Imaging and Analysis of the Actin Binding Protein, Fascin, in Lamellipodia of the Growth Cone

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Abbreviations

FWHM: Full width at half maximum

TPA: 12-*O*-tetradecanoylphorbol 13-acetate

CB: Cytochalasin B

FRAP: Fluorescence recovery after photobleaching

PKC: Protein kinase C

SIM: Structured illumination microscopy

STED: Stimulated emission depletion microscopy

AFM: Atomic force microscopy

Abstract

Fascin, an actin bundling protein, is present in the filopodia and lamellipodia of growth cones. Many studies have been performed on fascin associated with filopodial actin bundles, but few have examined lamellipodial fascin because it is difficult to observe. In recent years, advances in super-resolution microscopy have made it possible to observe the fine structures of the cell. I, therefore, decided to evaluate lamellipodial fascin.

First, I focused on the dynamics of fascin in lamellipodia. Turnover of fascin was elucidated in relation to its phosphorylation. Ser39 of fascin is a well-known phosphorylation site that controls the binding activity of fascin with actin filaments in filopodia. I performed fluorescence recovery after photobleaching experiments using confocal microscopy and found that fascin binding was controlled by Ser39 phosphorylation in lamellipodia. Moreover, I also found that 12-*O*-tetradecanoylphorbol 13-acetate (TPA), an agonist of protein kinase C, induced phosphorylation of fascin and its dissociation from actin filaments in lamellipodia.

In the next series of experiments, I examined the effects of fascin phosphorylation on the actin meshwork by using super-resolution microscopy. I visualized the actin meshwork of lamellipodia in living growth cones using SIM. Fascin was shown to co-localize with the actin meshwork in lamellipodia. I recorded a time series of images which showed that dissociation of fascin from the actin meshwork was induced by TPA. As the fascin dissociated from the actin filaments, their orientation became parallel with the leading edge. The angle of actin filaments against the leading edge changed from 73° to 15°.

To assess the contribution of actin bundles of fascin on the mechanical properties of the cell, I measured the elasticity of the cell using AFM, before and after the application of TPA. 20 min after the application of TPA, a 40% decrease in the elasticity of the lamellipodia was observed. These data suggest that actin bundles made by fascin contribute to the elasticity of the growth cone. I focused my research on fascin in the lamellipodial region, visualized it in the actin meshwork, and clearly showed the contribution of the fascin on the mechanical properties of the cell. These new findings lead to a greater understanding of neuronal elongation and/or migration of the cell, including in the metastasis of cancer cells.

General Introduction

Role and structure of the growth cone

A neuronal growth cone at the tip of a growing axon or dendrite, is endowed with motile activities for axonal guidance to a target cell (Harrison 1910). The growth cone crawls around, sensing for surrounding guidance cues such as attractive and repulsive factors, and finds a suitable path for axonal elongation (Vitriol & Zheng 2012). Finally, the growth cone arrives at a target cell and changes its shape into a synapse (Shen & Cowan 2010). These processes are important for neuronal network formation.

Growth cones were first observed in fixed brain samples in 1890 (Cajal 1899). The cytoskeletons growth cones consist of actin filaments and microtubules. The peripheral domain (P-domain) is rich in actin filaments, while the central domain (C-domain) is rich in microtubules. The P-domain has many filopodia and thin lamellipodia. Filopodia have thick (100–250 nm) and long actin bundles (Mattila & Lappalainen 2008), while lamellipodia have thin and wide actin meshworks (Bridgman & Dailey 1989; Lewis & Bridgman 1992; Vinzenz *et al.* 2012; Aramaki *et al.* 2016). The C-domain has microtubules, along which mitochondria and vesicles move. Some vesicles are transported from cell bodies to growth cones down the microtubules. The intermediate region between the P-domain and C-domain is called the transition zone (T-zone). In the T-zone, microtubules interact with actin filaments. Vesicles transported from the cell body on microtubules are transferred onto actin filaments.

Growth cone motility was first observed in primary cell cultures (Cajal 1905; Harrison 1910; Nakai 1956; Bray 1970). The motile activity of the

growth cone is supported by the dynamics of actin cytoskeletons (Dent *et al.* 2011).

Motion control of the growth cone by the actin skeleton

The P-domain of the growth cone shows a retrograde flow of actin (Smith 1988; Rinnerthaler et al. 1991). Globular actin are polymerized into actin filaments at the leading edge. The actin filaments are moved rearward and depolymerize in the C-domain (Forscher & Smith 1988). Many kinds of actin-associated proteins take part in this retrograde flow.

Retrograde flow is important for growth cone motility. Advance of the growth cone is dependent on the balance between retrograde flow and actin polymerization at the leading edge. In the present hypothesis, the growth cone moves forwards if the rate of polymerization is higher than that of the retrograde flow, and moves backwards if rate of retrograde flow is larger.

In addition to retrograde flow and actin polymerization, the orientation of the actin filaments is also important. The barbed end of the filament, the site of polymerization, is located near the leading edge, while the pointed end, the site of depolymerization, is located further inside the cell (Small *et al.* 1978). This orientation of actin filaments is important for the elongation of actin filaments and is correlated with growth cone advance.

Actin cytoskeleton control by the actin binding protein

The shape of the actin cytoskeleton is determined by various actin-associated proteins. A mass spectrometric approach identified 41 actin-associated proteins in the growth cone (Nozumi *et al.* 2009), which involved Arp2/3 at the branching point of actin filaments (Welch *et al.* 1997), talin at focal adhesions (Sydor *et al.* 1996), alfa-actinin at anti-parallel actin

filaments in stress fibers (Langanger *et al.* 1984), cofilin which breaks down filamentous actin (Muneyuki *et al.* 1985), fascin, and others.

One of the characteristic structures of the growth cone is the radially aligned actin bundles of filopodia, which are bundled by fascin. Fascin was first identified in sea urchin cell extracts (Kane 1975), and has been reported in numerous animals and cells, including sea urchin coelomocyte cells, human fibroblasts, and ß lymphocytes. Fascin is associated with membrane ruffling, pseudopodia elongation, and lamellipodia formation (Otto *et al.* 1979; Mosialos *et al.* 1994; Yamashiro *et al.* 1998). Fascin has three isoforms: Fascin-1 is widely expressed in the nervous system and mesenchymal tissues, fascin-2 is expressed specifically in the retina (Wada 2001), and fascin-3 is expressed only in the testis (Tubb *et al.* 2002). Throughout this paper, fascin-1 will be referred to as fascin.

Fascin is present in lamellipodia and is required for the formation of thin actin bundles, though their function remains unknown. Because of the resolution limit of conventional optical microscopy, it is difficult to observe the actin meshwork and fascin in lamellipodia. Super-resolution microscopy is therefore required to observe fascin in lamellipodia.

Recently, fascin-1 was reported as being a marker of metastasis of cancers (Hashimoto *et al.* 2005b). Fascin is not expressed in healthy epithelia but is expressed in metastatic cancers with high malignancy. (Hu *et al.* 2000; Pelosi *et al.* 2003; Hashimoto *et al.* 2005a). Increased expression of fascin is highly correlated with tumor malignancy (Vignjevic *et al.* 2007). The bundling activity of actin filaments by fascin in lamellipodia is likely to be involved in metastasis but the detailed mechanism of this remains unclear (Kim *et al.* 2009; Jacquemet *et al.* 2015). Thus, fascin is important for metastasis of cancer.

In this study, I examined lamellipodial fascin in the growth cones of NG108-15 cells. I found the following:

1) Super-resolution microscopy demonstrated the co-localization of fascin and the actin meshwork in lamellipodia, with high resolution.
2) Phosphorylation of fascin by TPA, a PKC agonist, induced a decrease in actin bundles by fascin in lamellipodia, and change in the orientation of actin filaments.
3) AFM measurements suggested that actin bundles made by fascin might contribute up to 40% of the elasticity of the lamellipodia.

General Discussion and Conclusion

Many researchers have reported that growth cone motility depends on the dynamics of actin-based cytoskeletal structures, filopodia, and lamellipodia. Actin bundles in filopodia and the actin meshwork in lamellipodia are controlled by actin-associate proteins. In this paper, I focused on fascin and analyzed its role in the lamellipodial region of the growth cone.
To investigate the details of the interaction between fascin and the actin meshwork in lamellipodia, I used two approaches: 1) FRAP experiments with confocal microscopy, to understand the turnover and dynamics of fascin, and 2) direct observation of fascin and the actin meshwork with super-resolution microscopy. Chapter 1, dealt with FRAP experiments and Chapter 2 dealt with observations using super-resolution microscopy.

I performed FRAP experiments on fascin and actin meshwork in lamellipodia with confocal microscopy in Chapter 1. I examined whether or not the turnover of fascin was similar to that of actin filaments in lamellipodia with FRAP experiments.

I first examined turnover and behavior of actin in lamellipodia of the growth cone. It has been well-reported that globular actin is polymerized into actin filaments at the leading edge, moving rearward by retrograde flow and is depolymerized in the central domain. Movies of GFP-actin and fascin show very similar retrograde flow, but the results of my FRAP experiments were largely different in fascin and in actin. Fascin showed fluorescent recovery, while actin did not. This suggests that fascin can be exchanged even after the actin bundles are made, but actin cannot be exchanged.

The binding of fascin to actin is regulated by phosphorylation, such as at the Ser39 site. Therefore, I used fascin mutated at Ser39 and wild type fascin to examine their differences in fluorescence recovery time. The recovery time of fluorescence intensity was shorter for the phosphorylated form (EGFP-fascin (S39D)) and longer for the dephosphorylated form (EGFP-fascin (S39A)). These results were similar to the data obtained from previous studies of filopodia.

TPA, a PKC agonist, activates PKC and induces phosphorylation of fascin. I therefore externally applied TPA and observed that the fluorescence recovery of fascin became faster. This result is consistent with the data of S39D and S39A mutant fascins. In the case of TPA, many portions of fascin might be phosphorylated. Moreover, there is a possibility that TPA might have pathological effects against unknown targets. However, it should be noted that TPA induced the phosphorylation of intrinsic fascin.

In Chapter 2, I observed fascin and the actin meshwork with super-resolution microscopy. Super-resolution microscopes have been developed in recent years, though the principle behind them was proposed in the 1990's (Hell & Wichmann 1994; Gustafsson *et al.* 1999; Gustafsson 2000). Their application to the field of biology started after 2000 (Willig *et al.* 2006; Schermelleh *et al.* 2008). The resolutions of super-resolution microscopes are different in different systems. For example, SIM can record time-lapse movies in live cells with a resolution of around 100–120 nm. STED can visualize fine structures of fixed samples with resolution of 30–70 nm. I thought that super-resolution microscopy was suitable for observing the actin meshwork in living and moving lamellipodia.

To record the actin meshwork and fascin simultaneously in living cells, I used a two-camera SIM. This microscope can record two images from two individual channels simultaneously. Using this system, the interaction between fascin and the actin meshwork was recorded before and after the

application of TPA. In control experiments, EGFP-fascin completely co-localized with the actin meshwork, labeled with mKO-Lifeact.

Previous research has demonstrated that TPA induced the phosphorylation of fascin and the disappearance of actin bundles. I therefore applied TPA to the external media and observed the cells with a two-camera SIM. 20 min after the external application of TPA, EGFP-fascin dissociated from the actin meshwork (mKO-lifeact). Actin bundles became much thinner and changed their angle against the leading edge from 73° to 15°. Previous reports may have omitted thin actin bundles due to the resolution of the standard optical microscopes being much lower than for super-resolution microscopes. Western blotting with Phos-tag SDS page showed a band shift 20 min after the application of TPA, suggesting that fascin had been phosphorylated.

TPA induced the loss of thick actin bundles in lamellipodia. This might have caused changes in the mechanical properties of the lamellipodia. I therefore measured the elastic modulus of lamellipodial regions using AFM. I measured a 40% decrease in the elastic modulus of the lamellipodia. This is the first experimental estimate of the contribution of the actin meshwork to the elasticity of lamellipodia. This elasticity may be important for penetration of growth cones into some tissues.

A similar role of fascin may also be present in different types of cells. Recently, fascin is reported as an invasion marker of several types of cancer (Hashimoto *et al.* 2005b; Jacquemet *et al.* 2015). Fascin appears at the leading edge of the cell when metastasis occurs (Kim *et al.* 2009; Li *et al.* 2010; Jacquemet *et al.* 2015). Fascin at the leading edge of cancer cells may contribute to elasticity and induce metastasis. While there is a large difference between the processes of cancer metastasis and growth cone migration, the mechanism of elasticity may be similarly controlled.

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