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# $Ca^{2+}$ regulates the subcellular localization of adenomatous polyposis coli tumor suppressor protein

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ABSTRACT

Microtubule (MT) plus-end tracking proteins (+TIPs) are involved in the regulation of MT plus-end dynamics and stabilization. It was reported previously that an increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) induced by disruption of the plasma membrane stimulates rearrangement of MTs [T. Togo, Disruption of the plasma membrane stimulates rearrangement of microtubules and lipid traffic toward the wound site, J. Cell Sci. 119 (2006) 2780–2786], suggesting that some +TIPs are regulated by $Ca^{2+}$. In the present study, the behavior of adenomatous polyposis coli (APC) following an increase in $[Ca^{2+}]_i$ was observed using *Xenopus* A6 epithelial cell expressing GFP-tagged APC. An increase in $[Ca^{2+}]_i$ by cell membrane disruption or by ionomycin treatment induced dissociation of APC without depolymerizing MTs. Inhibition of a tyrosine kinase and GSK-3β suppressed APC dissociation upon an increase in $[Ca^{2+}]_i$. Western blotting analysis showed that $Ca^{2+}$ transients activated GSK-3β through a tyrosine kinase. These results suggest that $Ca^{2+}$ stimulates redistribution of APC through a tyrosine kinase- and GSK-3β-dependent pathway.



## Introduction

Under physiological conditions mechanical stress can disrupt the plasma membrane of cells from many different animal tissues [1]. To survive, cells rapidly reseal the cell membrane by lipid bilayer reorganization, a process that requires reduction in cell membrane tension via $Ca^{2+}$-regulated exocytosis [2].

It has previously been shown that the rate of membrane resealing with repeated wounds at the same site is facilitated, and that this response requires both $Ca^{2+}$ and PKC activity, and is sensitive to brefeldin A [3,4]. Furthermore, same-site facilitation was found to be a polarized reaction only to the site where the $Ca^{2+}$-sensitive vesicle pool had been depleted by a previous membrane disruption [4,5]. These results strongly suggest that the direction of vesicle transport from the region of the trans-Golgi network to the plasma membrane is actively regulated upon cell membrane disruption. In fact, it was reported previously that disruption of the plasma membrane induces an increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and stimulates rearrangement of MTs [6].

Microtubule (MT) plus-end tracking proteins (+TIPs) are a diverse group of proteins involved in the regulation of MT plus-end dynamics and stabilization [7,8]. The dynamics of MTs are thought to be regulated by +TIPs and some of these may, in turn, be regulated by $Ca^{2+}$. A previous study indicated that recruitment of end binding protein 1 (EB1) to MTs around the site of cell membrane disruption is stimulated by an increase in $[Ca^{2+}]_i$ in PtK2 cells [6]. Many +TIPs have been shown to interact with one another to form a complex at MT plus ends [7,8]. Thus, it is possible that $Ca^{2+}$ also regulates other +TIPs, including adenomatous polyposis coli (APC).

APC is a 312 kDa protein that binds to many cytoskeletal components. In subconfluent cells, APC concentrates in clusters at the plus ends of MTs [9,10] and regulates cell migration [11,12]. In highly confluent epithelial cells, APC localizes at the basal cortex and near the lateral plasma membrane and plays an important role in the organization of cytoskeletons [10,13–15].

The aim of the present study was to elucidate the involvement of $Ca^{2+}$ in the regulation of APC. To observe the behavior of APC upon an increase in $[Ca^{2+}]_i$, *Xenopus* A6 epithelial cells expressing GFP-tagged APC were wounded by a glass needle or treated with the $Ca^{2+}$ ionophore ionomycin. The present study demonstrates that $Ca^{2+}$ stimulates redistribution of APC in addition to EB1.

## Materials and methods

*Cell culture.* *Xenopus* A6 epithelial cells and stable transfectants expressing GFP-tagged full length *Xenopus* APC (GFP-fAPC) [12] were kindly provided by Dr. Y. Mimori-Kiyosue (KAN Research Institute). These cells were grown in medium containing 50% Leibovitz's L-15 medium, 40% distilled water, 10% fetal bovine serum, and penicillin–streptomycin solution at 24 °C without $CO_2$. Cells for experiments were plated on glass-based 35 mm dishes (Iwaki).

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*Transfection.* Cellular Lights Tubulin-RFP and the expression vector for EGFP-α-tubulin were purchased from Invitrogen and Clontech, respectively. Cellular Lights Tubulin-RFP was transfected into A6 cells in accordance with the manufacturer's protocol. EGFP-α-tubulin was transfected into the cells using the CalPhos transfection kit (Clontech). Cells were then observed 48 h after transfection.

*Live imaging.* In some experiments, the $Ca^{2+}$ indicator Rhod-3 (Invitrogen) was introduced into the cells by AM-ester loading to monitor changes in $[Ca^{2+}]_i$. During observation, culture medium was replaced with imaging medium (50% L-15 medium without phenol red, 49% distilled water, 1% fetal bovine serum). This medium contained 0.63 mM $Ca^{2+}$, which is higher than the threshold level for membrane resealing [16]. Images were acquired at 24 °C with an LSM510 laser scanning confocal microscope (ver. 3.2; Carl Zeiss) equipped with Axiovert (C-Apochromat 40X/1.2 W Corr objective). To disrupt the plasma membrane, glass needles were made from Narishige G-1000 glass rods by pulling with a Narishige PC-10. Wounding of cells was performed using an InjectMan 5179 and a FemtoJet 5247 (Eppendorf) equipped with a microscope. The time setting for wounding was 0.3 s. For ionomycin treatment, 1 mM ionomycin dissolved in DMSO was diluted with imaging medium to 10 μM and then gently applied to the edge of the dish (final 1 μM). Images were exported as TIFF files and manipulated with GraphicConverter (Lemke Software) and Canvas (ACD Systems).

*Western blotting.* Cell lysates were prepared with M-PER protein extraction reagent (Thermo Scientific) containing Protease Inhibitor Cocktail (Calbiochem) and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific) at 4 °C, and lysates were centrifuged at 14,000*g* for 10 min to pellet the cell debris. The lysates were then separated by SDS–PAGE, electrophoretically transferred to PVDF membrane, and probed either with anti-GSK-3α[pY279]/β[pY216] phospho-specific antibody (Acris Antibodies) or anti-GSK-3β antibody (Santa Cruz Biotechnology).

## Results

### *Increase in intracellular $Ca^{2+}$ concentration induces dissociation of APC*

A previous study indicated that cell membrane disruption induced $Ca^{2+}$-dependent depolymerization of MTs, though depolymerization was restricted, especially around the site of membrane disruption [6]. This was also true in the present study using A6 cells (data not shown). Thus, to avoid observing the effect of MT depolymerization on the subcellular distribution of APC, the plasma membranes of *Xenopus* A6 epithelial cells expressing both tubulin-RFP and GFP-fAPC were wounded with a glass needle at sites far from APC clusters (e.g., Fig. 1A, asterisk). Cell membrane disruption induced dissociation of APC in 85% of wounded cells ($n = 20$). The intensity of GFP-fAPC began to decrease 78.6 ± 34.8 s ($n = 8$) after wounding and declined to 19.9 ± 8.59% of the initial value. However, MTs were still intact in regions where APC dissociated (Fig. 1B), indicating that membrane disruption specifically affected the localization of APC. Interestingly, 15% of wounded cells did not show APC dissociation.

Consistent with a previous study using PtK2 cells [6], cell membrane disruption of A6 cells stimulated recruitment of EB1 to MTs within 30 s, especially around the site of membrane disruption (Fig. 1C). Although cell membrane disruption induced dissociation of APC, there was no accumulation of APC around the site of membrane disruption (data not shown).

Unfortunately, it was impossible to observe the $Ca^{2+}$ dynamics and the behavior of GFP-fAPC in wounded cells simultaneously since cell membrane disruption induces efflux of various molecules including the $Ca^{2+}$ indicator from the cytosol. Thus, ionomycin was used to observe the relationship between $Ca^{2+}$ levels and APC dissociation. When cells were treated with 1 μM ionomycin to increase $[Ca^{2+}]_i$, 83% of cells ($n = 47$) showed APC dissociation as shown in Fig. 2A. On average, APC dissociation began 66.7 ± 11.8 s ($n = 18$) after the increase in $[Ca^{2+}]_i$, and intensity of APC-GFP declined to 11.2 ± 3.91% of the initial value. When cells were treated with DMSO (1/1000 dilution) as a control, there were no significant changes in the distribution of GFP-fAPC (Table 1). To determine whether dissociation of APC upon ionomycin treatment was due to depolymerization of MTs, A6 cells expressing EGFP-α-tubulin were treated with 1 μM ionomycin. Treatment with 1 μM ionomycin did not induce MT depolymerization (Fig. 2B) in all five cells examined. These data indicate that an increase in $[Ca^{2+}]_i$ directly enhances redistribution of APC protein.

### *Dissociation of APC after an increase in $[Ca^{2+}]_i$ requires the activities of a tyrosine kinase and GSK-3β*

It was previously reported that phosphorylation of APC by GSK-3β can cause APC to dissociate from MTs [17] and that phosphorylation

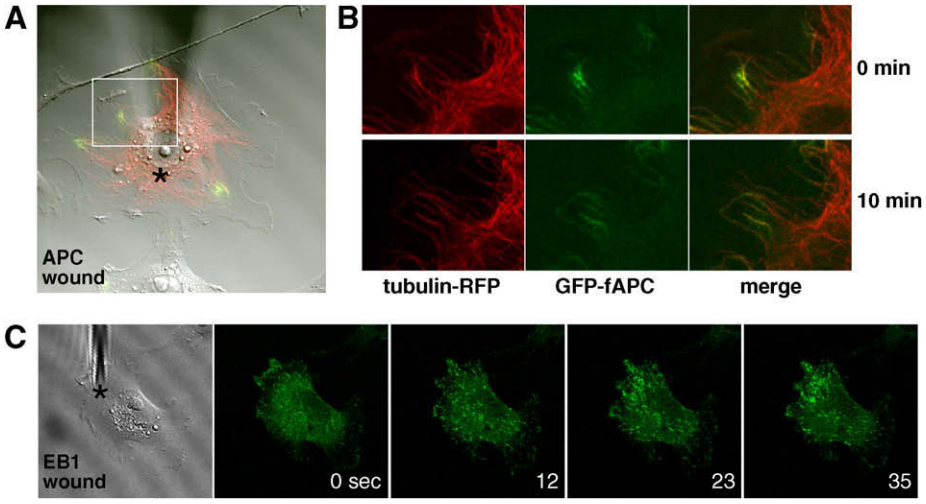


**Fig. 1.** Cell membrane disruption induces dissociation of APC and recruitment of EB1 in *Xenopus* A6 cells. (A,B) An A6 cell expressing both tubulin-RFP and GFP-fAPC was wounded by a glass needle to increase $[Ca^{2+}]_i$. Medium used in this study contained 0.63 mM $Ca^{2+}$. Asterisk indicates the site of cell membrane disruption. (B) Enlargement of the boxed area in (A). (C) Cells expressing EB1-GFP were wounded by a glass needle. Asterisk indicates the site of cell membrane disruption. A slight increase in the number of EB1-GFP comets was observed throughout the cell. Furthermore, numerous EB1-GFP comets appeared around wound site within 30 s after membrane disruption. This result is consistent with previous study using PtK2 cells [6].

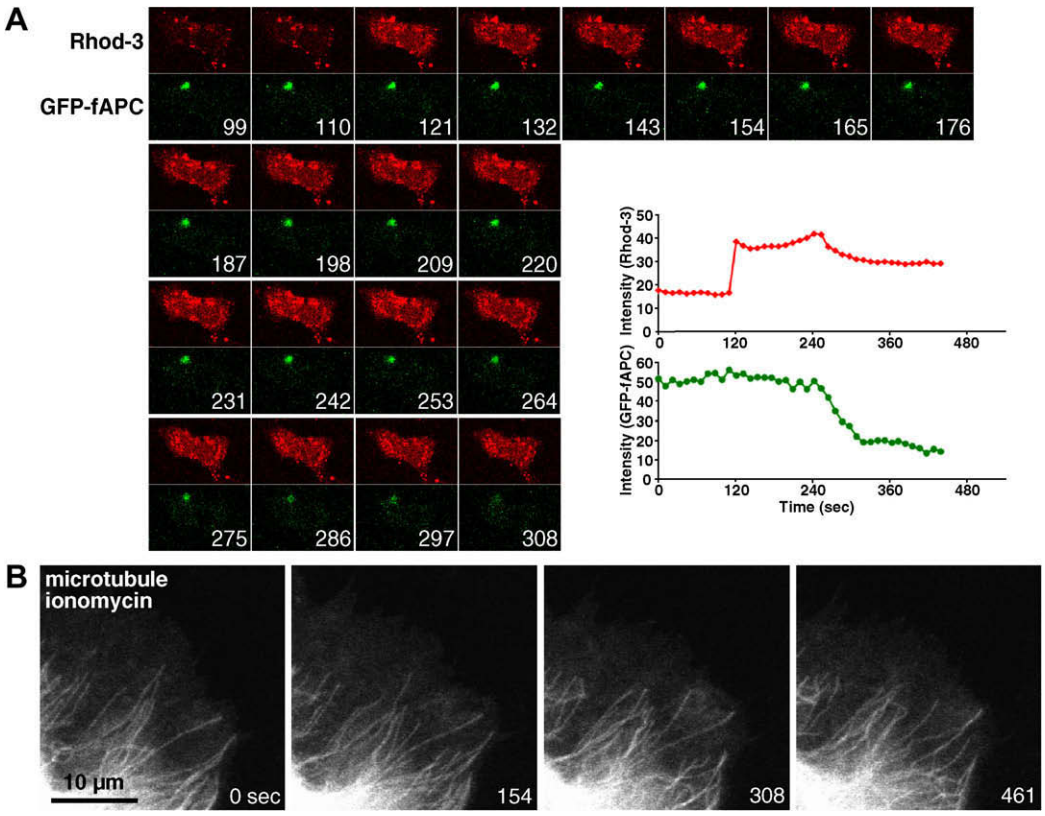


**Fig. 2.** An increase in $[Ca^{2+}]_i$ induces dissociation of APC. (A) A6 cells expressing GFP-fAPC were loaded with $Ca^{2+}$ indicator Rhod-3 and treated with 1 μM ionomycin. The intensity of GFP-fAPC began to decrease 66.7 ± 11.8 s ($n$ = 18) after the increase in $[Ca^{2+}]_i$. (B) A6 cells expressing EGFP-α-tubulin were treated with 1 μM ionomycin. Ionomycin (1 μM) did not induce depolymerization of MTs.

of Tyr276 and Tyr216 enhances the activity of GSK-3α and GSK-3β, respectively [18,19]. To analyze tyrosine phosphorylation of GSK-3 upon an increase in $[Ca^{2+}]_i$, parental A6 cells were incubated with or without the tyrosine kinase inhibitor genistein (50 μM) for 15 min and treated with 1 μM ionomycin for 5 min. Total cell lysates were then analyzed by Western blotting either with anti-GSK-3α[pY279]/β[pY216] phospho-specific antibody or anti-GSK-3β antibody. Ionomycin treatment induced phosphorylation on Tyr216 of GSK-3β (Fig. 3), whereas genistein suppressed this phosphorylation. On the other hand, phosphorylation of GSK-3α at Tyr279 did not increase upon ionomycin treatment. The same conclusion was obtained from three independent experiments. These data indicate that an increase in $[Ca^{2+}]_i$ stimulates GSK-3β through phosphorylation of Tyr216.

To observe the effect of GSK-3β inhibition on APC dissociation, A6 cells expressing GFP-fAPC were treated with a highly specific GSK-3 inhibitor, 6-bromoindirubin-3′-oxime (BIO) [20], for 15 min and wounded by a glass needle or treated with ionomycin. As shown in Fig. 4 and Table 1, 100 nM BIO suppressed APC dissociation after both wounding and ionomycin treatment. In contrast, ionomycin treatment induced APC dissociation in cells treated with 100 nM 1-methyl-BIO (MeBIO), an inactive analog of BIO [20]. Furthermore, a different GSK-3 inhibitor, SB216763 (2.5 μM), also prevented APC dissociation after ionomycin treatment (Table 1). To inhibit tyrosine phosphorylation of GSK-3β upon membrane disruption and ionomycin treatment, A6 cells expressing GFP-fAPC were treated with 50 μM genistein for 15 min, then wounded by a glass needle or treated with ionomycin. Genistein suppressed APC dissociation after both wounding and ionomycin treatment (Table 1). These results suggest that an increase in $[Ca^{2+}]_i$ stimulates dissociation of APC through a tyrosine kinase- and GSK-3β-dependent pathway.

**Table 1**
The effect of kinase inhibitors on APC dissociation.

| Treatment | Dissociation of APC (%) | $n$ |
|---|---|---|
| DMSO (control) | 0 | 33 |
| Wound by glass needle | 85 | 20 |
| Ionomycin alone | 83 | 47 |
| 50 μM Genistein, wound | 0 | 7 |
| 50 μM Genistein + ionomycin | 4.2 | 24 |
| 100 nM BIO, wound | 0 | 8 |
| 100 nM BIO + ionomycin | 1.9 | 52 |
| 100 nM MeBIO + ionomycin | 77.5 | 40 |
| 2.5 μM SB216763 + ionomycin | 2.7 | 37 |

A6 transfectants expressing GFP-fAPC were incubated with kinase inhibitors for 15 min, then wounded by a glass needle or treated with 1 μM ionomycin in the presence of inhibitors.

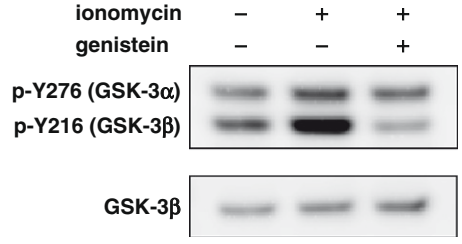


**Fig. 3.** An increase in $[Ca^{2+}]_i$ results in increased tyrosine phosphorylation of GSK-3β. Parental A6 cells were incubated with or without 50 μM genistein for 15 min, then treated with 1 μM ionomycin for 5 min. Total cell lysates were analyzed by Western blotting either with anti-GSK-3α[pY279]/β[pY216] phospho-specific antibody or anti-GSK-3β antibody. Ionomycin (1 μM) induced phosphorylation on Tyr216 of GSK-3β.

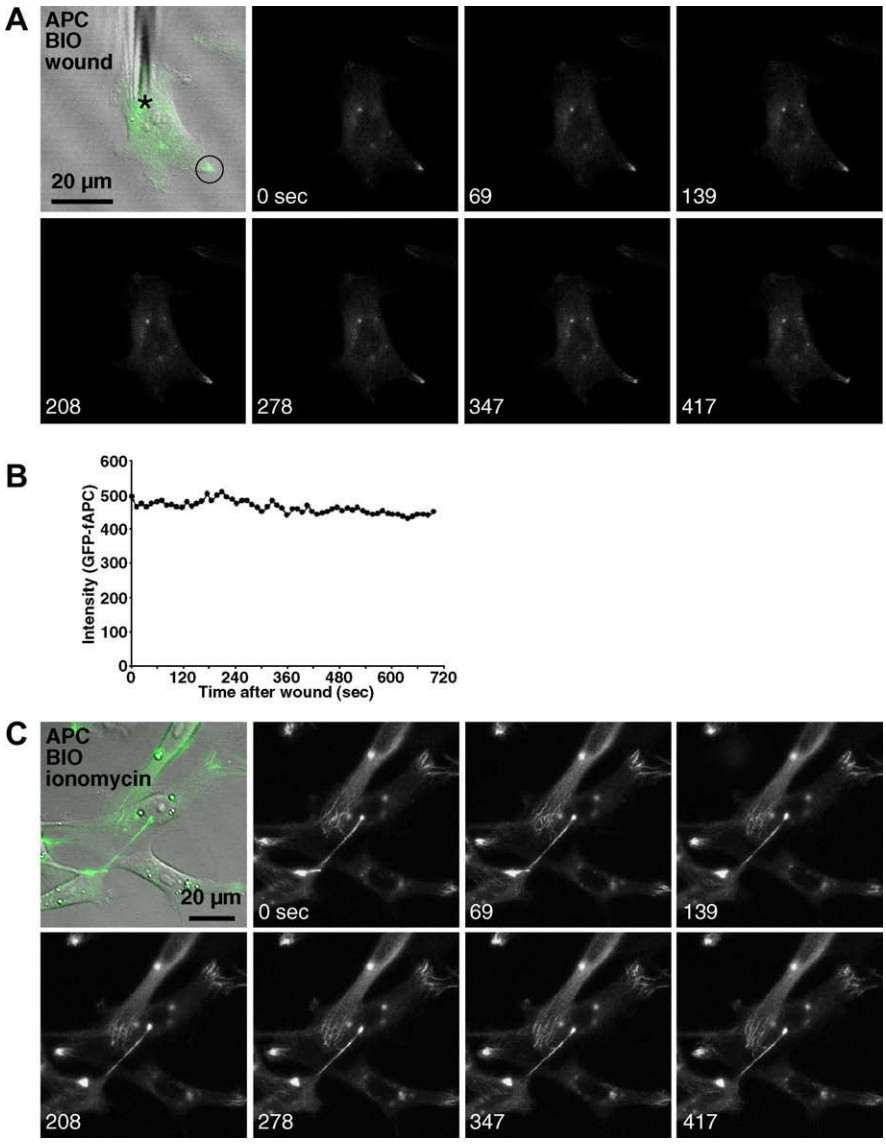


**Fig. 4.** Inhibition of GSK-3β suppresses APC dissociation. (A) A6 cells expressing GFP-fAPC were incubated with 100 nM BIO for 15 min, then wounded by a glass needle. (B) Fluorescent intensity of GFP at the region indicated by a circle in (A) was measured. Dissociation of APC upon cell membrane disruption was suppressed by BIO. (C) A6 cells expressing GFP-fAPC were incubated with 100 nM BIO for 15 min, then treated with 1 μM ionomycin. Ionomycin did not induce APC dissociation in BIO-treated cells.

## Discussion

The present study indicates that $Ca^{2+}$ transients stimulate dissociation of APC from MTs through a tyrosine kinase and GSK-3β. Although $Ca^{2+}$ signaling can dissociate APC from MTs in most cases, APC did not respond to $Ca^{2+}$ in 15% or 17% of wounded or ionomycin-treated cells, respectively. The reason as to why APC is $Ca^{2+}$-insensitive in some cases remains to be elucidated.

Previous studies and the present study show that cell membrane disruption stimulates recruitment of EB1 to MTs, especially around the site of membrane disruption, and that this response is important for facilitated cell membrane resealing of repeated wounds [6]. EB1 family members are known to interact with the other +TIPs to form the core of the +TIPs complex [7]. Thus, other +TIPs may also be recruited to MTs around the site of membrane disruption. The present study shows that cell membrane disruption and an increase in $[Ca^{2+}]_i$ trigger dissociation of APC from MTs, though there is no obvious accumulation of APC around the site of membrane disruption. Further studies are required to identify the +TIPs, in addition to EB1, that are recruited to MTs around the site of membrane disruption. The present study also suggests that $Ca^{2+}$ regulates the behaviors of EB1 and APC through different signal transduction pathways, although the signaling pathway that leads to recruitment of EB1 to MTs has not yet been fully elucidated.

Since APC is known to stabilize MTs [11,17], the present study suggests that $Ca^{2+}$ transients temporally destabilize MTs organization. When a monolayer of cells is scratched, the plasma membranes of the cells lining the scratch site is temporally disrupted, as shown previously [21]. Thus, it is possible that temporal destabilization of MT arrays induced by APC dissociation in polarized epithelial cells initiates reorganization of MTs for directional cell migration. Preliminary experiments showed that inhibition of APC dissociation from MTs by inhibiting tyrosine kinase and GSK-3β during scratching suppressed polarized elongation of MTs toward the scratch site and delayed cell migration (data not shown). Further studies are required to elucidate the role of APC dissociation in scratch-induced reorganization of MTs.

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