

## Involvement of Receptor-Mediated S1P Signaling in EGF-Induced Macropinocytosis in COS7 Cells

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**Macropinocytosis is a highly conserved cellular process of endocytosis by which extracellular fluid and nutrients are taken up into cells through large, heterogeneous vesicles known as macropinosomes. Growth factors such as epidermal growth factor (EGF) can induce macropinocytosis in many types of cells, although precise mechanism underlying EGF-induced macropinocytosis remains unclear. In the present studies we have shown the involvement of S1P signaling in EGF-induced macropinocytosis in COS7 cells. First, EGF-induced macropinocytosis was strongly impaired in sphingosine kinase isozymes, SphK1 or SphK2-depleted cells, which was completely rescued by the expression of the corresponding wild-type isozyme but not the catalytically inactive one, suggesting the involvement of sphingosine 1-phosphate (S1P) in this phenomenon. Next, we observed that EGF-induced macropinocytosis was strongly inhibited in S1P type 1 receptor (S1P<sub>1</sub>R)-knockdown cells, implying involvement of S1P<sub>1</sub>R in this event. Furthermore, we could successfully demonstrate EGF-induced trans-activation of S1P<sub>1</sub>R using one-molecular fluorescence resonance energy transfer (FRET) technique. Moreover, for EGF-induced Rac1 activation, a step essential to F-actin formation and subsequent macropinocytosis, S1P signaling is required for its full activation, as judged by FRET analysis. These findings indicate that growth factors such as EGF utilize receptor-mediated S1P signaling for the regulation of macropinocytosis to fulfil vital cell activity.**

### INTRODUCTION

Macropinocytosis is a cellular process of endocytosis whereby cells take up extracellular macromolecules and nutrients through the formation of macropinosomes, generated by the closure of lamellipodia at ruffling membrane domains (1). Remodelling of actin cytoskeleton leading to lamellipodia formation requires the Rho family GTPases such as Rac1 and phosphoinositide signaling in a spatiotemporally coordinated manner (2, 3). While macropinocytosis is constitutive in macrophages and dendritic cells, it can also be rapidly and synchronously induced by growth factors such as platelet-derived growth factor and epidermal growth factor (EGF) in other cell types (4, 5). Although the detailed molecular mechanism underlying growth factor-induced macropinocytosis is yet unclear, CtBP/BARS is required for EGF-induced macropinosome closure in A431 cells (6) and CtBP/BARS activation of phospholipase D is required for EGF-induced macropinocytosis in COS7 cells (7).

We have recently observed that activation of a receptor for sphingosine 1-phosphate (S1P) is important for subsequent Rac1 activation and actin polymerization (8). S1P is a phosphorylated product of sphingosine catalyzed by sphingosine kinase (SphK) and has emerged as a potent lipid mediator with diverse effects on multiple biological processes including cell growth, survival, differentiation, motility, and cytoskeletal organization (9, 10). Most of these processes are mediated by five S1P-specific G-protein-coupled receptors (S1P<sub>1-5</sub>R) and show distinct expression in tissues and cells, and also unique G-protein-coupling patterns suggesting distinctive functions (11-13). These findings led us to ask whether S1P signal is involved in EGF-induced macropinocytosis.

In the present study, we have shown that the trans-activation of S1P<sub>1</sub> receptor (S1P<sub>1</sub>R) is necessary for EGF-induced macropinocytosis in COS7 cells. Molecular mechanism underlying EGF-induced trans-activation of S1P<sub>1</sub>R is described and its pathophysiological relevance is discussed herein.

## MATERIALS AND METHODS

**Cell culture**

COS7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO<sub>2</sub>. Cells were plated on 24-well plates (Corning, NY) or on glass-bottomed 35mm dishes (MatTek, Ashland, MA) 2 to 3 days prior to the experiment day.

**Plasmides and mutations**

mCherry-mSphK1(WT) and mCherry-kinase-dead-mSphK1(G81D) (mCherry-mSphK1(KD)) were constructed from GFP-mSphK1(WT) and GFP-mSphK1(KD) (14), respectively, by inserting into pmCherry-C1. These mouse SphK1 constructs are resistant to human/monkey SphK1 siRNA-mediated knockdown. mCherry-hSphK2(WT) and mCherry-kinase-dead-hSphK2(G248D) (mCherry-hSphK2(KD)) were constructed from GFP-hSphK2(WT) and GFP-hSphK2(KD) (15), respectively, by inserting into pmCherry-N1. Site-directed mutagenesis was performed using QuikChange protocol for siRNA-resistant SphK2 silent mutant as described previously (15).

**Transfection of siRNA**

For RNA interference, the following oligonucleotides (Japan Bio Services, Saitama, Japan) were used: sense 5'-GGGCAAGCCUUGCAGCUCdTdT-3' and antisense 5'-GAGCUGCAAGGCCUUGCCdTdT-3' for SphK1, sense 5'-GCUGGGCUGUCCUUAACCUdTdT-3' and antisense 5'-AGGUUGAAGGACAGCCCAGCdTdT-3' for SphK2, sense 5'-CUGACUUCAGUGGUGUUCAdTdT-3' and antisense 5'-UGAACACCACUGAAGUCAGdTdT-3' for S1P<sub>1</sub>R, sense 5'-CUCUACGGCAGUGACAAAAdGdC-3' and antisense 5'-UUUUGUCACUGCCGUAGAGdCdT-3' for S1P<sub>2</sub>R and sense 5'-CCCUCUACUCCAAGAAAUAdCdA-3' and antisense 5'-UAUUUCUUGGAGUAGAGGGdGdC-3' for S1P<sub>3</sub>R. All the siRNA sequences of green monkey (COS7 cells) correspond to the ones identical with human. The siRNAs were transfected by using Lipofectamine RNAiMax reagent (Invitrogen Corp).

**Real-time quantitative reverse transcription-PCR**

Total RNA was extracted from COS7 cells that had been transfected with S1P<sub>1</sub>R-, S1P<sub>2</sub>R-, S1P<sub>3</sub>R-, SphK1-, SphK2- or control siRNA two days prior using the NucleoSpin kit (Macherey-Nagel). Eighteen hours prior to RNA extraction cells were incubated in serum free medium to match the conditions under which the macropinocytosis experiments were done. One µg of total RNA was then used to synthesize cDNA using the reverseTra Ace qPCR-RT kit (TOYOBO) according to the manufacturer's instruction. Quantitative PCR was performed in quadruplicates using the SYBR Premix Ex Taq kit (TaKaRa). The reaction and quantification was performed using the ABI prism 7000 qPCR machine (Applied Biosystems). Control and knockdown sample copy number was computed based on the standard DNA samples which were titrated to predetermined copy numbers. Monkey and human compatible primers (sense and antisense) were as follows: S1P<sub>1</sub>R: 5'-TGTTAGCTGTGCTCAACTCC-3' and 5'-GAATTTGCCAGCAGAGTCTC-3'; S1P<sub>2</sub>R: 5'-CGCCATTGTGGTGGAAAAC-3' and 5'-CCCAGAAACAGGTACATTGC-3'; S1P<sub>3</sub>R: 5'-ACCTGCAGCTTACTGGCCAT-3' and 5'-ATTGTGCAGGCAGTTCCAGC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-GCCATCAATGACCCCTTCATT-3' and 5'-TCTCGTCTCTGGAAGATGG-3' SphK1: 5'-CCAGGCCACTGTAGGGAAC-3' and 5'-CAGCTCCTTATCGGTGCTG-3'; SphK2: 5'-ATGAATGGACACCTTGAAGCAG-3' and 5'-CATGGCCTTAGCCCTGACCAG-3'.

**Macropinocytosis**

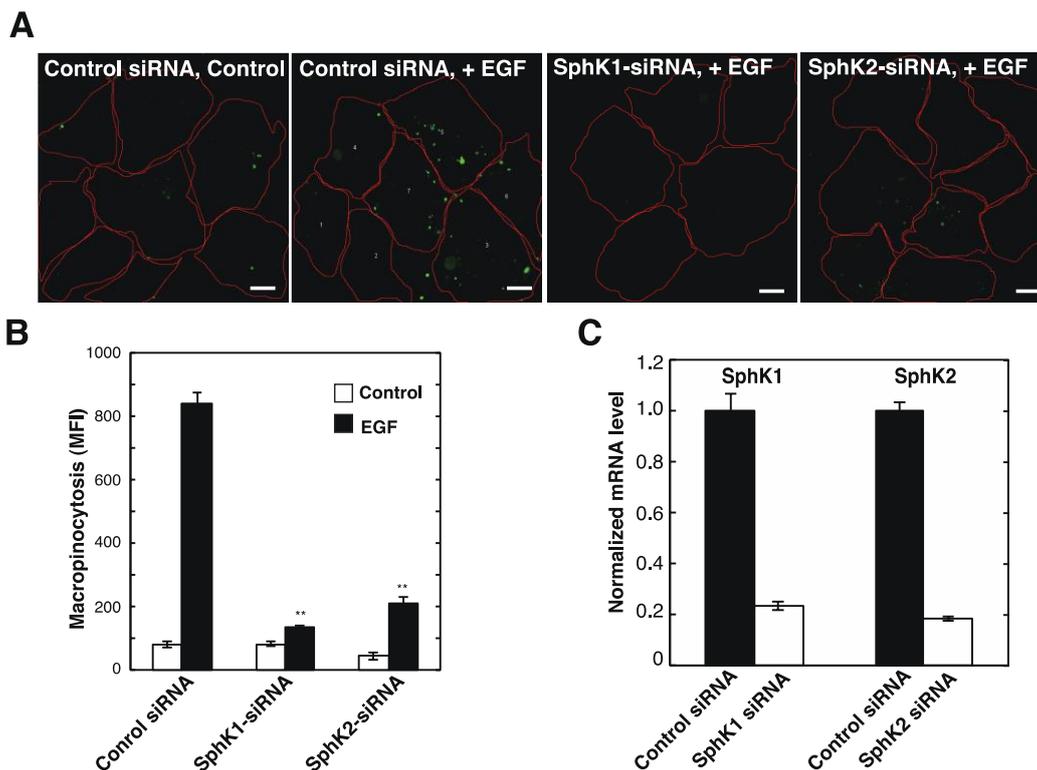
COS7 cells cultured on 24-well plate or glass-bottomed dishes were serum-starved for 18 hours in DMEM followed by a 1 hour incubation in 0.1% BSA-containing serum-free DMEM. Cells were then stimulated with 10 ng/ml EGF for 10 minutes in the presence of 0.5 mg/ml FITC-labelled 70-kDa dextran (Sigma). Cells were washed with phosphate-buffered saline (PBS) twice and then either fixed with 4% paraformaldehyde for imaging using LSM 700 confocal microscope or trypsinized and kept on ice for analysis by flow cytometry using the FACSCalibur (BD). The fluorescent intensity of the cells not treated with FITC-dextran was subtracted as background.

**Fluorescence resonance energy transfer (FRET) analysis**

S1P<sub>1</sub>R activation was detected by monitoring agonist-induced conformational change of the receptor using a one-molecular FRET technique as described previously (16). Rac1 activity was measured using a Raichu-Rac1 probe and ratiometric FRET analysis was performed in living cells as described in (17).

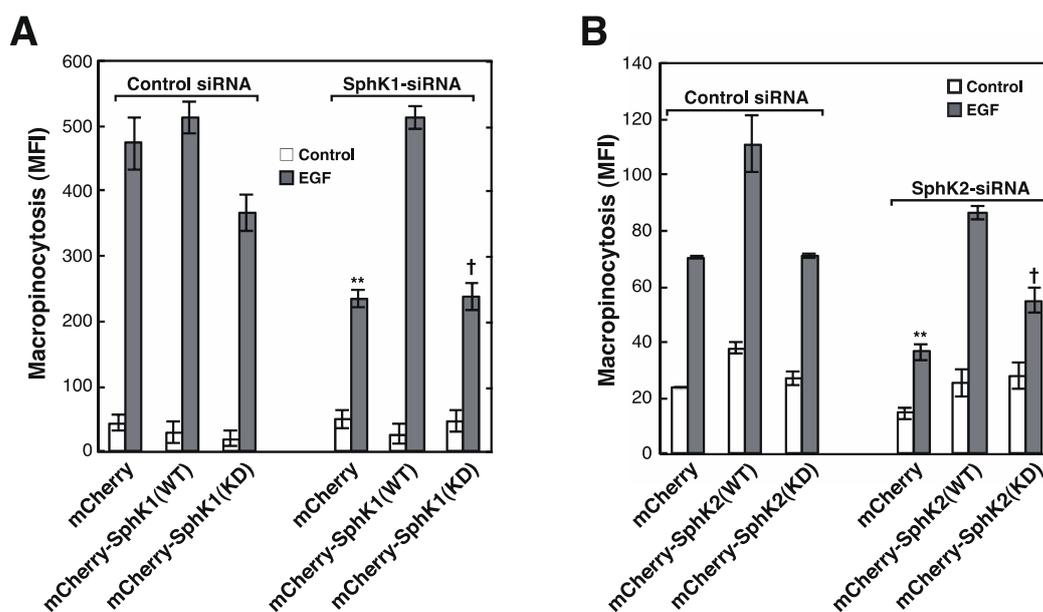
## RESULTS

Involvement of S1P signaling in EGF-induced macropinocytosis was tested first. Forty eight hours following transfection with control, SphK1 or SphK2 isoforms- small interference RNA (siRNA) and after 18 hours of serum starvation, COS7 cells were stimulated by 10 ng/ml EGF to enhance macropinocytosis. A comparison was made in the absence or presence of EGF treatment. EGF caused a robust enhancement of macropinocytosis as judged by the uptake of FITC labeled dextran (Fig. 1A). Interestingly, EGF-induced macropinocytosis was much weaker in SphK1- or SphK2-depleted cells compared to control siRNA-treated cells. A quantitative analysis of fluorescence of uptaken dextran using a flow cytometer showed that EGF-induced enhancement of macropinocytosis (10 fold in control siRNA) became much weaker (1.6 fold and 4 fold) in SphK1- or SphK2-siRNA treatment, respectively (Fig. 1B). Both siRNAs were verified for their ability to down-regulate each mRNA level (Fig. 1C). These results imply that sphingosine kinases may play a stimulatory role in EGF-induced macropinocytosis in COS7 cells.



**Figure 1. siRNA-mediated depletion of SphK1 or SphK2 expression in COS7 cells causes reduction in EGF-induced macropinocytosis.**

COS7 cells transfected with control, SphK1- or SphK2-siRNA were serum-starved and stimulated with 10 ng/ml EGF in the presence of FITC-labelled dextran in the culture medium for 10 min, washed, fixed and analyzed for macropinocytosis by confocal microscopy (A). Representative data from 3 independent experiments are shown. Scale bars, 10  $\mu$ m. Cells treated as in (A) were trypsinized and analyzed by flow cytometry. The extent of macropinocytosis was expressed as mean fluorescence intensity (MFI). Data are means  $\pm$  s.e. from at least three independent experiments carried out in triplicate. Statistical significance was analyzed by Student's t test (\*\*,  $P < 0.01$  versus control siRNA transfected and EGF-treated cells) (B). Reverse transcription was performed on total RNA isolated from COS7 cells. The DNAs were used for real-time PCR with primers specific for various SphKs and for housekeeping gene, GAPDH. Values for mRNA amounts were normalized to GAPDH expression and expressed relative to each subtype expression from control siRNA-treated cells and are the mean  $\pm$  s.e. from three independent experiments done in quadruplicate (C).

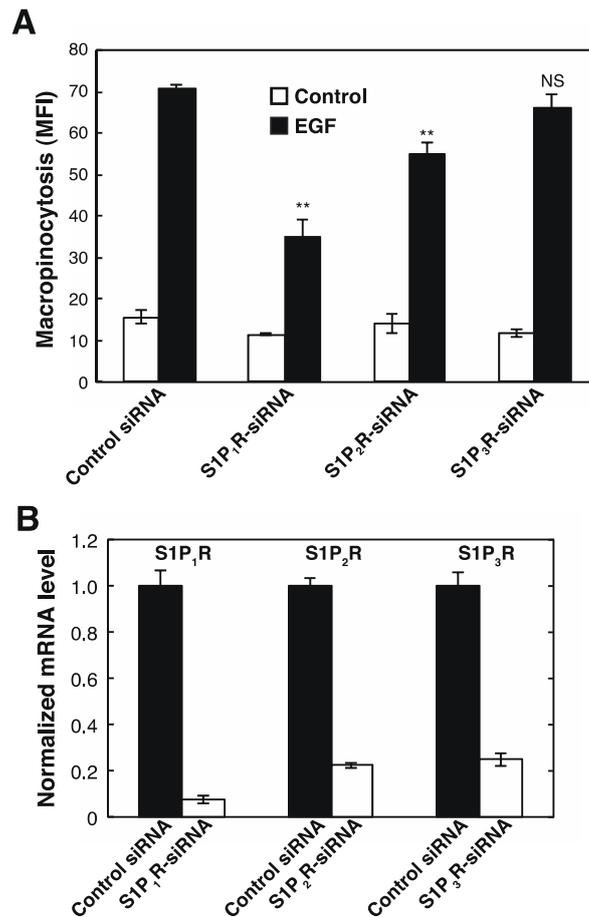


**Figure 2. SphK plays a role in EGF-induced macropinocytosis in an activity-dependent manner.**

COS7 cells cotransfected with SphK1-siRNA and siRNA-resistant mCherry-SphK1(WT) or mCherry-SphK1(KD) were treated with 10 ng/ml EGF and FITC-dextran for 10 min. Macropinocytosis was measured by flow cytometry using mCherry gating (A). COS7 cells cotransfected with SphK2-siRNA and siRNA-resistant mCherry-SphK2(WT) or mCherry-SphK2(KD) were treated with 10 ng/ml EGF and FITC-dextran for 10 min, followed by macropinocytosis assay (B). FITC-dextran signal only in mCherry expressing cells were analyzed by using appropriate gate in flow cytometry guaranteeing the expression of siRNA-resistant SphK1 or SphK2. Data are means  $\pm$  s.e. from at least three independent experiments carried out in triplicate. Statistical significance was analyzed by Student's *t* test (\*\*,  $P < 0.01$  against control siRNA and mCherry transfected and EGF-treated cells; †,  $P < 0.05$  against control siRNA and mCherry-SphK1(KD) transfected and EGF-treated cells in (A); \*\*,  $P < 0.01$  against control siRNA and mCherry transfected and EGF-treated cells; †,  $P < 0.05$  against control siRNA and mCherry-SphK2(KD) transfected and EGF-treated cells in (B)).

To further characterize the role of SphK in macropinocytosis, the effect of expression of mCherry-fused SphK1 and SphK2 was compared with mCherry only in each isoform-depleted COS7 cells. Suppression in EGF-induced macropinocytosis observed in SphK1-depleted cells was almost completely rescued by the expression of wild-type mCherry-SphK1 (mCherry-SphK1(WT)) as compared with only mCherry expression (Fig. 2A). Importantly, kinase activity-dead variants of mCherry-SphK1 (mCherry-SphK1(KD)) expression were shown to be significantly lower. Similar results were obtained in the SphK2-depleted cells. The diminished EGF-induced macropinocytosis in SphK2-depleted cells was overcome by mCherry-SphK2(WT) but not by mCherry-SphK2(KD) expression (Fig. 2B). These results suggest the importance of the catalytic activity of SphK, *i.e.*, production of S1P, rather than the protein/protein interaction for the EGF-induced enhancement of macropinocytosis.

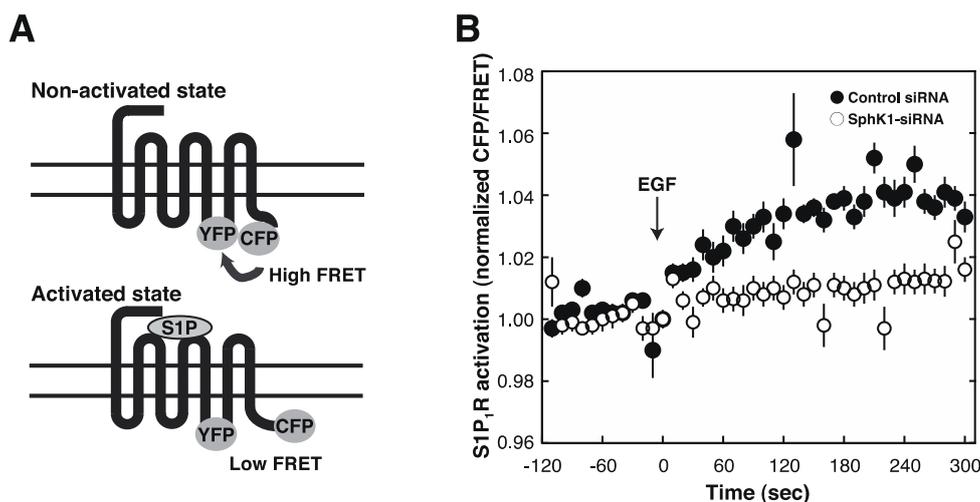
The finding that catalytic activity of sphingosine kinases is involved in mediating EGF induced macropinocytosis facilitated us to study the mode of actions of S1P in this phenomenon. Since many of the S1P actions were shown to be mediated by S1P receptors, we tested the three most ubiquitously expressed receptors, S1P<sub>1-3</sub>R out of S1P<sub>1-5</sub>R. S1P<sub>1</sub>R, S1P<sub>2</sub>R or S1P<sub>3</sub>R was depleted using the respective siRNA and EGF-induced macropinocytosis was analyzed by flow cytometer. The results suggested the involvement of S1PR in EGF-induced macropinocytosis in this cell line, with S1P<sub>1</sub>R being the major and S1P<sub>2</sub>R as a weaker but significant role in EGF-induced macropinocytosis (Fig. 3A). S1P<sub>3</sub>R seems to have a minimal role in this phenomenon. All the siRNAs were verified for their ability to down-regulate each mRNA level (Fig. 3B). These results indicate that receptor-mediated S1P action plays an important role in EGF-induced enhancement of macropinocytosis.



**Figure 3. Requirement of S1P<sub>1</sub>R in EGF-induced macropinocytosis in COS7 cells.**

COS7 cells transfected with control, S1P<sub>1</sub>R-, S1P<sub>2</sub>R- or S1P<sub>3</sub>R-siRNA were serum-starved and stimulated with 10 ng/ml EGF in the presence of FITC-labeled dextran for 10 min, washed, trypsinized and measured for macropinocytosis by flow cytometry. Statistical significance was analyzed by Student's *t* test (\*\*,  $P < 0.01$  versus control siRNA transfected and EGF-treated cells). NS, not significant (A). Reverse transcription was performed on total RNA isolated from COS7 cells. The DNAs were used for real-time PCR with primers specific for various S1PR subtypes and for housekeeping gene, GAPDH. Values for mRNA amounts were normalized to GAPDH expression and expressed relative to each subtype expression from control siRNA-treated cells and are the mean  $\pm$  s.e. from three independent experiments done in quadruplicate (B).

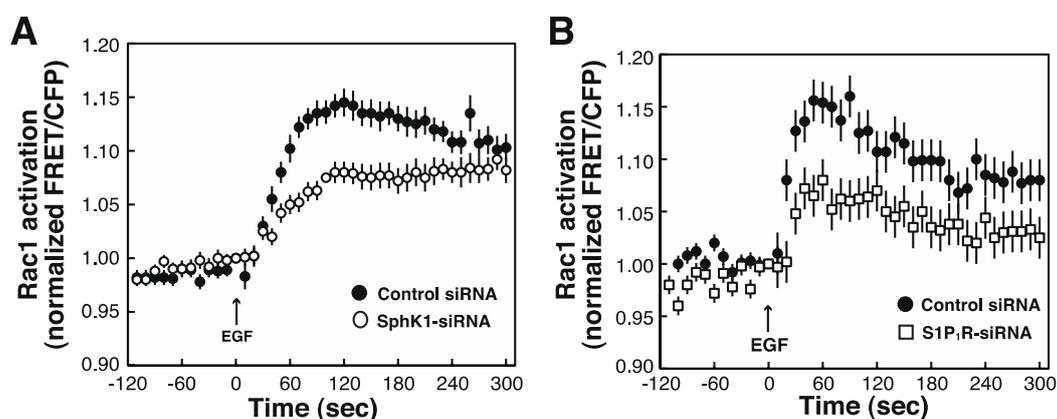
To prove the hypothesis that receptor-mediated S1P action is important during EGF-induced enhancement of macropinocytosis, it is essential to show the activation of S1P receptor during EGF stimulation. To address this issue, EGF-induced S1PR transactivation was studied next. Since knockdown of S1P<sub>1</sub>R showed the strongest effect in impairing EGF-induced macropinocytosis, this subtype was chosen for this experiment. To detect S1P<sub>1</sub>R activation, we employed a one-molecular FRET tool as previously reported (16), where the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were separately fused in the same S1P<sub>1</sub>R molecule (Fig. 4A). Conformational changes associated with receptor activation elicited by S1P caused an increase in normalized CFP/FRET ratio, which was counteracted by a S1P<sub>1</sub>R-selective blocker, W146 (16). Using cells transiently expressing this reporter probe, EGF stimulation actually induced conformational changes of S1P<sub>1</sub>R in a manner inhibitable by SphK1 knockdown (Fig. 4B). This indicates that EGF actually causes S1P<sub>1</sub>R transactivation through SphK-catalyzed production of S1P, which plays an important role in EGF-induced enhancement of macropinocytosis.



**Figure 4. EGF induces transactivation of S1P1R as judged by its conformational changes.**

COS7 cells cotransfected with S1P1R FRET probe and either control or SphK1 siRNA were serum-starved and stimulated with 10 ng/ml EGF (arrow) and analyzed for FRET in living cells. A representative emission ratio of the 2 fluorophores from 5 independent experiments is shown.

Macropinocytosis is an actin-dependent process initiated by surface membrane ruffles that is controlled by the Rho family of small GTPases, Rac1 (2, 3). To further strengthen our hypothesis that S1P/S1P1R signaling plays an important role in EGF-induced enhancement of macropinocytosis, we studied the effect of SphK1 or S1P1R depletion on EGF-triggered Rac1 activation, which is essential to macropinocytosis. Rac1 activation was assessed by a FRET-based, Raichu-Rac1 probe. In SphK1-depleted Raichu-Rac1 expressing cells Rac1 activation was significantly weaker than in control cells, as denoted by a smaller normalized FRET/CFP ratio for the SphK1 knockdown cell than corresponding controls (Fig. 5A).



**Figure 5. Requirement of receptor-mediated S1P signaling in EGF-induced Rac1 activation in COS7 cells.**

Inhibition of EGF-induced Rac1 activation by SphK1 siRNA (A) and S1P1R siRNA (B). COS7 cells cotransfected with Raichu-Rac1 and control and either SphK1- siRNA (A) or S1P1R-siRNA (B) were serum-starved and stimulated with 10 ng/ml EGF (arrow) and analyzed for ratiometric FRET in living cells. A representative emission ratio of the 2 fluorophores from 5 independent experiments is shown.

Similar results were obtained from S1P1R knockdown experiment (Fig. 5B). These results not only strongly suggest that SphK1 and S1P1R are involved in EGF induced macropinocytosis but also posits them as intermediate actors in a pathway that encompasses EGF initiated to Rac1 signaling cascades that leads to actin polymerization/membrane ruffling toward macropinocytosis.

## DISCUSSION

We have shown in the present study that either SphK1 or SphK2 knockdown causes inhibition of EGF-induced macropinocytosis in COS7 cells (Fig. 1). These siRNA effects were fully rescued by corresponding subtype of wild-type SphK but not by kinase-dead form of SphKs, suggesting the catalytic activity of SphK is essential for EGF-induced macropinocytosis (Fig. 2). Actually knockdown of S1P<sub>1</sub>R strongly reduced EGF effect, although S1P<sub>2</sub>R was also involved (Fig. 3). Our original probe for detecting S1P<sub>1</sub>R conformational changes could demonstrate for the first time that EGF causes conformational changes of S1P<sub>1</sub>R in a catalytic activity of SphK1-dependent manner (Fig. 4), suggesting that EGF elicits S1P<sub>1</sub>R trans-activation presumably through an autocrine fashion. It has been well known that Rac1 activation plays an important role in actin-rearrangement required for membrane ruffling and ultimately macropinocytosis induced by EGF. Our FRET analysis using Raichu-Rac1 probe showed that receptor-mediated S1P signaling is required for EGF-induced Rac1 full-activation in COS7 cells (Fig. 5). It has been previously suggested that both Akt and S1P<sub>1</sub>R activation are necessary for full activation of Rac1. In this scenario transactivation of S1P<sub>1</sub>R is caused by Akt-mediated phosphorylation at threonine 236 residue of the receptor (18). In the present study we have demonstrated that EGF-induced transactivation of S1P<sub>1</sub>R required SphK1 (Fig. 4B), indicating the involvement of S1P in this phenomenon. At present, it is uncertain whether the S1P<sub>1</sub>R FRET probe used in the present studies can detect conformational changes induced by phosphorylation at threonine 236 residue. It may be plausible to assume that both agonist (S1P)-dependent and Akt-mediated transactivation of S1P<sub>1</sub>R may transmit signal for the full activation of Rac1, which may account for the subtle changes between control and SphK1-knockdown cells in the later phases of EGF stimulation (Fig. 5A) presumably through the action of SphK2 (Figs. 1, 2). Another line of evidence from our laboratory showed that agonist-stimulated S1P<sub>1</sub>R provides the G $\beta\gamma$  subunits dissociated from G<sub>i</sub> on multivesicular endosomes. The G $\beta\gamma$  subunits directly binds to and activate PLEKHG2 and P-Rex1, GTP exchange factors for Rac1, promotes F-actin formation on the surface of multivesicular endosomes, which facilitates cargo sorting (8). We also showed that S1P<sub>1</sub>R is mainly involved in the EGF-induced macropinocytosis with S1P<sub>2</sub>R having a moderate but significant role (Fig. 3). In many cell types S1P<sub>1</sub>R and S1P<sub>2</sub>R couple with distinct G-proteins and have unique functions. Macropinocytosis is a complex process starting from engulfment of extracellular fluid, internalization and trafficked through fission and fusion events. Each S1P<sub>1</sub>R subtype may be involved in the particular step of the phenomena. Further studies are necessary to identify the S1P<sub>1</sub>R subtype-specific role in macropinocytosis using selective antagonists.

In the present study either SphK1 or SphK2 siRNA almost completely inhibited EGF-induced macropinocytosis, suggesting there is no redundancy in these SphK isozymes. One possibility for these results would be that these isozymes may be regulated in different spatio/temporal manners and be required at the different steps of macropinocytosis. Further studies are necessary to elucidate molecular mechanism underlying how and where these isozymes are activated by EGF stimulation and exert their role in EGF-induced macropinocytosis. In addition to physiological processes, cancer cells utilize macropinocytosis as a means of metabolic adaptations where macropinocytic uptake of extracellular proteins, and their further degradation provides them with enough amino acids that fuel cancer cell metabolism and tumor growth (19). Our present findings that receptor-mediated-S1P signal plays an indispensable role in EGF-induced macropinocytosis may provide a new therapeutic tool for signaling molecule-targeted cancer treatment.

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