Direct binding of SWAP-70 to non-muscle actin is required for membrane ruffling

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Summary
Membrane ruffling induced by growth factor stimulation is caused by actin remodeling, which is mediated by various signaling molecules including Rac. We have shown that SWAP-70, which binds phosphatidylinositol trisphosphate, is one such molecule required for membrane ruffling in mouse kidney cells. Here, we show that SWAP-70 directly binds to F-actin. The bacterially expressed C-terminal region of SWAP-70 co-sedimented with non-muscle F-actin, suggesting direct binding of SWAP-70 to F-actin. The binding was much weaker in muscle F-actin. A truncated mutant of SWAP-70 containing only the C-terminal region readily colocalizes with F-actin, supporting this idea. Full-length SWAP-70 does not colocalize with F-actin unless cells are stimulated with growth factors, suggesting the presence of a stimuli-dependent regulatory mechanism for actin-binding activity in vivo. Overexpression of the mutant SWAP-70 lacking this binding domain inhibits the membrane ruffling induced by epidermal growth factor stimulation in COS7 cells. This dominant-negative effect is also observed in membrane ruffling induced by a dominant-active Rac, suggesting that SWAP-70 cooperates with Rac. These results suggest that the binding activity of SWAP-70 to non-muscle F-actin is required for membrane ruffling.

Key words: SWAP-70, Actin, Membrane ruffling, Rac

Introduction
Actin is a major component of the cytoskeleton, which mediates many cellular responses such as motility, morphological change and cytokinesis (Mitchison and Cramer, 1996; Pollard et al., 2000; Schmidt and Hall, 1998). Actin is involved in these responses through two major processes: first, assembly and disassembly through monomeric G-actin and filamentous F-actin; second, the organization of filamentous actin into higher order structures, which allows for the diversity of actin filament forms. These processes are regulated by multiple actin-binding proteins (Ayscough, 1996; dos Remedios et al., 2003; Winder and Ayscough, 2005).

Vertebrates express three types of actin isoforms: α, β and γ. α-Actin is expressed in muscle, whereas non-muscle cells contain cytoplasmic β- and γ-actin isoforms (Herman, 1993; Khaitlina, 2001; Rubenstein, 1990). Compartmentalization has been observed in cells expressing several types of these isoforms (Herman, 1993; Hoock et al., 1991). For example, β-actin accumulates at the membrane cytoskeleton interface in regions of moving cytoplasm such as membrane ruffles, whereas localization of γ-actin is suggested to be restricted to stress fibers. These actin isoforms have generally been believed to play distinct roles, particularly as there is evidence that they cannot be substituted for each other; however, functional differences have not been fully explained.

Actin remodeling dependent on signal transduction for growth factor stimuli has been studied extensively. One example is membrane ruffling induced by growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Hall, 1998; Mellstrom et al., 1988; Ridley et al., 1992). Among the signaling molecules activated by these growth factors, phosphoinositide 3-kinase (PI 3-kinase) (Hawkins et al., 1995; Wennstrom et al., 1994) and Rac (Ridley et al., 1992) have been considered to play crucial roles in inducing membrane ruffling. PI 3-kinase is required for membrane ruffling by producing phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P₃], which is suggested to activate Rac through PtdIns(3,4,5)P₃-dependent guanine nucleotide exchange factor (GEF) for Rac (Nimmul et al., 1998; Yoshii et al., 1999). The signaling pathways downstream of Rac that lead to formation of membrane ruffling have been explored intensively (Bishop and Hall, 2000; Burridge and Wennerberg, 2004). Among many effector proteins that interact with activated Rac, WAVE has been suggested to be one that is crucial for inducing membrane ruffling (Miki et al., 1998; Suetsugu et al., 2003). After activation, it can initiate actin polymerization either de novo or at the barbed end or sides of pre-existing filaments by activating the Arp2/3 complex (Machesky et al., 1999; Takenawa and Miki, 2001). This polymerization of actin and the branching of actin filaments has been suggested to be the major driving sources of cell movement (Pollard and Borisy, 2003).

We identified SWAP-70 as a PtdIns(3,4,5)P₃-binding protein that is required for formation of membrane ruffling by growth factor stimulation (Shinohara et al., 2002). SWAP-70 is a 70 kDa protein that was originally isolated from activated B lymphocytes (Borggreve et al., 1998). This protein contains an EF-hand motif in its N-terminal domain, a pleckstrin-homology (PH) domain in its central region, and a coiled-coil domain that overlaps with a domain that is weakly homologous...
to Db1 homology (DH) domains near the carboxyl terminal region. Although a high level of expression was observed in activated B cells and immature mast cells, SWAP-70 is widely expressed in various cell types and tissues (Borggrefe et al., 1998; Gross et al., 2002; Hilpela et al., 2003). In COS7 cells, SWAP-70 translocates from the cytoplasm to the membrane ruffles upon PI 3-kinase activation through growth factor stimulation, and overexpression of SWAP-70 enhances growth-factor-induced membrane ruffling. Kidney cells lacking SWAP-70 show impaired membrane ruffling after growth factor stimulation (Shinohara et al., 2002). We previously suggested that SWAP-70 is involved in the formation of membrane ruffling by activating Rac as a GEF.

In this study, we found that SWAP-70 can bind to F-actin directly. Our results demonstrate that the binding activity is specific for non-muscle actin. In addition, the binding activity was required for growth-factor-induced membrane ruffling as well as for that induced by constitutively active Rac, implying that SWAP-70 cooperates with Rac not only as a GEF.

**Results**

**The C-terminus of SWAP-70 binds to F-actin in vitro**

We previously demonstrated that SWAP-70 translocates to peripheral membrane ruffles and colocalizes with F-actin after EGF stimulation in COS7 cells, which suggests the possible binding ability of SWAP-70 to F-actin. We found a putative actin-binding sequence composed of basic amino acids homologous to that found in gelsolin in the C-terminus of SWAP-70 (Fig. 1A), supporting this hypothesis. To test this, the C-terminal portion of SWAP-70 was expressed as a His-tagged protein, His–SWAP-70(448-585) (Fig. 1A), in *Escherichia coli*. The protein was purified and tested for actin-binding activity in a co-sedimentation assay using highly purified, platelet-derived, non-muscle actin, in accordance with the finding that expression of SWAP-70 has hardly been detected in muscle tissue (Hilpela et al., 2003). As shown in Fig. 1B, His–SWAP-70(448-585) sedimented only in the presence of F-actin. By contrast, His–SWAP-70(448-564), which lacks the putative actin-binding site, failed to interact with F-actin, suggesting that the C-terminal region covering 21 amino acids was crucial for binding. When the co-sedimentation assay was performed with muscle actin, binding of His–SWAP-70(448-585) was hardly detectable, suggesting that SWAP-70 binds to F-actin in an isospecific manner. To determine the stoichiometry at which SWAP-70(448-585) binds to non-muscle F-actin, increasing amounts of His–SWAP-70(448-585) were incubated with a fixed amount of actin, and the ratio at which binding became saturated was determined. As shown in Fig. 1C,D, binding of His–SWAP-70(448-585) to F-actin increased up to a ratio of approximately 1:1. The estimated dissociation constant (Kd) for F-actin was 0.74 μM.

**Fig. 1.** The C-terminal region of SWAP-70 binds to non-muscle actin in an isospecific manner. (A) The structure of SWAP-70 and its truncation mutants. The amino acid sequence of the putative actin-binding region in SWAP-70 was aligned with those of the homologous region seen in proteins of the gelsolin family. Basic amino acids are shown with a gray background; PH, pleckstrin-homology domain. (B) Binding of the C-terminal region of SWAP-70 to F-actin. His–SWAP-70(448-585) or His–SWAP-70(448-564) was mixed with or without (–) 10 mM polymerized actin derived from human platelets (non-muscle) or rabbit skeletal muscle (muscle), and ultracentrifuged. Proteins in the supernatant (S) and the pellet (P) were analyzed by SDS-PAGE followed by staining with Coomassie Blue. (C) Quantitative analysis for binding of the C-terminal region of SWAP-70 to F-actin. A co-sedimentation assay was performed by mixing of 5 μM polymerized non-muscle actin with various amounts of His–SWAP-70(448-585) at the indicated final concentrations. (D) Similar experiments as in C with a wide range of concentrations of His–SWAP-70(448-585) were performed, and the amounts of protein on the gel were quantified by an imaging analyzer.
Full-length SWAP-70 binds to F-actin only weakly

Full-length His–SWAP-70 and its mutant, His–SWAP-70(1-564), which lacks the putative binding region, were tested for actin-binding ability (Fig. 2A). Full-length SWAP-70 co-sedimented with non-muscle F-actin, although the affinity appeared to be much weaker than that of His–SWAP-70(448-585). Indeed, the binding plot obtained from co-sedimentation with various amounts of full-length SWAP-70 was quite different from that of His–SWAP-70(448-585), and the ratio of bound protein to unbound protein remained unchanged even if the amount of His–SWAP-70 was decreased to 1 μM (Fig. 2B).

In addition, this binding was not isospecific, giving virtually the same profile when muscle actin was used (Fig. 2C). These results suggest that this binding was somewhat different from that seen with the C-terminal portion of SWAP-70. Supporting this idea, deletion of the actin-binding domain found above did not completely abolish the activity (Fig. 2A), suggesting that another binding region, which exhibits non-isospecific binding for actin, is present in the full-length SWAP-70. Several proteins with a PH domain have been reported to be able to bind F-actin (Thacker et al., 2004; Yao et al., 1999). Therefore, we tested whether the PH domain of SWAP-70 binds to F-actin. As shown in Fig. 2D, His–SWAP-70 PH co-sedimented with F-actin, but preferential binding to non-muscle F-actin was not observed. The binding property of His–SWAP-70 PH to F-actin was similar to that of full-length His–SWAP-70 (Fig. 2E). Therefore, the actin-binding property seen for full-length SWAP-70 might be reflected in this binding property of the PH domain to F-actin. The binding properties of the two actin-binding regions are summarized in Fig. 2F. The results imply that the actin-binding activity of the C-terminal region might be more important than that of the PH domain for the physiological function of SWAP-70. Considering the fact that full-length SWAP-70 did not exhibit the binding property of the C-terminal actin-binding region, this region might be masked without stimulation.

The C-terminal portion of SWAP-70 colocalizes with F-actin

The behavior of the C-terminal portion of SWAP-70 containing the actin-binding domain was examined. SWAP-70(448-585), which was used in the in vitro actin-binding assay, was expressed in COS7 cells (Fig. 3A). As shown in Fig. 3B, GFP–SWAP-70(448-585) colocalized with F-actin at the cortical region and at the stress fibers when cells were not stimulated. Membrane ruffling was rarely observed under this condition. A similar result was obtained with another mutant, GFP–SWAP-70(500-585), which contained only 86 C-terminal amino acids (data not shown). Such colocalization was seen neither in GFP–SWAP-70(448-564) lacking the C-terminal 21 amino acids nor in GFP–SWAP-70 PH, supporting the idea that the C-terminal binding site for F-actin is indeed important for colocalization of SWAP-70 with F-actin. However, full-length SWAP-70 did not colocalize with F-actin in unstimulated cells even though the C-terminal region was present [Fig. 3C, EGF(−)]. Membrane ruffling was observed in COS7 cells after EGF.

![Fig. 2. The actin-binding property of SWAP-70. (A) Binding of full-length SWAP-70 to F-actin. His–SWAP-70 or His–SWAP-70(1-564), lacking the actin-binding domain, was mixed with (+) or without (−) 10 μM polymerized non-muscle actin. Proteins in the supernatant (S) and the pellet (P) were analyzed by SDS-PAGE followed by Coomassie Blue staining. The band seen at the position of actin in the lane of actin (−) is the degradation product of His–SWAP-70. The same protein may be present in the lane of actin (+). (B) Quantitative analysis for binding of His–SWAP-70 to F-actin. A co-sedimentation assay was performed by mixing of 5 μM polymerized non-muscle actin with various amounts of His–SWAP-70 (0 μM to 5 μM). (C) Binding property of full-length SWAP-70. A co-sedimentation assay was performed by mixing of His–SWAP-70 with polymerized 10 μM actin derived from human platelets (non-muscle) or rabbit skeletal muscle (muscle). (D) Binding of the PH domain of SWAP-70 to F-actin. A co-sedimentation assay was performed by mixing of His–SWAP-70 PH with polymerized 10 μM muscle actin, non-muscle actin, or without (−) actin. (E) Quantitative analysis for binding of the PH domain of SWAP-70 to F-actin. A co-sedimentation assay was performed by mixing 5 μM polymerized non-muscle actin with various amounts of His–SWAP-70 PH (0 μM to 5 μM). Quantitative analysis was done as in B. (F) Actin-binding properties of the various mutants of SWAP-70. The structure of SWAP-70 and its truncation mutants is shown. All constructs were tagged with His at the N-terminus. The actin-binding property of each protein is summarized on the right.](image-url)
Binding of SWAP-70 to actin in membrane ruffling stimulation. The terms of membrane ruffles and lamellipodia are often used interchangeably. In this paper, we define membrane ruffles as undulating plasma membrane that folds back, which is transported rearwards and fails to adhere, as described by others (Wells et al., 2004; Kurokawa and Matsuda, 2005). Membrane ruffles were distinguished from lamellipodia by scanning the dorsal plane of focus using confocal microscopy or analyzing the x/z plane [Fig. 3C, EGF(+), arrowheads]. After stimulation with EGF, the full-length SWAP-70 clearly co-localized with F-actin at the membrane ruffles [Fig. 3C, EGF(+)]. The C-terminal region of SWAP-70 was also found at the membrane ruffles because it always colocalizes with F-actin (data not shown). These data support the idea from the in vitro study that the C-terminal actin-binding site might not be fully accessible in the full-length protein and that some signal might facilitate the conformational change that exposes the binding site.

Mutant SWAP-70 lacking the C-terminal actin-binding domain acts as a dominant-negative reagent for membrane ruffling induced by EGF

We previously reported that cells from SWAP-70-deficient mice show impaired ruffling, indicating that SWAP-70 is responsible for membrane ruffling (Shinohara et al., 2002). To examine the significance of the C-terminal binding site for F-actin of SWAP-70 in membrane ruffling, we tested the effect of GFP–SWAP-70(1-564), the mutant lacking the region, in EGF-treated COS7 cells. As shown in Fig. 4A, overexpression of GFP–SWAP-70(1-564) inhibited membrane ruffling induced by EGF, whereas nontransfected cells showed clear membrane ruffling (indicated by arrowheads in Fig. 4A). Expression of GFP did not show any effect on the formation of membrane ruffles (indicated by arrowheads in Fig. 4A). Quantification of the membrane ruffles (Fig. 4B) showed that expression of GFP–SWAP-70(1-564) reduced the extent of ruffles to about 30% of that for GFP-expressing cells, indicating a dominant-negative effect of GFP–SWAP-70(1-564) on membrane ruffling induced by EGF stimulation.

Membrane ruffling induced by dominant-active Rac is also suppressed by the mutant SWAP-70

We also tested the effect of SWAP-70(1-564) on the dominant-active Rac-induced membrane ruffling. COS7 cells were cotransfected with the expression vectors for dominant-active Rac and either GFP or GFP–SWAP-70(1-564). SWAP-70(1-564) inhibited Rac-induced membrane ruffling (Fig. 5A,B),
implying that activity of SWAP-70 is required for membrane ruffling induced by the activated Rac. Expression of SWAP-70(1-564) showed no effect on filopodia formation induced by a dominant-active Cdc42 (Fig. 5B).

SWAP-70 binds to activated Rac

The results shown above suggest the possibility that SWAP-70 is downstream of Rac. To examine this hypothesis, we tested the nucleotide preference of SWAP-70 expressed in mammalian cells in binding to Rac. Pull-down assays using GST-Rac, loaded with GTPγS or with GDP, or using GST-Rac

Fig. 4. The actin-binding ability of SWAP-70 through the C-terminal region is crucial for membrane ruffling induced by EGF. (A) COS7 cells expressing GFP or GFP–SWAP-70(1-564) were treated with (+) or without (−) EGF (100 ng/ml) for 5 minutes and stained with Phalloidin to detect F-actin. Localization of SWAP-70 (green) and F-actin (red) was observed under the confocal microscope. Stacked images are indicated by ‘S’, and dorsal planes of focus by ‘D’. Membrane ruffles are indicated by arrowheads. Bar, 20 μm. (B) The actin-binding ability of SWAP-70 through the C-terminal region is crucial for membrane ruffling induced by dominant-active Rac. (A) COS7 cells were transfected with RFP-RacV12 together with GFP or GFP–SWAP-70(1-564). Localization of SWAP-70 (green) and Rac (red) was observed under the confocal microscope. Stacked images are indicated by ‘S’, and dorsal planes of focus by ‘D’. Membrane ruffles (indicated by arrowheads) were observed in the cells that did not express GFP–SWAP-70(1-564), but not in those that expressed the protein. Bar, 20 μm. (B) Ruffling formation observed in the experiments illustrated in A was quantified by scores defined in the Materials and Methods (left panel). Formation of filopodia was monitored by similar analysis as in A by use of RFP-Cdc42V12 instead of RFP-RacV12 and quantified by scores defined in the Materials and Methods (right panel).
In this study, we found that SW AP-70 exhibits binding activity for F-actin. In co-sedimentation assays, we found that two regions, the PH domain and the C-terminal region, are responsible for the binding activity. Measurement of the binding stoichiometry of the C-terminal region or the PH domain to actin (Fig. 1C,D and Fig. 2E) showed that the binding activity of the former was strong, but that of the latter was relatively weak. The C-terminal binding region showed preferential binding activity for non-muscle actin. Together with the study of the expression pattern of SWAP-70 among various tissues showing that expression of SWAP-70 is hardly detectable in muscle (Hilpela et al., 2003), these results suggest that the C-terminal region plays a significant role in the physiological function of SWAP-70. Usually, rabbit skeletal-muscle actin is used as a universal substrate for actin-binding assays because the difference of amino acids between actin isotypes is only about 10%, although there have been several reports showing differences in actin-binding activity for actin isotypes (Prassler et al., 1997; Shuster and Herman, 1995; Yao et al., 1996). In addition to these reports, our finding supports the idea that the specific actin isotype is regulated in some cases by an iso-specific actin-binding protein, which might explain the fact that a given isotype cannot be substituted by others. Colocalization of SWAP-70 with F-actin has been reported previously by others (Hilpela et al., 2003); however, direct binding was not observed in our study. This might be because of the very weak affinity of SWAP-70 to rabbit skeletal-muscle actin, which is commonly used in the actin-binding assays.

When expressed in COS7 cells, the C-terminal actin-binding portion [GFP–SWAP-70(448-585)] colocalized with F-actin, whereas its derivative lacking the binding region [GFP–SWAP-70(448-564)] or the PH domain [GFP–SWAP-70 PH] did not, suggesting that the C-terminal binding domain appears to be important for the actin-binding ability of SWAP-70 in vivo (Fig. 3B). At present, the role of the PH domain for the actin-binding ability of SWAP-70 is not clear. Colocalization of full-length SWAP-70 with F-actin is observed only when cells are stimulated by growth factors. This observation agrees with in vitro results showing that full-length SWAP-70 does not exhibit the actin-binding property of the C-terminal region, suggesting that the region might be masked in the unstimulated state. It is possible that the exposure of the C-terminal actin-binding site might be regulated by a conformational change accompanied by extracellular stimuli. Because the translocation of SWAP-70 is dependent on PI3-kinase and SWAP-70 is capable of binding to PtdIns(3,4,5)P3 through its PH domain, PtdIns(3,4,5)P3 might affect the binding property of SWAP-70 to F-actin. Further work is needed for testing this possibility.

We previously suggested, using kidney cells derived from SWAP-70−/− mice, that SWAP-70 is required for membrane ruffling induced by growth factor stimulation. In this study, a deletion mutant of SWAP-70 that lacks the C-terminal actin-binding region [SWAP-70(1-564)] acted as a dominant-negative reagent for membrane ruffling in COS7 cells stimulated by EGF. This result suggests that the role of SWAP-70 in the formation of membrane ruffling is explained by its ability to bind to actin. Although the structure and molecular composition of membrane ruffles remained largely unknown, in contrast to that of lamellipodia and filopodia, a recent study showed that membrane ruffles are composed of densely packed bundles of actin filaments (Borm et al., 2005). It is possible that SWAP-70 plays some role in the formation of these actin structures.

The dominant-negative effect of SWAP-70(1-564) was also observed in membrane ruffling induced by dominant-active
Rac, implying that SWAP-70 might cooperate with activated Rac to induce membrane ruffling. In fact, interaction of SWAP-70 derived from mammalian cells with GTPγS-loaded Rac was detected, and the binding was mediated by the effector domain of Rac. Because the binding of the two molecules in vivo has not been detected by immunoprecipitation (data not shown), the interaction might be transient or might need other molecules for formation of a stable complex. So far, the significance of the binding of activated Rac and SWAP-70 remains unclear. To understand fully the function of SWAP-70 in the formation of membrane ruffling, in which the coordinated remodeling of actin and the membrane is necessary, further analysis of the regulation of SWAP-70 by interacting molecules such as Rac and PtdIns(3,4,5)P3 may be required.

Materials and Methods

Cell culture and transfection

The COS7 cells used in our experiment were derived from COS7 cells described by Sawano et al. (2002). The cells were subcloned and their response to EGF treatment was tested. One out of 20 cell lines that gave strong peripheral ruffling was used. For 50% of the cells after EGF treatment was selected and used in this study. These cells showed minimum membrane ruffling when cultured without any stimuli. HEK-293T cells and COS7 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 5% FCS. Transfection into HEK-293T cells was performed by the calcium phosphate precipitation method as described previously (Tanaka et al., 1999). DNA was introduced into COS7 cells by electroporation as described previously (Shinohara et al., 2002).

Plasmids and protein expression

Point mutations were introduced into Rac by the method described by Sawano and Miyawaki (2002). Various deletion mutants of SWAP-70 were constructed by use of restriction enzyme sites present in the cDNA. pRFP-Swap-70 (pEGFP-C1, Clontech) with that of monomer RFP, respectively. His-Swap-70, His-Swap-70(1-564), His-Swap-70 PH, His-Swap-70(448-585) and His-Swap-70(448-564) were constructed in pQE-30 (Qiagen) and purified through His-Bind Resin (Novagen).

Co-sedimentation of SWAP-70 with F-actin

Rabbit skeletal-muscle actin (≥99% purity) and non-muscle actin (≥99% purity, 5:1 ratio of β/γ isoforms) derived from human platelets were purchased from Cytoskeleton. His-SWAP-70 or its truncation mutants were clarified by centrifugation for 30 minutes at 4°C at 109,000 g prior to the assay.

Actin was polymerized in F-buffer (50 mM KCl, 2 mM MgCl2, 0.2 mM ATP, and 0.2 mM DTT in 2 mM imidazole, pH 6.5) at room temperature for 30 minutes and then incubated with His-SWAP-70 or its mutants for 1 hour at room temperature. After ultracentrifugation at 109,000 g for 30 minutes at 25°C, the proteins in the supernatant or pellet were analyzed by SDS-PAGE followed by Coomassie Blue staining. The amount of the protein on the gel was quantified by the image analyzer LAS1000 plus (Fuji Photo Film).

Assay for binding of SWAP-70 and Rac

HEK-293T cells were transfected with expression vectors for GFP-SWAP-70 or its mutants. Thirty-six hours after transfection, cells were lysed in a lysis buffer containing 1% NP40, 20 mM Tris HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 10% glycerol and centrifuged at 15,000 g for 5 minutes. The supernatants were used as sources of SWAP-70.

Bacterially expressed GST-Rac was immobilized on glutathione-Sepharose and loaded with 200 μM GTP or GTPγS in a loading buffer containing 25 mM Tris HCl (pH 8.0), 1 mM DTT, 40 μg/ml BSA, 4.7 mM EDTA, 0.16 mM MgCl2 for 20 minutes at 30°C. The reaction was stopped by addition of MgCl2 (final concentration of 10 mM) and placed on ice. The beads were then mixed with the cell lysates containing SWAP-70 and incubated for 30 minutes at 4°C. After washing with lysis buffer three times, proteins trapped on the beads were eluted by boiling with SDS sample buffer and analyzed by SDS-PAGE. For detection of GFP-tagged SWAP-70, western blotting was performed with anti-GFP antibody (Santa Cruz Biotechnology).

Fluorescence microscopic analysis

COS7 cells harboring the expression vectors were fixed with 3.7% formaldehyde for 5 minutes at room temperature, followed by permeabilization with 0.1% Triton X-100 in PBS for 5 minutes. F-actin was stained with tetramethylrhodamine-isothiocyanate-conjugated Phalloidin (Sigma). Cells were analyzed by confocal fluorescence microscopy (Fluoview 30, Olympus) equipped with lasers for excitation at wavelengths of 488, 546 and 633 nm. The images shown in figures are stacked images of each plane, unless otherwise indicated.

Quantification of ruffling or filopodia formation

Membrane ruffles were defined as an undulating membrane protrusion, folding back and transported rearwards, that fails to adhere. Cells do not detach from the dish at the lamellipodia. Therefore, membrane ruffles can be distinguished from lamellipodia by observing the dorsal planes. The dorsal planes contain the edge of the cells when membrane ruffling is present. The extent of ruffling induced by EGF or dominant-active Rac in COS7 cells was quantified as described before, with slight modifications (Wells et al., 2004): 0 = no ruffles, 1 = isolated areas of ruffling covering no more than 50% of the peripheral area, 2 = extensive ruffling covering no more than 50% of the peripheral area. Total scores for more than 100 cells were calculated, and the average score per cell is indicated in graphs. Scores for filopodia formation were defined similarly. 0 = without filopodia, 1 = with filopodia.

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