading edge in migrating cells. This study suggests that WasC plays a key role in F-actin polymerization at the leading edge of the cells.

Electrotaxis is a directional cell migration in an electric field (EF) and occurs in a variety cell types. The directed cell migration by EF is involved in diverse physiological processes including embryogenesis, immune response, and wound healing. The molecular mechanism of electrotaxis is beginning to be revealed, although the major signaling pathways are expected to be shared between electrotaxis and chemotaxis. Recent papers showed that cell migration directionality and speed are independently regulated by RasG and Gβ in *Dictyostelium* in electrotaxis. *Dictyostelium* contains only one Gβ subunit and one Gγ subunit, while 12 Gα subunits have been identified. To examine the regulation of cell migration speed by G-proteins in electrotaxis, here I analyzed cell motilities of *Gα2* null cells as well as *Gβ* null cells. Wild-type cells display a distinct acceleration/deceleration kinetics of trajectory speed and directionality upon EF exposure and the significant induction of the speed and directionality within 5-10 min after EF stimulation. In contrast, *Gβ* null cells showed that no acceleration of the trajectory speed upon EF stimulation and almost constant migration speed as reported previously. *Gα2* null cells showed similar acceleration/deceleration kinetics of speed to those of *Gβ* null cells in response to EF stimulation. Cells lacking Gα2 displayed relatively constant migration speed without significant induction of speed on EF exposure. These results indicated that not only Gβ but also G-protein complex are involved in electrotaxis, suggesting that Gα2 and Gβ is required for the induction of migration speed in response to EF.

국문초록

딕티오스텔리움에서 WasC에 의한 액틴 세포 골격 및 G-단백질에 의한 전기 자극 매개 세포 이동 조절

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액틴 세포 골격은 세포 형태 및 세포 이동의 조절에 관여하며 특히 주화성 이동을 하는 세포에서 매우 양극화 되어있다. Wiskott-Aldrich 증후군 단백질 (WASP)은 Arp 2/3 복합체를 활성화시킴으로써 액틴 중합을 조절하는데 중요한 역할을 하며, 액틴 세포 골격을 조절하는 다양한 신호 전달 경로에 중요한 조절인자로 알려져 있다. 본 연구에서는, *Dictyostelium* 의 세포 이동과 발생 과정에서 3가지 WASP 단백질 중 WasC의 기능을 조사하기 위해 상동재조합에 의해 WasC가 없는 세포를 준비하였다. *wasC* null 세포는 야생형과 비교했을 때 퍼져 있는 형태를 가지고 강한 세포 부착이 나타났으며 주화성 물질의 자극 시 야생형에 비해 1.8배의 증가된 F-액틴 중합을 보여주었다. *Dictyostelium* 세포의 식세포작용과 세포 이동에 관한 WasC의 역할을 조사하였고, *wasC* null 세포는 식세포작용과 주화성 세포 이동에 부정

적인 영향을 미친다는 것을 발견하였다. *wasC* null 세포의 이러한 현상은 GFP-WasC를 과발현 함으로써 회복되었다. 위치조사에서 WasC는 F-액틴의 의존적으로 세포 피질에 위치하였고 주화성 물질의 자극 시 세포 피질로 전위되며 이동하는 세포의 앞쪽 가장자리에 위치함을 보여주었다. 본 연구에서는 WasC가 세포의 앞쪽 가장자리에서 증가된 F-액틴 중합에 중요한 역할을 수행한다는 것을 시사한다.

Electrotaxis는 전기장(EF)에서의 방향성 세포 이동이며 다양한 세포에서 발생하는 메커니즘이다. EF에 의한 세포 이동은 발생 과정, 면역 반응, 상처 치유를 포함한 다양한 생리학적 과정에 관여한다. 최근 연구는 electrotaxis에서 *Dictyostelium* 세포의 방향성과 속도가 RasG와 Gβ에 의해 독립적으로 조절됨을 보여주었다. *Dictyostelium* 세포에서는 1개의 Gβ, Gγ, 12개의 Gα 서브 유닛이 확인되었으며 electrotaxis에서 G-단백질의 속도에 대한 메커니즘을 조사하기 위해 *Gα2* and *Gβ* null 세포를 이용하여 세포 이동을 분석하였다. 야생형 세포는 EF 자극 시 이동 속도와 방향성의 특정한 가속 및 감속을 보여주었고 특히 EF 자극 후 5-10분 이내에 속도와 방향성이 크게 증가하는 것을 보여주었다. 반면, *Gβ* null 세포는 이전에 보고된 바와 같이 EF 자극 시 속도의 가속을 나타내지 않았으며 거의 일정한 속도를 나타내었고 *Gα2* null 세포 역시 *Gβ* null 세포의 같이 EF 자극에서 일정한 이동 속도를 보여주었다. 연구결과는 Gβ 뿐 아니라 G-단백질 복합체 자체가 electrotaxis에 영향을 미칠 수 있음을 암시하며 본 연구에서, Gα2와 Gβ가 EF에서의 세포 이동 속도의 증가를 유도할 수 있다는 것을 시사한다.

❖ **This part is to be submitted for publication as Pyeonghwa Jeon and Taeck J. Jeon, 2019.**

Part I. WasC is Involved in Cell Adhesion and Cell Migration by Regulating F-actin Polymerization in *Dictyostelium*

I. INTRODUCTION

Cell migration is involved in many biological and pathological processes, such as embryonic development, wound healing, inflammatory responses, and tumor metastasis (Jin et al., 2009; Lee and Jeon, 2012; Ridley et al., 2003). Cell migration is facilitated by reconstitution of actin cytoskeleton; therefore, it is necessary to understand the signaling pathway. However, it is difficult to understand cell migration because cell migration requires the integration and temporal coordination of many different processes that appear in spatially different locations within the cells (Lee and Jeon, 2012).

The social amoeba *Dictyostelium discoideum* has been used as a powerful model system for investigation of chemotaxis, directional cell movement towards chemoattractants, over the past 40 years. Therefore, *Dictyostelium* is a good model organism for studying cellular process such as cell migration, cell division, and multicellular developmental process. *Dictyostelium* is a free-living soil amoeba that feeds on bacteria. These organisms follow bacteria by chemotaxing towards folic acid, which is secreted by the bacteria (Chisholm and Firtel, 2004; Lee and Jeon, 2012). This process

is very similar to macrophages or neutrophils chasing bacteria. In the state of starvation, *Dictyostelium* experiences a strictly regulated multicellular developmental process in which they secrete cAMP and move toward aggregation centers through chemotaxis, finally forming fruiting bodies (Chisholm and Firtel, 2004).

The actin cytoskeleton is involved in almost every aspects of cell behavior and particularly involved in cell migration, cell adhesion, cell division, and endocytosis (Insall and Machesky, 2009). F-actin polymerization at the leading edge of the cells are regulated by lots of actin-modifying proteins such as Arp2/3 complex, WASP, WAVE / SCAR and Cofilin (Cha et al., 2011; Sasaki and Firtel, 2006). Among these, Arp 2/3 complex is one of the important proteins required for the assembly of new F-actin filaments (Pollard, 2007). The production of effective actin nuclei requires the interaction of WASP family proteins with, Cdc42 and Rac effector (Raftopoulou and Hall, 2004) and existing actin filament (Cha et al., 2011; Firat-Karalar and Welch, 2011). Actin polymerization is mediated by the Arp2/3 complex, the activity of which is regulated by adapter proteins such as WASP and SCAR / WAVE family proteins.

WASP family proteins were first discovered in 1994 as the gene mutated in Wiskott-Aldrich Syndrome Patients (Derry et al., 1994). The WASP family proteins share a similar domain structure and were involved in signal transduction from the cell surface receptor to the actin cytoskeleton. *Dictyostelium* have a single, conserved member of each of the WASP (Myers et al., 2005), SCAR (Bear et al., 1998), and WASH (Carnell et al., 2011) families. WASP family protein in *Dictyostelium* are involved in F-actin polymerization. These proteins directly or indirectly binds to a small GTPase Cdc42 and Arp2/3 complex to regulate the formation of actin filaments. The binding of active Cdc42 enhances the ability of N-WASP (Neural WASP) to activate Arp2/3, and thus a signaling pathway leading to actin-filament nucleation (Rohatgi et al., 1999). The C-terminus of WASP family proteins are composed of domains that bind to and activate the Arp2/3 complex.

This domain is called the VCA domain, containing a Verprolin homology, a Central, and an Acidic region, which is the criteria for defining WASP protein. A more correct name for the Verprolin homology domain is WASP Homology2 (WH2) motif, and it binds to actin monomers (Dominguez, 2007; Veltman and Insall, 2010). C motif is similar to the corresponding region of cofilin in function (Boczkowska et al., 2008; Marchand et al., 2001). The Central region has binding affinities to both actin and Arp2/3 and play a role in the transfer of the actin monomer to the Arp2/3 complex (Kelly et al., 2006; Marchand et al., 2001). The Acidic region binds to, and activates the Arp2/3 complex. Three WASP family proteins have been identified in *Dictyostelium*. WasA was suggested to regulate the F-actin level and F-actin assembly during chemotaxis. *wasA* null cells showed reduced F-actin levels and defects in organizing polarized F-actin assembly, resulting in decreased cell motility during chemotaxis, and delayed development. Rac1b, RacA, RacB and RacC of Rho family proteins was identified as an interacting protein with WasB in yeast two-hybrid screening. *wasB* null cells showed loss of F-actin organization polarity, resulting in increased F-actin polymerization and aberrant protein localization. Cells lacking of WasB exhibited abnormal cell motility due to defective pseudopod formation at the lateral side of the cells during chemotaxis (Chung et al., 2013).

Here, I investigated the functions of WasC, one of the WASP family proteins, in cell migration and F-actin polymerization by using cells lacking or overexpressing WasC.

II. MATERIALS AND METHODS

II-1. Cell culture and strains

The *Dictyostelium discoideum* KAx-3 cells were cultured axenically in HL5 medium at 22°C (Nellen et al., 1984). The HL5 medium contained 6ml of 100X antibiotic-antimycotic solution and 16ml of 50% glucose in 600 ml. The cell expressing green fluorescent protein (GFP)-WasC and other transformants were maintained with 10 μg/ml G418.

II-2. Plasmids

The coding sequence of WasC (cDNA) was obtained by reverse transcription-polymerase chain reaction (RT-PCR) and then was cloned into the *EcoR*I-*Xho*I site of the expression vector pEXP-4(+) containing a GFP fragment, respectively (Jeon et al., 2009). The *wasC* knockout construct was prepared by inserting the blasticidin resistance cassette (*bsr*) into *BamH*I site created at nucleotide 500 of *wasC* gDNA and used for a gene replacement in KAx-3 parental strains. Randomly selected clones were screened for a gene disruption by PCR. The primers used in the screening for a gene replacement are stated in Table 1.

II-3. Cell adhesion assay

Log-phase growing cells on plates were washed with 12 mM Na/K phosphate buffer (pH 6.1) and resuspended at a density of 2×10^6 cells/ml. Cells (4×10^5 in 200 μl) were plated and attached on the 6-well culture dishes. Before shaking the plates, cells were photographed and counted for calculating the total cell number. The plates were constantly shaken at 150 rpm for 1h to detach the cells from the plates, and the attached

cells were photographed and counted (attached cells) after the medium containing the detached cells was removed. Cell adhesion was shown as a percentage of attached cells compared with total cells.

II-4. Development

Development assay was performed as described previously (Jeon et al., 2009). Growing cells were harvested and washed twice with 12 mM Na/K phosphate buffer (pH 6.1) and resuspended at a density of 3.5×10^7 cells/ml and then 50 μl of the cells were dropped on Na/K phosphate agar plates and developed for 24 hours. The developmental morphology of the cells was examined under a phase-contrast microscope.

II-5. Phagocytosis assay

Phagocytosis was analyzed as described previously (Benghezal et al., 2006) and based on a published protocol (Pontel et al., 2016; Rivero and Maniak, 2006). Exponentially growing bacteria (*K. aerogenes* cells) in SM media were placed on the SM plate and then 50, 100, and 150 *Dictyostelium* cells were seeded. Photographs were analyzed using Image J software (National Institutes of Health).

II-6. Chemotaxis assay

Chemotaxis of the cells toward cAMP and changes in the subcellular localization of GFP-fusion proteins in response to chemoattractant stimulation were examined as described previous (Jeon et al., 2007b; Mun et al., 2014). Aggregation-competent cells were prepared by incubating cells at a density of 5×10^6 cells/ml in 12 mM Na/K phosphate buffer (pH 6.1) for 10 hours with a constant shaking (110 rpm). Cell migration was analyzed using a Dunn Chemotaxis Chamber (SVDCC100, UK). The images of chemotaxing cells were taken at time-lapse intervals of 1 min for 1 hour. The data were

analyzed using the NIS-Elements software (Nikon) and Image J software (National Institutes of Health).

II-7. Electrotaxis assay

Dictyostelium cells were starved in DB buffer for 3 hours and dropped into an electroctaxis chamber as described previously (Zhao et al., 1996a). Before EF stimulation, two agar salt bridges were prepared of no less then 15cm long. The two agar salt bridges were used to connect $AgCl_2$ electrodes in beakers with Steinberg's solution to pools of buffer at either sides of chamber. For electric field application, the electric field strength of 15V/cm was used. The field strength was measured at the beginning and the end of the experiment (Shanley et al., 2006).

II-8. Phalloidin staining

Exponentially growing cells were placed on the coverslip and then fixed with 3.7 % formaldehyde for 5 min. The fixed cells were washed with phosphate-buffered saline (PBS buffer, pH 7.4), permeabilized with 0.1% Triton X-100 for 1 min, and then stained with 0.5 μg/ml of TRITC-Phalloidin dye. Images were captured using NIS-elements software (Nikon).

II-9. F-actin assay

Log-phase vegetative cells were washed with 12 mM Na/K phosphate buffer (pH 6.1) and resuspended at 5 x 10^6 cells/ml in 12 mM Na/K phosphate buffer. The cells were starved for 10 hours. Before cAMP stimulation, cells were pretreated with 1 mM caffeine. The cells were washed twice with 12 mM Na/K phosphate buffer, stimulated with 0.5 μM cAMP, and collected at different time points. The samples were subjected to SDS-PAGE and stained with Coomassie Blue. The densities of F-actin bands were quantified using Wasabi software (Software Lab).

II-10. Quantitative analysis of membrane or cortical localization of GFP fusion proteins

Localization of the proteins was analyzed as described previously (Jeon et al., 2007b; Sasaki et al., 2004). Aggregation competent cells were allowed to adhere to the plate for 10 min. Cells were then uniformly stimulated with cAMP by quickly pipetting 250 μl of 150 μM cAMP into the plate containing cells. Fluorescence images were taken at time-lapse intervals of 1 sec for 1 min using an inverted microscope. The frames were captured using NIS-elements software (Nikon) and analyzed using Image J software (National Institutes of Health). The intensity of fluorescence at the cell cortex was measured, and the level of cortical GFP was calculated by dividing the intensity at each time point (Et) by the intensity before stimulation (Eo).

Table 1. PCR primers used in *wasC* knock-out construct

Primer name	Direction	Sequence (5´ → 3´)	Location
I	F	GAATTCATGACAAATCAATCGAATTGC	1 - 27
II	R	GTAGTAGTAGTAGGTTTAGTCCTAGG	479 - 504
III	F	TCAGGATCATCATTTATGAG	499 - 518
IV	R	TTTGCAGAGGTTGCAACTGG	859 - 879

F – Forward, R - Reverse

Table 2. VCA domains used in this study.

Species	Gene name	Abbreviation	Gene banker name
Dictyostelium discoideum	WasA	Dd_A	G0293834, Dictybase
Dictyostelium discoideum	WasB	Dd_B	G0272811, Dictybase
Dictyostelium discoideum	WasC	Dd_C	G0283827, Dictybase
Homo sapiens	N-Wasp	Hs	NP_003932, Genbank
Xenopus tropicalis	Wasl	Xt	L7N342_XENTR, UniProt
Danio rerio	Wasl	Dr	NP_957353, Genbank
Strongylocentrotus purpuratus	Wasl	Sp	XP_001181262.1, Genbank
Drosophila melanogaster	Wasp isoform A	Dm	NP_651637.1, Genbank
Nematostella vectensis	Wasl	Nv	NP_001635928.1, Genbank
Trichoplax adhaerens	Triaddrafr_54962	Ta	B3RTH0_TRIAD , UniProt
Batrachochytrium dendrobatidis	Batdedrafr_21300	Bd	F4NS90_BATDJ , UniProt

III. RESULTS

III-1. Characterization of the gene encoding WasC

There are 3 genes encoding WASP proteins in the *Dictyostelium*. The domain structures are described (Fig. 1A). Most of WASP proteins have similar domain structure, while WasB and WasC do not have the WH1 domain at the N-terminus and the N-terminus of WasC is longer than other two genes. Among of them, WasA and WasB have been characterized previously (Chung et al., 2013; Myers et al., 2005), and their functions have been studied in various biological processes. However, the function of WasC has not been studied yet. Here, I investigated one of 3 WASP proteins WasC. *Dictyostelium* WasC has 905 amino acids (expected molecular mass of 99.5kDA) and contains domains of GBD (GTPase Binding Domain), PPR (Poly Proline-Rich region), and VCA (Verprolin homology, Central, and Acidic region) (Fig. 1A). The phylogenetic tree with VCA domains of Wasp subfamily proteins show that WasC is closely related to WasB (Fig. 1B).

To investigate the similarity of WasC to other Wasp subfamily proteins, the VCA domain sequence of WasC was compared to other proteins with VCA domains of Wasp proteins by multiple alignment (Fig. 2). These results show that the critical amino acids (**LK**X**V**) of the domain which are involved in F-actin polymerization were conserved in WasC as in other Wasp subfamily proteins.

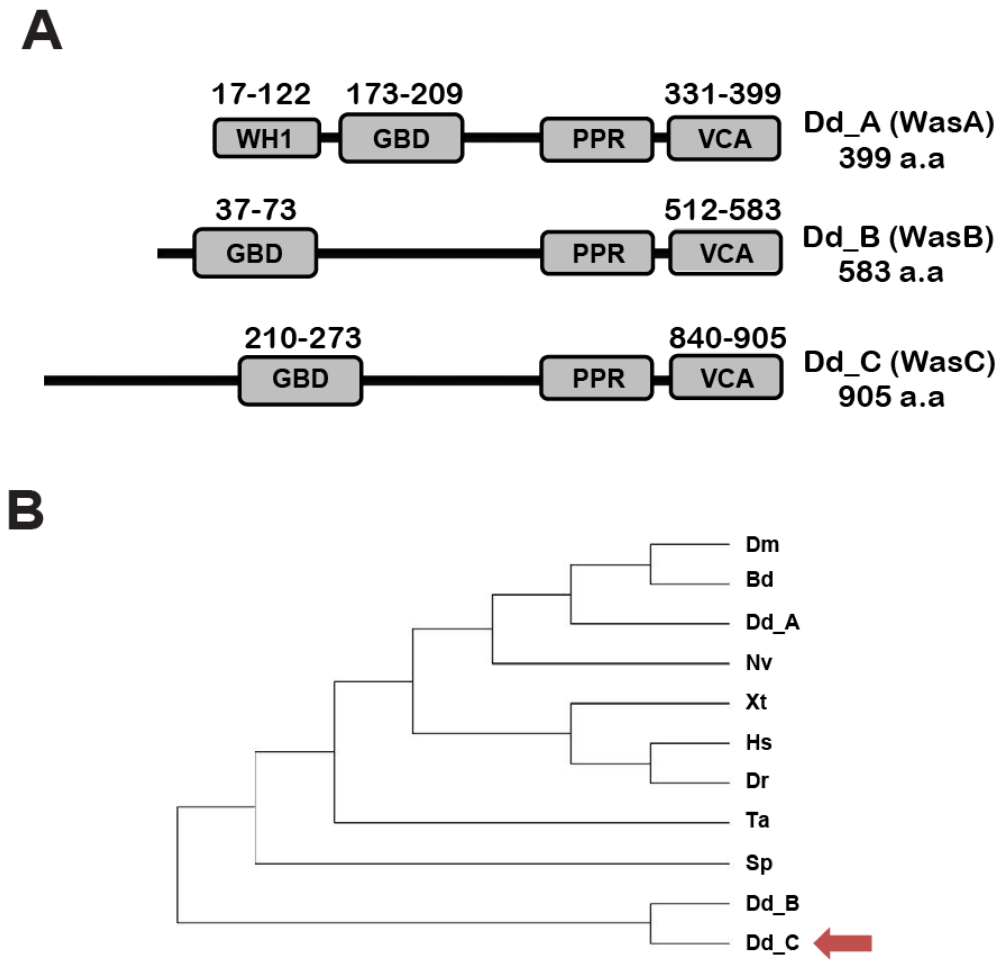

Fig 1. Domain structure of Wasp proteins in *Dictyostelium*, and phylogenetic tree of VCA domains

(A) Domain structure of Wasp proteins. (B) Phylogenetic tree analysis of VCA domains in Wasp proteins. The amino acid sequences of VCA domain were compared with those of other Wasp proteins. The arrow indicates WasC. The amino acid sequences of Wasp are available at www.dictybase.org. *Dictyostelium discoideum* WasA, (G0293834); WasB, (G0272811); WasC, (G0283827).

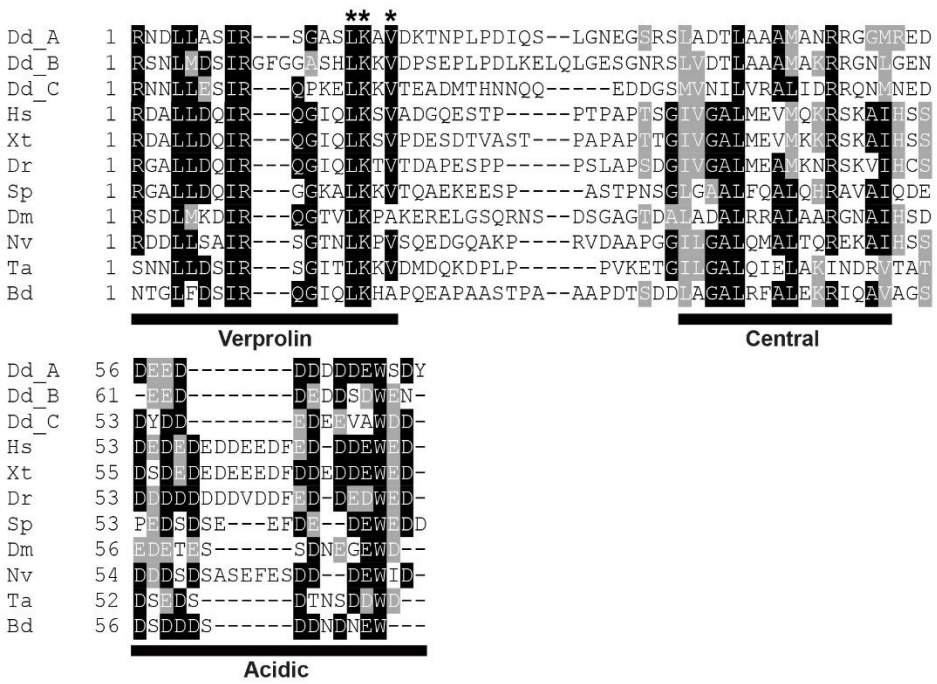

Fig 2. Multiple alignment of VCA domains

The amino acid sequence of Wasp proteins were compared with those of other Wasp containing VCA domain. The identical amino acid residues were highlighted in dark gray or light gray. The conserved residues in the VCA domain were marked with asterisks.

III-2. Confirmation of *wasC* knock-out and WasC-overexpressing cells

To understand the functions of WasC in *Dictyostelium*, I prepared the *wasC* knock-out strain by homologous recombination. The *wasC* knock-out construct was prepared by inserting the blasticidin resistance (*bsr*) antibiotic cassette into the *wasC* cDNA and used for a gene replacement in KAx-3 parental strains (Fig. 3A). Randomly selected clones were screened by polymerase chain reaction (PCR) with sets of primers (Table 1). PCR with a set of primers, III /IV and I/IV, produced bands of 380 and 880 bp in wild-type cells and bands of 380 and 2180 bp in *wasC* null cells, respectively. The increased size (2180 bp in *wasC* null cells) was consistent with the insertion of the *bsr* cassette into the gene (Fig. 3B). These results indicated that the *wasC* gene was replaced with the *wasC* knock-out constructs in *wasC* null cells. To investigate the functions of WasC, cells overexpressing GFP-WasC fusion protein (expected molecular mass of 126 kDa) were prepared, and protein expression was confirmed by western blotting using anti-GFP antibodies (Fig. 3C).

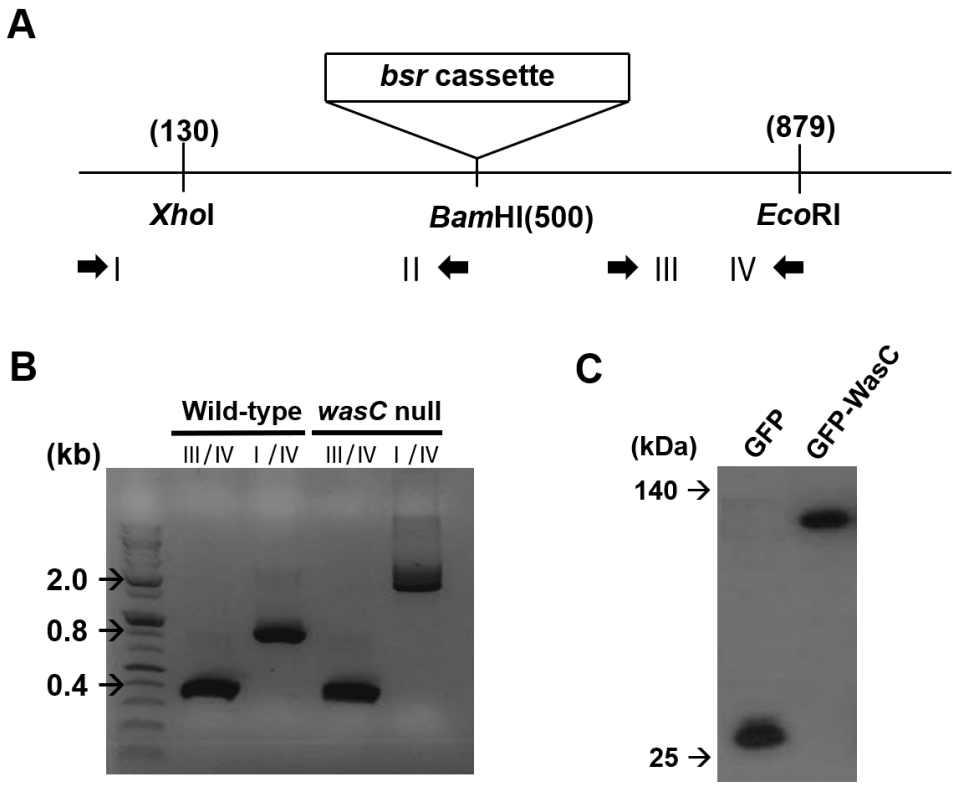

Fig 3. Confirmation of *wasC* knock-out and WasC-overexpressing cells

(A) Schematic diagram of *wasC* knockout construct. The *bsr* cassette was inserted into the *wasC* cDNA that was cloned into a cloning vector. Roman numerals and arrows indicate the location of the primers used in this study. (B) Confirmation of the *wasC* knock-out construct substitution in *wasC* null cells. Genomics DNAs were extracted from wild-type and *wasC* null cells and then subjected in PCRs with primers shown in panel A. (C) Confirmation of WasC-overexpressing cells GFP-WasC was expressed in *wasC* null cells, and the expression of the proteins was examined by western blotting with anti-GFP antibodies (expected size of GFP-WasC, 126 kDa).

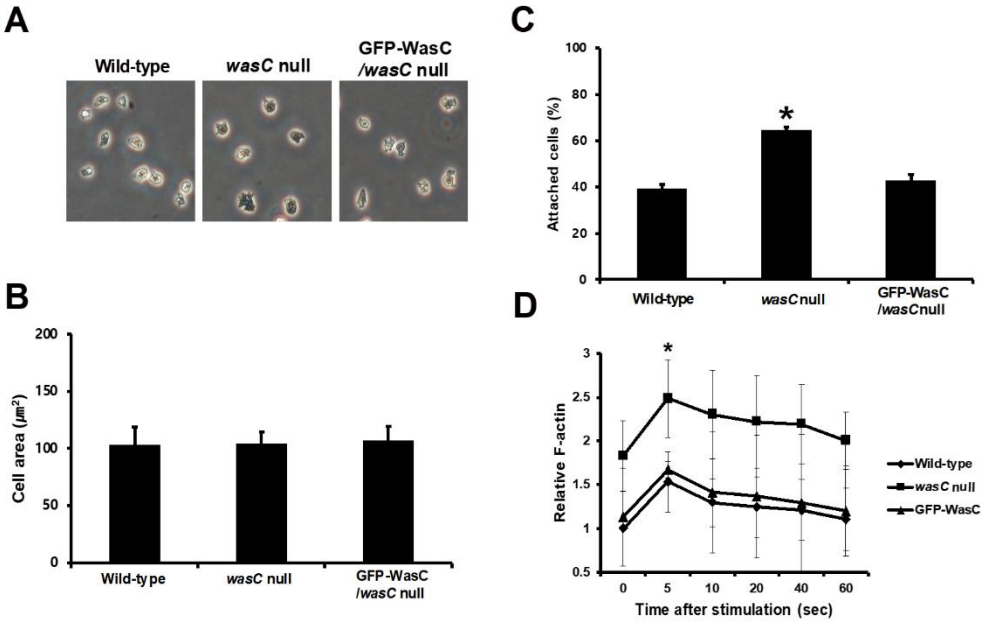

Fig 4. WasC is involved in the control of cell adhesion and F-actin assembly

(A) Morphology of wild-type cells, *wasC* null cells, and *wasC* null cells expressing GFP-WasC cells. The exponentially growing cells were photographed using NIS-element software. (B) Measurement of cell area. The cell area was measured using Image J software. The error bar on each panel indicates the SD value. (C) Cell-substrate adhesion. Adhesions of cells were presented as the percentage of attached cells to the total cells. The values indicate the mean value ± SD from three independent experiments (*p<0.05 compared to the control). (D) Kinetics of F-actin polymerization in response to chemoattractant stimulation. Error bar represents SD. (*p<0.05 compared to the control by the student's t-test).

III-3. WasC is involved in the regulation of cell adhesion and actin cytoskeleton

To understand the functions of WasC, I first examined the morphology of *wasC* null cells and GFP-WasC overexpressing cells (Fig. 4A). *wasC* null cells seemed to be larger than wild-type cells. However, measurement of cell areas using the NIS-element software showed that there was no significant difference between *wasC* null cells and wild-type cells (Fig. 4B). Next, I examined cell adhesion by measuring the fraction of cells that attached to the plate during agitation. Compared to wild-type cells, *wasC* null cells showed significantly increased cell-substrate adhesion (Fig. 4C).

I then examined the effects of loss of WasC on chemoattractant–mediated reorganization of the cytoskeleton. Wild-type cells showed a transient and rapid F-actin polymerization with a peak at 5 sec in response to chemoattractant stimulation. In contrast, the loss of WasC resulted in 1.8-fold increase in the basal level of F-actin. However, the kinetics of the response was similar to those of wild-type cells with the level of F-actin proportionally higher in *wasC* null cells (Fig. 4D). These results suggest that WasC is involved in reorganizing the actin cytoskeleton and cell-substrate attachment.

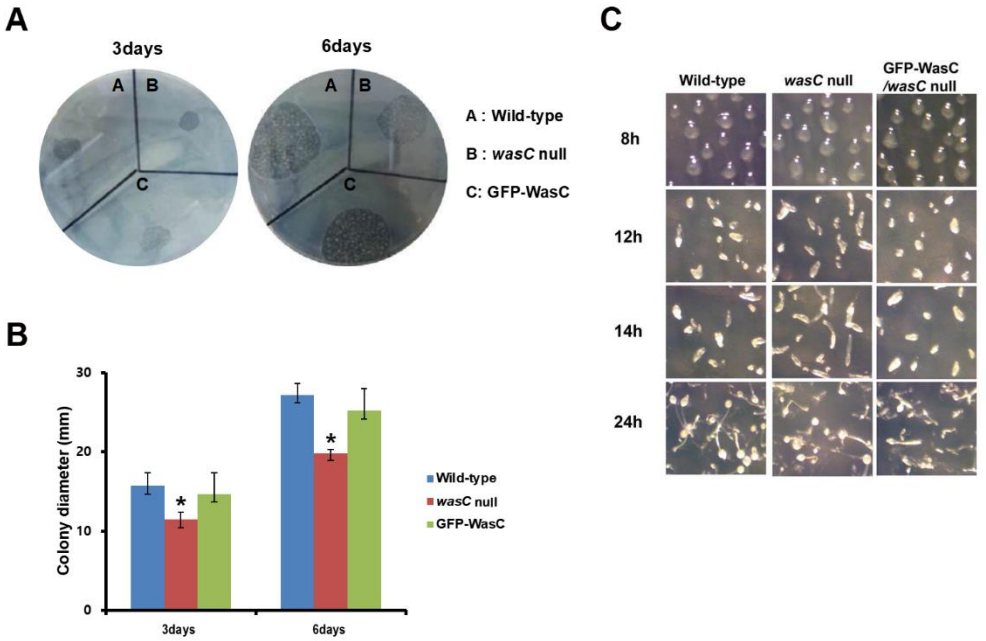

Fig 5. Phagocytosis and development of wild-type, *wasC* null, and GFP-WasC cells

(A) Wild-type, *wasC* null, and GFP-WasC cells were plated on agar plates coated with *K. aerogenes*. Photographs were taken at the indicated days after plating. (B) Measurement of colony diameter. The diameters of the colonies were measured using Image J software. The values are the means ± SD of three independent experiments (*p<0.05 compared to the control by the t-test). (C) Development of wild-type, *wasC* null, and *wasC* null cells expressing GFP-WasC. Exponentially growing vegetative cells were washed and plated on non-nutrient agar plates. Photographs were taken at the indicated times after plating. The images of multicellular developmental structures at 8 hours (aggregation stage), 12 hours and 14 hours (slug and mound stage), 24 hours (fruiting body stage) are shown.

III-4. WasC is essential for phagocytosis

Phagocytosis is actin-dependent and clathrin-independent processes mainly performed by cells like neutrophils. Phagocytosis consists of a number of stages including attachment of particles to cell surface receptors, engulfment of the particle dependent on actin polymerization, and formation of phago-lysosomes (Cardelli, 2001).

Wasp proteins were known to be involved in F-actin polymerization. To investigate the functions of WasC in the phagocytosis, I performed phagocytosis assay using the plates on which *K. aerogenes* bacteria were placed (Fig. 5A). As time goes on, the colony size of *wasC* null cells were found to be smaller than wild-type and GFP-WasC cells (Fig. 5B). These results suggest that WasC is required for mediating phagocytosis.

In a state of starvation, *Dictyostelium* cells release cAMP, causing surrounding cells to migrate toward the cAMP source and initiate their multicellular developmental process (Chisholm and Firtel, 2004). To investigate the role of WasC during development, I performed development assay with wild-type cells, *wasC* null cells, and WasC overexpressing cells (Fig. 5C). *wasC* null cells and GFP-WasC cells showed normal developmental processes compared to wild-type cells, forming a mound at 8 hours and fruiting body within 24 hours similar to wild-type cells. These results showed that WasC is not required for developmental process.

III-5. Roles of WasC on cell migration

1) Loss of WasC causes defects in chemotactic response

The previous data showed defects of phagocytosis in *wasC* null cells. These data propose that *wasC* null cells may have defects in chemoattractant–directed cell migration. To examine whether *wasC* null cells are defective in cAMP – mediated chemotaxis, I performed the chemotaxis assay with wild-type, *wasC* null, and GFP-WasC cells using a Dunn chemotaxis chamber (Fig. 6A). Aggregation – competent cells were prepared by starving the cells in Na/K phosphate buffer for 10 h. Wild-type cells moved at 8.5 μm/min toward the cAMP source. In contrast, GFP-WasC cells showed slightly high moving speed with 10.5 μm/min and *wasC* null cells had significantly low moving speed with 5.3 μm/min (Fig. 6B). In directionality, which is the index showing how straight the cells move, all of the cells including wild-type, *wasC* null cells, and GFP-WasC cells showed no significant difference (Fig. 6B). These results suggest that WasC is involved in the regulation of the speed but not the direction of migration during chemotaxis.

2) WasC is not involved in electrotaxis.

It is known that cells display electrotaxis by migrating directionally in gradients of specific electrical potential and *Dictyostelium* show conspicuous electrotaxis in an applied direct EF. F-actin was polymerized at the leading edge of cells during electrotaxis (Zhao et al., 2002). To investigate the roles of WasC in EF-directed cell migration, I performed electrotaxis and examined the directedness and trajectory speed of wild-type, *wasC* null, and GFP-WasC cells (Fig. 7). Wild-type cells moved at 3.51 μm/min, *wasC* null cells at 3.46 μm/min, and GFP-WasC cells at 3.37 μm/min in response to EF (Fig. 7B). No significant difference was found in the directedness and the speed of migration (Fig.7B). These results suggest that WasC is not involved in electrotaxis.

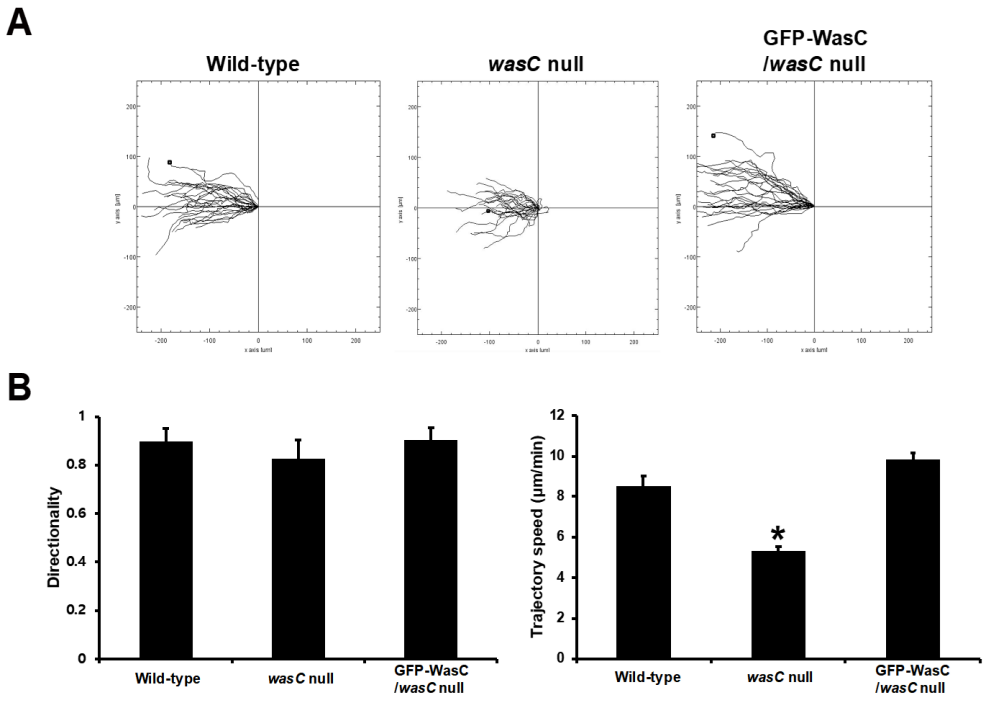

Fig 6. Analysis of cell motility in chemotaxis

Aggregation competent wild-type cells or *wasC* null cells were placed in a Dunn chemotaxis chamber, and the motility of cells towards cAMP gradient were analyzed. The cell migration was recorded at time lapse intervals of 1 min for 1 hour. (A) Trajectories of migrating cells in the Dunn chemotaxis chamber towards cAMP. Each line indicates the track of single cell chemotaxing towards cAMP. (B) Quantification of the cell motility of chemotaxing cells. The recorded images were analyzed by NIS-element software. Directionality measures how straightly a cells move. The direction of cell moving in a straight line is 1. Trajectory speed is the total distance traveled by the cell divided by the time. These experiments were performed at least three times. Error bar indicated SD. (*p<0.05 compared to the control by the t-test).

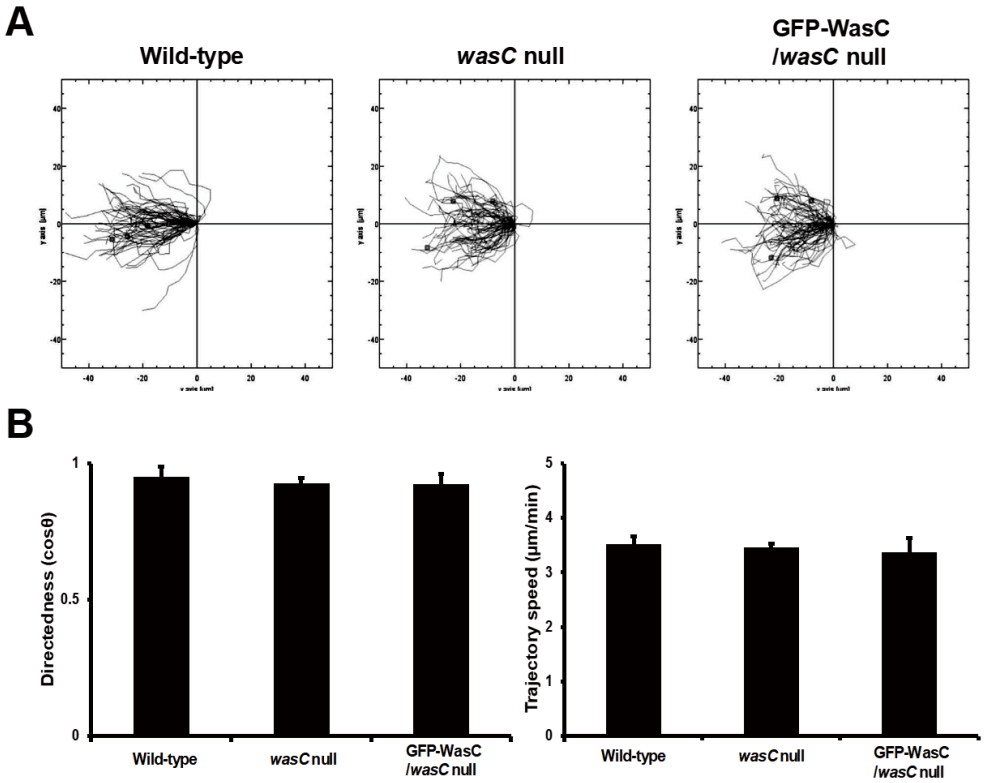

Fig 7. Analysis of cell motility in electrotaxis

Wild-type, *wasC* null, and GFP-WasC cells starved for 3 hours were placed in an electrotaxis chamber, and then EF of 15 V/cm was applied. The motility of the cells in response to EF was recorded at time-lapse interval of 1 min for 1 hours. (A) Trajectories of cell migration toward the cathode in an electrotaxis chamber. (B) Quantification of the motility of electrotaxing cells. The speed and directedness were explained in the previous experiments. Directedness was measured as cosine θ, a cell moving directly to the cathode would have a directedness of 1, whereas a cell moving directly to the anode would have directedness of -1.

III-6. WasC cortical localization is dependent on F-actin

Coronin is an actin-binding protein that accumulates at the cell cortex of moving cells and contributes to the dynamics of the actin cytoskeleton (Gerisch et al., 1995). Another actin-binding protein, ArpD plays a key role in the regulation of actin assembly at the leading edge of cells (Bretschneider et al., 2004).

To understand the dynamic of WasC, I investigated WasC localization using GFP-WasC in *wasC* null cells. GFP-WasC was located at the cell cortex, at which F-actin is accumulated (Fig. 8A). To examine whether WasC is co-localized with F-actin in the cell cortex, I performed indirect immunofluorescence microscopy with Hoechst dye and for staining nuclei TRITC-phalloidin dye for staining F-actin (Fig. 8B). The results showed that both GFP-WasC and F-actin were observed at the same cell cortex. To examine the mechanism by which WasC is located at the cell cortex, I examined the localization of WasC in the presence of Latrunculin B (Lat B) and LY294002, which are inhibitors of F-actin assembly and PI3K signaling pathway, respectively (Fig. 8C). GFP-WasC at the cell cortex disappeared in the presence of LatB but not LY294002, suggesting that the cortical localization of GFP-WasC is dependent on F-actin assembly.

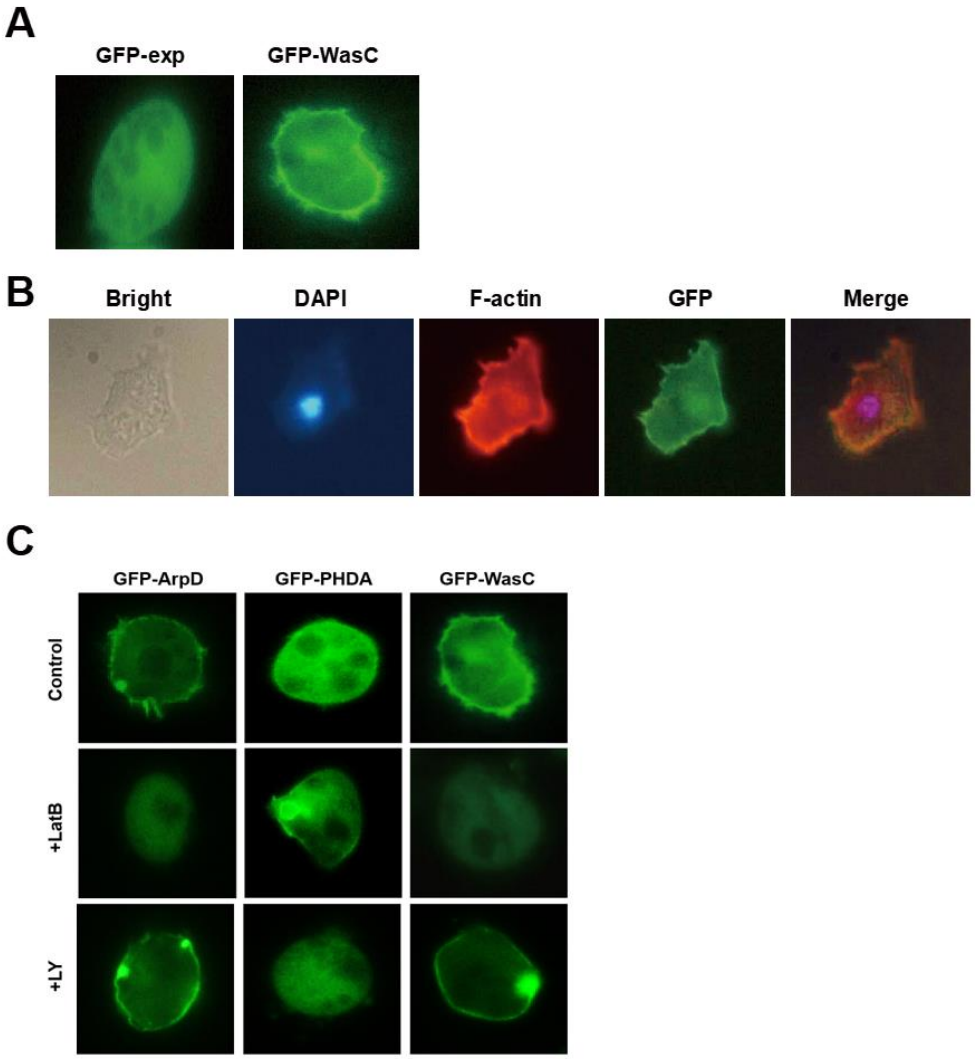

Fig 8. Co-localization of WasC with F-actin

(A) Localization of GFP-WasC. GFP-exp cells were used as a control. (B) Co-localization of WasC with F-actin. (C) Effect of LatB and LY294002 on localization of GFP-ArpD, GFP-PhdA and GFP-WasC. GFP-ArpD and GFP-PhdA were used as controls the localizations of which are dependent on F-actin and PI3K, respectively.

III-7. Translocation of GFP-WasC into the cortex

I analyzed the translocation kinetics of WasC to the cell cortex in response to uniform chemoattractant stimulation to understand the temporal mechanism for dynamic localization of WasC to the cell cortex in chemotaxing cells. Unstimulated cells showed low level of GFP-WasC at the cell cortex. Upon uniform chemoattractant stimulation, GFP-WasC transiently and rapidly translocated to the cell cortex with a peak at 10 sec, followed by decrease to the basal level within 30 sec (Fig. 9A and 9B). To understand temporal translocation kinetics of WasC in more detail, the translocation kinetics of WasC were compared with those of other F-actin binding proteins such as ArpD, a subunit of Arp 2/3 complex (Rodal et al., 2005), and Coronin. WasC showed similar translocation kinetics to the cell cortex with a peak at 10 sec on chemoattractant stimulation compared to Coronin, while much slower than those of ArpD with a peak at 5 sec (Fig. 9A and 9B). The Arp 2/3 complex and Coronin are progressively involved in regulating F-actin polymerization at the cell cortex upon chemoattractant stimulation. In addition, I investigated the dynamic localization of GFP-WasC in moving cells by changing the location of the pepette filled with chemoattractants. WasC was located at the leading edge of cells during random movement and chemoattractant or EF-directed cell migration (Fig. 9C). These results suggest that WasC is likely to play some roles at the leading edge of migration cells in the next step of the Arp2/3 complex in the regulation of actin cytoskeleton.

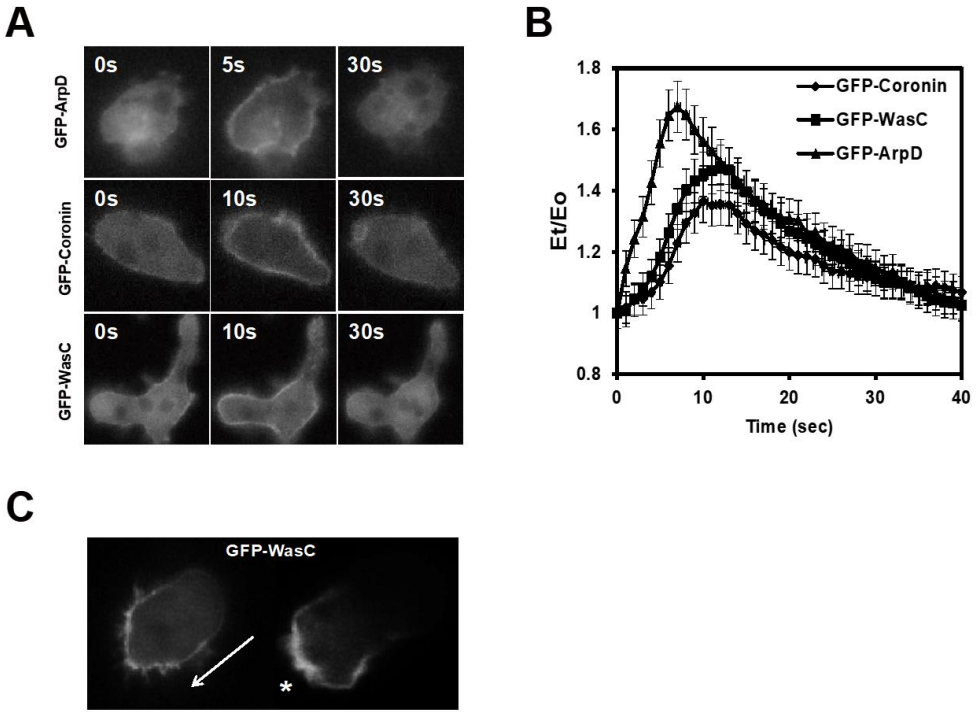

Fig 9. Temporal localization of GFP-WasC in response to chemoattractant stimulation

(A) Translocation of GFP-WasC cells to the cell cortex in response to chemoattractant stimulation. GFP-ArpD and GFP-Coronin cells were used as controls. Cells were stimulated with cAMP, and the images were taken every sec for 1 min. Three representative frames from time-lapse recordings are shown. (B) Translocation kinetics of GFP-WasC, GFP-Coronin and GFP-ArpD. The fluorescence intensity of cortex-localized GFP-WasC, GFP-Coronin and GFP-ArpD was quantified using Image J software as described previously (Jeon et al., 2007a). (C) Localization of GFP-WasC in chemotaxing cells. The arrow shows the direction of movement.

IV. DISCUSSION

It is previously known that WasA and WasB are involved in cell migration and F-actin polymerization. *wasA* null cells showed that decreased cell motility and reduced F-actin polymerization (Myers et al., 2005). *wasB* null cells showed defects of chemotaxis to cAMP and increased F-actin polymerization (Chung et al., 2013).

In this study, I investigated the functions of WasC, a WASP family protein, in the regulation of F-actin assembly and cell migration. WASP proteins are involved in F-actin polymerization, and the VCA domain of the proteins is known to play an important role. Thus, to determine whether the VCA domain of the *Dictyostelium* Wasp proteins play a role, I examined multiple alignment of the VCA domains of Wasp proteins and performed the phylogenetic analysis using Mega 7.0 programs. These results showed that WasC has similar domain structure to the other two Wasp proteins, WasA and WasB; however, WasC is longer than the other two proteins (Fig. 1A). In the phylogenetic tree, the VCA domain of WasC is most closely related to WasB (Fig. 1B). In support of this conclusion, multiple alignment shows that the critical amino acids of the VCA domain, LKXV, were conserved in WasC and WasB (Fig. 2). These data suggest that WasC and WasB might have similar functions.

Wasp protein is known to be involved in F-actin polymerization. I examined morphology of wild-type, *wasC* null cells, and WasC overexpressing cells. Additionally, I investigated F-actin level in response to chemoattractant stimulation using wild-type cells, *wasC* null cells, and WasC overexpressing cells (Fig. 4). This result showed no significant difference in cell morphology (Fig. 4B). However, loss of WasC resulted in strong cell adhesion and increased level of F-actin (Fig. 4C and 4D). In agreement with these results, wild-type cells showed a transient and rapid F-actin polymerization within a peak at 5 sec. However, loss of WasC caused 1.8-fold increase in the basal level of F-

actin. These data suggest that WasC might play a role in the regulation of cytoskeleton reorganization.

When phagocytosis occurs in cells, a network of actin fibers is formed. I investigated the functions of WasC in phagocytosis by using knock-out *wasC* and overexpressing WasC. As a result, it was confirmed that the size of colony was decreased in *wasC* null cells compared to wild-type and overexpressing WasC. These data suggest that WasC plays an important role in phagocytosis (Fig. 5B).

Cells lacking WasC exhibited increased F-actin assembly compared to wild-type (Fig. 4D). All types of the cells showed similar migration behaviors in response to chemoattractant stimulation. However, *wasC* null cells were defective in the control of migration speed in the chemoattractant-directed cell migration. In contrast, overexpressing WasC cells showed increased speed (Fig. 6B). These results suggest that chemotaxis defects of *wasC* null cells are likely to results from abnormal regulation of F-actin assembly.

GFP-WasC was located at the cell cortex. Actin filaments in the cell are organized into several different forms and functional arrays by various actin-binding proteins, resulting in F-actins with different biochemical and structural properties (Galkin et al., 2010; Uyeda et al., 2011). Generally, the mechanism by which the proteins are located in the cell cortex includes two cases, an F-actin dependent and a PI3K dependent pathway. To determine which mechanism drove WasC to be located at the cell cortex. I examined the localizations of GFP-WasC in the presence of LatB or LY294002. These results suggest that WasC is located at cell cortex by an F-actin dependent manner (Fig. 8C).

The Arp 2/3 complex, PhdA, and Coronin are progressively involved in regulating F-actin polymerization at the cell cortex upon chemoattractant stimulation (Cha et al., 2011). In present study showed that high level of WasC was found at the cell cortex before stimulation and then transiently translocated to the cell cortex with a peak 10 sec by

chemoattractant stimulation, followed by decrease to the basal level within 30 sec (Fig. 9A and 9B). Compared this result with those of other F-actin binding proteins such as ArpD and Coronin, translocation kinetics of WasC and Coronin were similar, translocated to the cell cortex with a peak 10 sec, while translocation of ArpD was 4~5 sec faster than WasC and Coronin. These data suggest that WasC is involved in F-actin polymerization at the cell cortex probably in the next step of the Arp 2/3 complex.

Conclusions

WASP proteins are involved in various cellular processes including F-actin assembly. These WASP proteins have a highly conserved VCA domain that activates Arp 2/3 complex involved in F-actin polymerization. Loss of WasC, one of three WASP proteins in *Dictyostelium*, resulted in defects in various cellular processes including cell adhesion, F-actin assembly, phagocytosis, and chemotaxis. Cells lacking WasC showed increased levels of F-actin compared to wild-type cells and severe defects in phagocytosis. In addition, *wasC* knock-out cells exhibited defects in chemotaxis, which is mediated by proper regulation of F-actin polymerization. This study suggests that defective cell migration of *wasC* null cells might be originated from abnormal regulation of F-actin assembly.

Part II. Electric Field-Directed Cell Migration by G-proteins in *Dictyostelium*

I. INTRODUCTION

Directional cell migration is fundamental in pathology and development (Zhao et al., 2002) and is a highly adjusted process of directional sensing, migration speed and polarity (Jeon et al., 2019). Directional sensing and polarity establishment are coordinated by a system that senses temporal and spatial stimuli and biases motility in a particular direction (Artemenko et al., 2014; Jeon et al., 2019). Over the decades, these processes have been studied and several signaling molecules related to the directional cell migration have been characterized (Jeon et al., 2019). However, the underlying molecular mechanisms of these sophisticated processes remain unknown.

Electrotaxis is a directional cell migration in an electric field (EF) and occurs in a variety of cell types, from bacteria to mammalian cells, including *Dictyostelium discoideum*, skin keratinocytes, and epithelial cells (Jeon et al., 2019; Zhao et al., 1996b; Zhao et al., 2002). Many papers reported the importance of EFs in physiology. Disruption of EF impairs wound healing and results in developmental abnormalities of embryonic nervous and skeletal systems. These defects could be the results of impaired directional cell migration (Sta. Iglesia et al., 1998; Zhao et al., 2002). Electric fields were measured at wounds of human skin about one half centuries ago. Disruption of an epithelial layer immediately generates endogenous electric fields. Most types of cells migrate directionally in an electric field, a phenomenon called electrotaxis. Endogenous electric fields which naturally occurring may be an important signal that regulates directional cell migration in vivo. EFs may have a potential clinical role guiding cell migration in wound

healing. (Tai et al., 2009).

The social amoeba *Dictyostelium discoideum* is a powerful model organism for studying cell migration and shows strong electrotaxis (Zhao et al., 2002). Although these amoebae would not naturally encounter EFs, they offer a powerful system for molecular and genetic analyses of the mechanisms underpinning electrotaxis (Zhao et al., 2002). Studies using *Dictyostelium* have provided substantial mechanistic insights into the signaling mechanisms underlying cell polarity and migration (Gao et al., 2015).

Activation of G-protein coupled receptors (GPCRs) is at the very base of the signaling pathways that enable this very sensitive and broad chemotaxis response. Chemotaxis starts with binding of the chemoattractant to GPCRs at the cell surface (Kamp et al., 2016). The receptors transmit these signals into the interior of the cell by activation and dissociation of the heterotrimeric G protein complex. This subsequently results in the activation of a complex network of signaling molecules and the coordinated remodeling of the cytoskeleton. Studies with the chemotaxis experimental model *Dictyostelium discoideum* have provided important insights into chemoattractant GPCR signaling dynamics (Islam et al., 2018). Twelve Gα subunits have been identified in *Dictyostelium*. When cAMP is bound to the receptor, the activated receptor stimulates exchange of GDP for GTP on Gα2. The trimeric G-protein dissociates into the active α subunit and β/γ dimer as long as cAMP bound to the receptor (Janetopoulos et al., 2001). Gα2 plays an important role in cAMP relay and prestalk differentiation during aggregation (Carrel et al., 1994). Lacking Gα2 results in loss of cAMP receptor-mediated signaling (Kumagai et al., 1991; Okaichi et al., 1992), abolished chemotaxis to cAMP and decreased activation of Ras GTPase (Kortholt et al., 2013). *gpbA*(Gβ) encodes the evolutionally conserved β subunit which dimerized with the gamma subunit in the presence of the phosducin-like protein PhLP1 (Knol et al., 2005; Pupillo et al., 1988). Cells lacking Gβ grow axial direction but defective in phagocytosis of bacteria (Peracino et al., 1998). Also, mutant

cells in Gβ have reduced levels of the cAMP receptor cAR1 and do not response to the chemoattractant cAMP. As a result, *Gβ* null cells are defective in the developmental process and showed increased oscillation of F-actin required for efficient directional migration.

PI3K signaling pathway is a key component of many signaling pathways regulating a broad range of fundamental cellular processes including cell migration, growth, differentiation, and survival in response to extracellular stimulation (Sun et al., 2004). LY294002 and Wortmanin are inhibitors for the PI3K signaling pathway. These inhibitors have importance in understanding the biological functions of the PI3K signaling pathway in diverse physiological processes. LY294002 is a synthetic compound derived from the broad area kinase inhibitor quercetin and inhibits PI3K by competing with ATP for the active site of catalytic subunit p110 (Sun et al., 2004).

In the present study, I examined the roles of Gα2 and Gβ, one of the G-protein subunits in the regulation of cell migration speed in response to electric fields, and the effects LY294002 on random cell motility to determine if the PI3K signaling pathway is involved in EF-directed cell migration.

II. MATERIALS AND METHODS

II-1. Strains and cell culture

Dictyostelium discoideum cells were obtained from the Dictybase Stock Center; wild-type KAx-3 cells (DBS0236487), *Gα2* null (DBS0236575), and *Gβ* null (DBS0236531). All cells were cultured axenically in HL5 medium at 22°C. The knock-out strains were maintained in 10 μg/ml blasticidin.

II-2. Random motility assay

In random movement assay, vegetative cells growing on plates were harvested and seeded onto a chambered coverglass in starvation buffer (Sasaki et al., 2007). Before experiment, cells were washed three times with development buffer and starved for 1 hour or 3 hour. Images were captured using a phase-contrast microscope (Olympus IX71) equipped a with camera (Nikon) and analyzed with Image J (National Institutes of Health). The moving cells were photographed at time-lapse intervals of 1 min for 1 hour.

II-3. Cell preparation and electrotaxis assay

Growing cells on plates were washed three times and starved for 3 hour in development buffer (DB; 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$ and 0.2 mM $CaCl_2$), and then placed on the electrotaxis chamber (Jeon et al., 2019). Before electric field stimulation, two agar salt bridges were prepared of no less then 15cm long. For electric field application, the electric field strength of 15V/cm was used. Time-lapse images of migration cells were obtained using a microscope (Olympus IX71) equipped with camera (Nikon).

II-4. Quantitative analysis of electrotaxis

Time-lapse records of cell migration with an interval of 1 min for 1 hour were analyzed using Image J (National Institutes of Health) as previously described (Jeon et al., 2019; Zhao et al., 2002). Directedness means how directionally cells migrated in response to an EF and measured cosine θ in which θ was the angle between the direction of the field and a straight line connecting the start and end points of cells. Moving cells directly toward to the anode or cathode would have a directedness of -1 or 1, respectively, and randomly movement cells would have a value of directedness close to 0. Trajectory speed is the total distance travelled of the cells divided by time. Kinetics of trajectory speed and directedness of the cells were calculated by measuring cell migration for every 2 min period in time-lapse recordings.

III. RESULTS

III-1. *Gβ* null cells have defects in random movement

The binding of the chemoattractant to the cell-surface G-protein coupled receptors (GPCRs) initiates the chemoattractant-mediated directional movement. The activated GPCRs expedite dissociation of the three subunits of G-protein as a GFP-Gα and a Gβγ dimer, both of these mediate downstream signaling pathways for directional cell migration (Artemenko et al., 2014).

There are 14 genes encoding G-protein subunits in *Dictyostelium* (14 genes; 12 Gα, one Gβ, and one Gγ). Cells lacking Gβ showed defect in random movement (Jeon et al., 2019; Sasaki et al., 2007; Zhao et al., 2002). It has been previously suggested that this phenotype occurred by disruption of the PI3K signaling pathway (Sasaki et al., 2007).

To determine whether the G-protein subunits are involved in random movement, I examined the phenotypes of wild-type, *Gα2* null cells, and *Gβ* null cells in random movement after starvation of 1 hour or 3 hours. *Gβ* null cells displayed defects in random motility after starvation for 1 or 3 hours (Fig. 1 and Fig. 2). Taken together with the previously reported data, it is postulated that defect of *Gβ* null cells in random motility might come from misregulation of the PI3K signaling pathway.

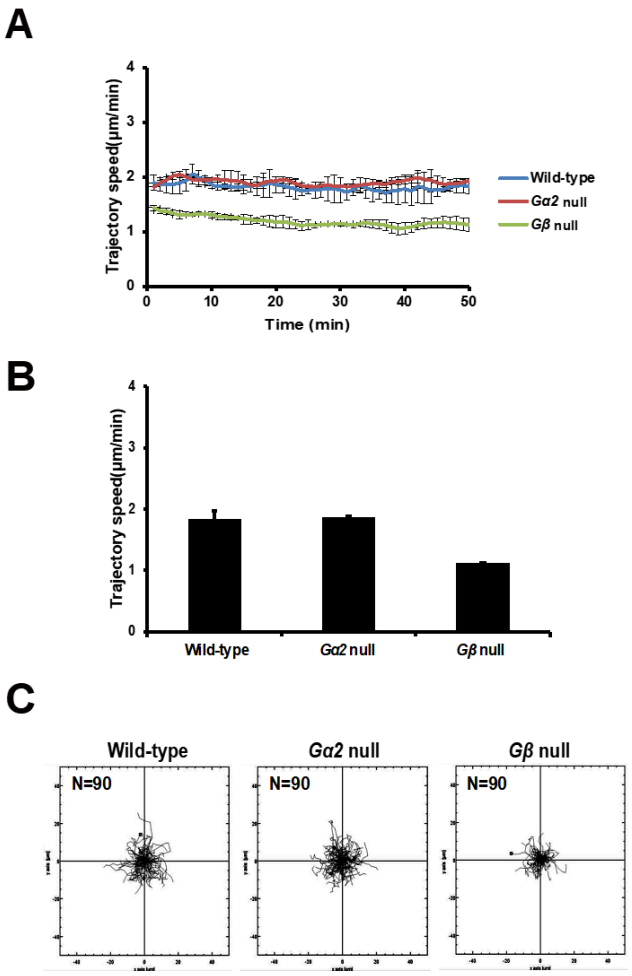

Fig 1. Random motility of cells 1 hour after development

The cells on plates, wild-type, *Gα2* and *Gβ* null cells, were washed twice with a development buffer and incubated in the development buffer deprived of nutrient for 1 hour, and then subjected to the random movement assay. (A) Kinetics of trajectory speed in random motility. (B) Quantitative analysis of the random migration of cells. (C) Trajectories of wild-type KAx3, *Gα2* and *Gβ* null cells in random movement. Plots show migration paths of the cells with the start position of each centered at point 0,0. The movements of the cells were recorded by time lapse photograph for 1 hour at 1 min intervals. The value indicated the mean ± SD of three independent experiments.

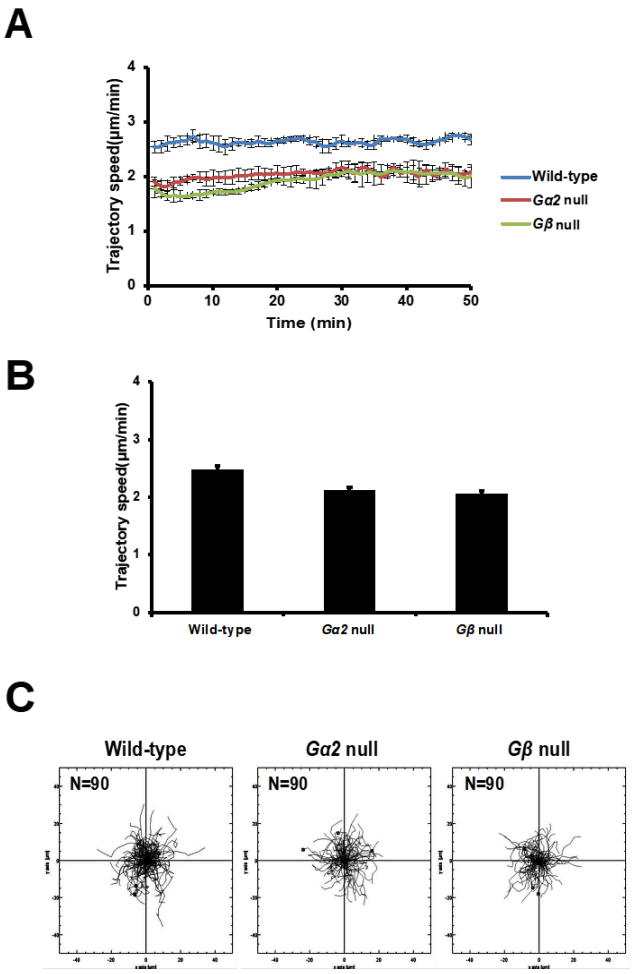

Fig 2. Random motility of cells 3 hours after development

Wild-type, *Gα2,* and *Gβ* null cells, which were starved for 3 hours in the development buffer, were used. (B) Quantitative analysis of the random migration of cells. (C) Trajectories of wild-type KAx3, *Gα2* and *Gβ* null cells in random movement.

III-2. *Gα2* and *Gβ* null cells display no specific acceleration / deceleration kinetics of migration speed in response to EFs

Dictyostelium cells show specific acceleration / deceleration kinetics of directedness and trajectory speed in response to EFs (Jeon et al., 2019).

To understand the roles of G-proteins in electric-field directed cell migration, I examined the kinetics of trajectory speed and directedness of *Gα2* and *Gβ* null cells in responses to EF. Recently it was reported that *Gβ* null cells exhibit no acceleration / deceleration of the trajectory speed upon EF stimulation compared to wild-type (Jeon et al., 2019). The same results were obtained in our present study (Fig. 3). *Gβ* null cells moved at 2.65 μm/min before EF stimulation, 2.88 μm/min in an EF of 15V/cm, and 2.69 μm/min after EF off (Fig. 3B). Even though *Gβ* null cells seemed to have a slightly increased migration speed on in EF stimulation (15V/cm), the trajectory speed of *Gβ* null cells showed no significant acceleration / deceleration kinetics in migration speed compared to wild-type (Fig. 3A). Gα2 subunit is critical in cAMP relay and prestalk differentiation during aggregation (Carrel et al., 1994). *Gα2* null cells moved at 2.22 μm/min before EF stimulation, 2.52 μm/min in an EF of 15V/cm, and then 2.35 μm/min after EF off (Fig. 3B). These results suggest that both Gα2 and Gβ subunits are essential for induction of the speed in EF-directed cell migration.

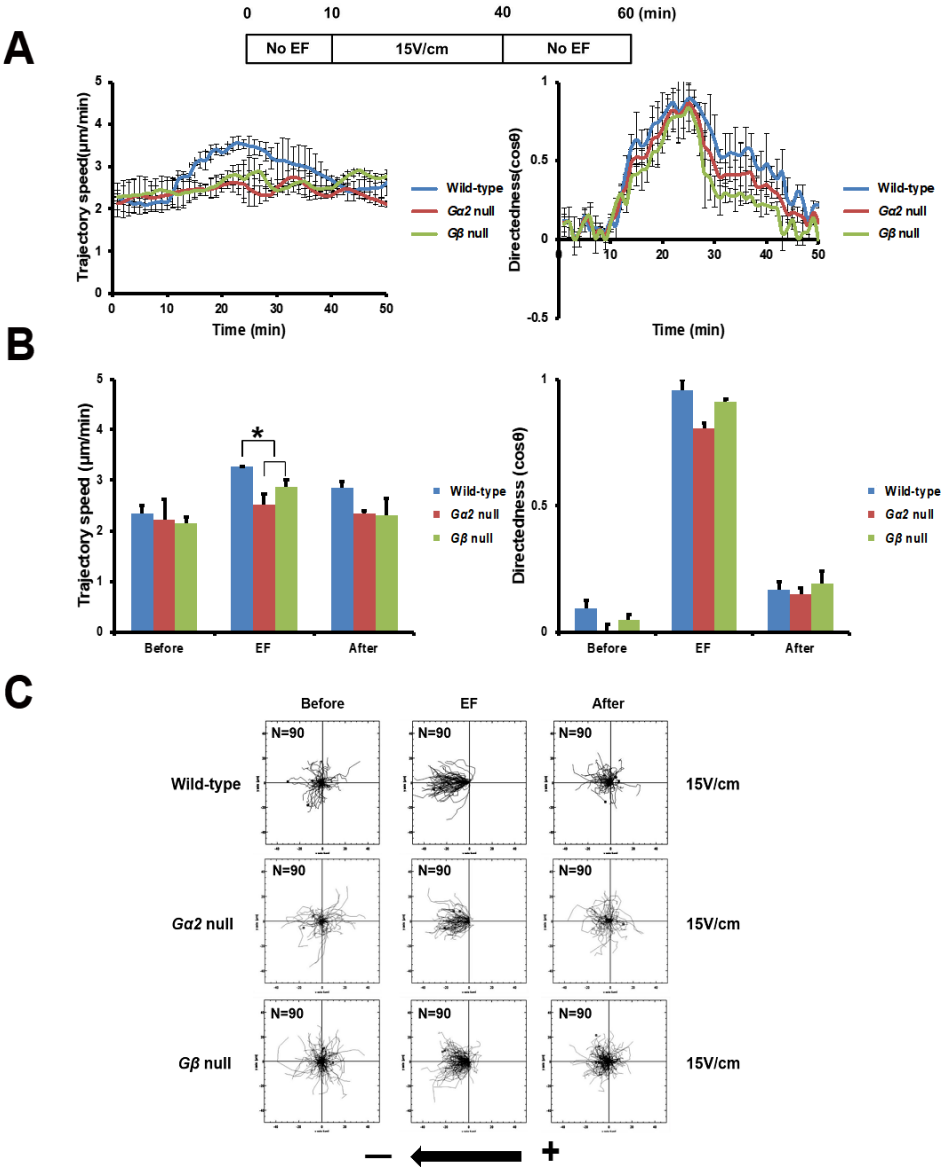

Fig 3. Electrotactic response of wild-type, *Gα2,* and *Gβ* null cells

Wild-type Ax3, *Gα2,* and *Gβ* null cells, which were starved for 3 hours in the development buffer, were placed in an electrotaxis chamber. Elctrotaxing cells were recorded at time-lapse intervals of 1 min for 60 min (A) Kinetics of trajectory speed and directedness in EF-directed cell migration. (B) Quantification of the direction and the speed of migrating cells. Trajectory speed and directedness in an EF of 15 V/cm were measured Image J software and compared with those before applying an EF and after switching off. 'Before' indicates the values of trajectory speed and directedness for 10 min before EF stimulation. 'EF' indicates 10 min after EF stimulation (20 min to 30 min). 'After' indicates the 10 min after EF stimulation off. Directedness was measured as cosine θ, a cell moving directly to the cathode would have a directedness of 1, whereas a cell moving directly to the anode would have directedness of -1. Data are the means ± SEM. from three independent experiments in an EF. **P*<0.05, Student's *t*-test. (C) Trajectories of the cells in an EF. Plots show migration paths of the cells with the start position of each centered at point 0,0. An arrow indicates the direction of EF. Cells migrate toward the cathode on the left in an EF.

III-3. LY294002, an inhibitor of PI3Ks, increases the migration speed in random motility

LY294002 is a synthetic compound derived from the wide area kinase inhibitor quercetin and inhibits PI3Ks by competing with ATP for the active site of p110.

Previously it was suggested that the defect in the speed of *Gβ* null cells in random movement might be caused by unproper regulation of the PI3K signaling pathway. To determine whether the defect in speed of *Gβ* null cells is related to the PI3K signaling pathway, I examined random movement of the cells in the presence of LY294002, a PI3Ks inhibitor.

Interestingly, the results showed that all types of the cells used in this study had an increased speed in random motility in the presence of LY294002 (Fig. 4A and 4B). Wild-type cells moved at 2.26 μm/min in 0.3% DMSO (control), and 2.61 μm/min when treated with 30μM LY294002. *Gα2* null cells moved at 2.0 μm/min in 0.3% DMSO, and 2.56 μm/min when treated with 30μM LY294002. *Gβ* null cells moved at 2.07 μm/min in 0.3% DMSO and 2.56 μm/min when treated with 30μM LY294002 (Fig. 4B). In addition, I examined random motility of *pi3k 1/2* null cells in the presence of LY294002 (Fig. 5). The result also showed an increase of the speed in random motility in presence of LY294002 (Fig. 5A and 5B). *pi3k 1/2* null cells moved at 1.6 μm/min in normal media, 1.76 μm/min in 0.3 % DMSO, and 2.52 μm/min when treated with 30μM LY294002 (Fig. 5B). These results are in conflicts with the previous results, suppression of cell motility in the presence of LY294002 through inhibiting the PI3K signaling pathway, described in other papers. I do not know yet the reasons why all of the cells in our experiments showed increased migration speed in the presence of LY294002, but suspect the possibility of the side effects of LY294002 on membrane potential. This possibility are under examination.

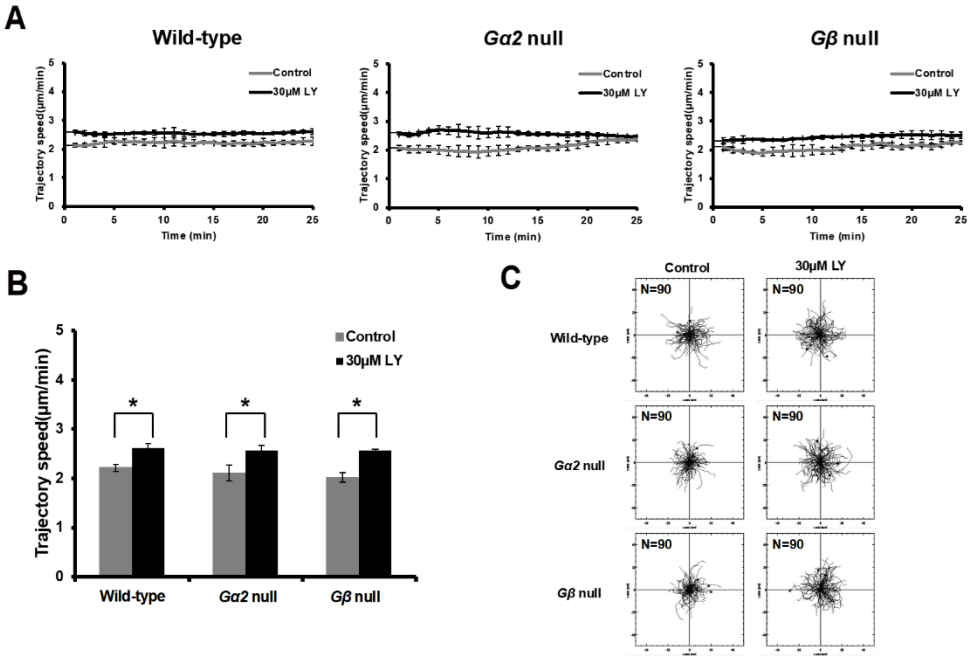

Fig 4. Random motility of cells in the presence of LY294002

Wild-type KAx3, *Gα2* null, and *Gβ* null cells, which were starved for 3 hours, were treated with 30μM LY294002 for 30 min, and then photographed for additional 30 min. (A) Kinetics of trajectory speed in random movement of cells. (B) Quantification of the random motility. **P*<0.05, Student's *t*-test. (C) Trajectories of wild-type, *Gα2* null and *Gβ* null cells in random migration. Plots show migration paths of the cells with the start position each centered at point 0.0. Control cells were treated with 0.3% DMSO for 30 min instead of LY294002. The movements of the cells were recorded by time-lapse photograph for 30 min at 1 min intervals. The value indicated the mean ± SD of three independent experiments.

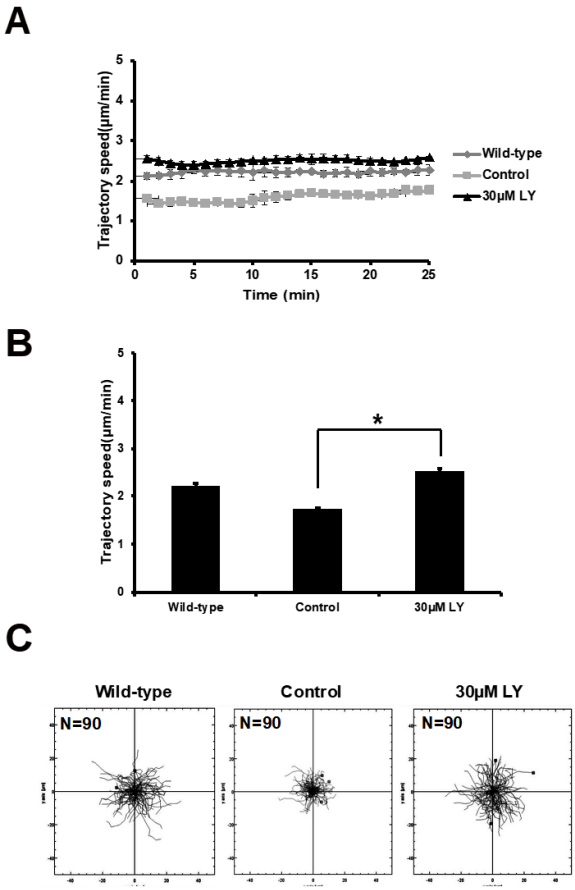

Fig 5. Random motility of *pi3k 1/2* null cells in the presence of LY294002

pi3k 1/2 null cells, which were starved for 3 hours, were treated with 30μM LY294002 for 30 min, and then photographed. (A) Kinetics of trajectory speed in random motility of cells. (B) Quantification of the random motility. **P*<0.05, Student's *t*-test. (C) Trajectories of *pi3k 1/2* null cells in random migration. Plots show migration paths of the cells with the start position each centered at point 0.0. Control cells were photographed after incubation for 30 min with 0.3% DMSO. Treated of LY294002 was also tested in the same way. The movements of the cells were recorded by time lapse photograph for 30 min at 1 min intervals. The value indicated the mean ± SD of three independent experiments.

IV. DISCUSSION

In the present study, I investigated the roles of G-protein subunits, Gα2 and Gβ, in the regulation of migration speed upon EFs, and the effect of LY294002, an PI3K inhibitor, on random cell motility. The binding of chemoattractant to cell surface G-protein coupled receptors (GPCRs) initiates the chemoattractant-mediated directional migration and activates downstream signaling pathways including the PI3K signaling pathway which is involved in diverse cellular processes such as cell migration and cancer metastasis.

It has been previously reported that impairment of the PI3K signaling pathway caused the cells defective in random movement. To investigate the roles of G-protein subunits in random movement, I examined and compared random movement of wild-type, *Gα2* null cells, and *Gβ* null cells after starvation for 1 hour or 3 hours. *Gα2* null cells and *Gβ* null cells showed decreased migration speed (Fig. 1 and Fig. 2).

Gα2, one of 12 Gα subunits in *Dictyostelium*, plays a critical role in chemotaxis through relaying cAMP to the downstream signaling pathway. Lack of Gα2 show loses cAMP receptor-mediated signaling process. Gβγ subunits are essential for directional sensing in chemotaxis. Effective chemotaxis requires both increased migration speed and directionality (Jeon et al., 2019). In neutrophil chemotaxis, Gβγ signaling is known to be primarily involved in regulating cell migration (Kamakura et al., 2013). In the present study, *Gα2* and *Gβ* null cells showed unexpectedly no increase of migration speed in response to EF (Fig. 3A and 3B). Analysis of the kinetics of the speed and directionality of *Gα2* and *Gβ* null cells in an EFs suggest that Gα2 and Gβ are required for the induction of migration speed on EF.

LY294002 is an inhibitor of the PI3K signaling pathway by competing with ATP for the active site of p110. Here, I demonstrate that random motility of the cells was increased in the presence of LY294002 which is contrary to the expected results (Fig. 4B and 5B).

These results suggest the possibility of the side effect of LY294002 in cell motility increasing the migration speed.

Conclusions

G-protein coupled receptors (GPCRs) are very base of the signaling pathway that enables broad chemotaxis response. G-proteins play an important role in regulating a diverse of physiological processes including ion channels, cell motility, development and homeostasis. Cells lacking Gα2 and Gβ, one of 14 G-protein subunits in *Dictyostelium*, showed defects in random movement probably by defective PI3K signaling pathway. Interestingly, when treated with LY294002, a PI3K inhibitor, the speed of motility was increased. Loss of Gα2 and Gβ showed no specific acceleration / deceleration kinetics in the migration speed on EF stimulation compared to wild-type cells. This study will provide further insights into understanding the roles of the PI3K signaling pathway in cell migration and suggest that Gα2 and Gβ subunits are required for the induction of migration speed on EF stimulation.

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처음 연구실 생활을 했을 때 많이 낯설고 특히나 실험결과가 원하는 대로 나오지 않아 많이 속상했고 아쉬워하며 포기하고 싶을 때도 많았지만 그 때마다 옆에서 많은 분들께서 도움을 주셔서 학위를 무사히 마칠 수 있었습니다. 실험을 하다 보면 스스로는 열심히 한다고 하지만 다양한 어려움과 스트레스를 받을 때 마다 많은 도움을 준 동주, 지현이, 원범이까지 모두 고맙고 특히 연구실에 들어온지 얼마 안된 지성이, 상철이 지금도 열심히 하

고 있지만 조금 더 열심히하면 좋은 연구 결과가 나올거라 믿으니 조금만 더 힘내길 바랄게 !! 특히나 실험 알려주라고 전화하면 '공부 먼저 하고 물어봐', 때로는 저녁 늦게 일 끝나고 실험실로 달려와 가장 많이 도와주고 가끔은 바람도 쐬러 가자는 등 실험적인 면이나 다양한 면에서 내 생각해주고 걱정해주는 동엽이형, 형 덕분에 정말 많은 걸 배우게 해줘서 고마워. 아마 형이 없었다면 학위과정 포기했을 것 같다는 생각이 들어.

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