Requirement of autophosphorylated tyrosine 992 of EGF receptor and its docking protein phospholipase Cγ1 for membrane ruffle formation

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Abstract Stimulation of the epidermal growth factor receptor (EGFR) produces membrane ruffles through the small G protein Rac1; however, the signaling pathway from EGFR to Rac1 has not yet been clarified. Here, we show that autophosphorylation of EGFR at tyrosine 992 is required for EGF-induced membrane ruffle formation in CHO cells. Signaling from the autophosphorylated tyrosine 992 appears to be mediated by phospholipase C (PLC) γ1. Furthermore, activation of Rac1 by EGF is inhibited by a PLC inhibitor. These results, taken together, suggest that autophosphorylation of EGFR at tyrosine 992 and the subsequent PLCγ1 activation transduce the signal to Rac1 to induce membrane ruffle formation.

Key words: Epidermal growth factor receptor; Tyrosine phosphorylation; Phospholipase Cγ1; Membrane ruffle; Rac1; CHO cell

1. Introduction

Epidermal growth factor (EGF) specifically binds to and activates the EGF receptor (EGFR), resulting in stimulation of cellular responses such as cell proliferation [1,2] and membrane ruffle formation [3–5], the latter of which is critical for cell movement. Upon EGF stimulation, the activated EGFR is autophosphorylated at specific residues in its cytoplasmic region [6,7]. Five tyrosine residues at positions 992, 1068, 1086, 1148, and 1173 are major autophosphorylation sites [8–11]. Autophosphorylated tyrosine residues serve as binding sites for docking proteins with Src homology 2 (SH2) and phosphotyrosine-binding domains to transduce signals from EGFR to downstream signaling pathways [12,13]. These proteins include enzymes, such as Src, phospholipase Cγ1 (PLCγ1), and phosphatidylinositol 3-kinase that are phosphorylated and thereby activated, and adapter molecules, such as Shc, Grb2, and Nck that interact with and activate downstream effectors [12–14].

Interaction of Grb2 with the autophosphorylated tyrosine 1068 of EGFR through its SH2 domain triggers the sequential activation of the small GTPase Ras and mitogen-activated protein kinase cascade, which in turn promotes cell proliferation [15–17]. On the other hand, another small GTPase, Rac1, is the key molecule in the signaling pathway of EGF-dependent membrane ruffle formation [18]. We have recently reported that phosphatidylinositol 4-phosphate [PI(4)P] 5-kinase, which produces the versatile phospholipid phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] by phosphorylation of PI(4)P at the D5′ position, is directly activated by ADP-ribosylation factor (ARF) and that the ARF-dependent PI(4)P 5-kinase activation functions as a downstream signaling pathway of Rac1 in membrane ruffle formation [19]. Although, thus, the signaling pathway downstream of Rac1 in EGF-induced membrane ruffle formation becomes clearer, the molecular mechanism of signal transduction from EGFR to Rac1 remains to be elucidated.

In the present study, we first investigated which tyrosine residue of EGFR, of five major autophosphorylation sites, is required for EGF-induced membrane ruffle formation, and then examined which protein(s) or enzyme(s) play a role as a docking molecule for the autophosphorylated EGFR in membrane ruffle formation. The results obtained suggest that the autophosphorylation of EGFR at tyrosine 992 is required for EGF-induced membrane ruffle formation and that the docking enzyme PLCγ1 mediates the signaling from the autophosphorylated EGFR to Rac1.

2. Materials and methods

2.1. Plasmids and antibodies

cDNAs for wild-type EGFR (WT-EGFR) and its mutants, termed SM992, SM1068, SM1086, SM1148, and SM1173, in which the single tyrosine residues at positions 992, 1068, 1086, 1148, and 1173 were replaced by phenylalanine, were constructed and subcloned into vector pRc/CMV as previously described [20]. Additional EGFR mutants, QM992 and F5, in which four tyrosine residues other than tyrosine 992 and the five tyrosine residues described above were re-

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Abbreviations: ARF, ADP-ribosylation factor; CHO, Chinese hamster ovary; DAG, diacylglycerol; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PI(4)P, phosphatidylinositol 4-phosphate; PKC, protein kinase C; PLCγ1, phospholipase Cγ1; SH2, Src homology domain 2; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

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placed by phenylalanine, were similarly prepared. cDNA for the myc-tagged dominant negative PLCγ1 fragment, designated PLC-Z, which consists of SH2 and SH3 domains (amino acids 517–901) of PLCγ1 [21], was subcloned into the pcDNA3-myc vector, a generous gift of Dr. K. Nakayama. pEF-BOS-Rac1G12V and pEF-BOS-Rac1T17N were generous gifts of Dr. K. Kaibuchi. Mouse monoclonal anti-EGFR antibodies for Western blotting (6F1) and immunofluorescence (R1), both of which detect WT-EGFR and its mutants used in this study, were purchased from MBL and Santa Cruz, respectively. Rabbit polyclonal anti-PLCγ1 antibody was a generous gift of Dr. C.H. Heldin. Anti-phospho extracellular signal-regulated kinase (ERK), anti-ERK, and mouse monoclonal and rabbit polyclonal anti-myc antibodies (9E10 and A-14, respectively) were obtained from Santa Cruz. Rat monoclonal anti-hemagglutinin (HA) (3F10) and mouse monoclonal anti-Rac1 antibodies were from Boehringer Mannheim and Transduction Laboratories, respectively.

2.2. Cell culture, transfection of plasmids and preparation of stable cell clone

Chinese hamster ovary (CHO) cells were maintained in a culture medium comprising Ham/F-12 nutrient medium (Sigma), 10% fetal bovine serum (Nissui), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco BRL). Cells were cultured at 8.0×10^4 cells/ml on glass coverslips in 8-well chamber plate (Nunc) in the same medium at 37°C for 30 h, and transfected with 1 μg/ml of each expression plasmid DNA in Opti-MEM-I (Gibco BRL) using Lipofectamine PLUS (Invitrogen). After incubation at 37°C for 3 h, these cells were further cultured at 37°C for 12 h, starved of serum for 24 h, and then used for experiments unless otherwise noted. In an experiment for Rac1 activation, clones that stably express QM992 were selected using G418 (Nakarai) after CHO cells were transiently transfected with pRc/CMV-QM992. Stable clones were cultured in the culture medium described above at 37°C for 36 h, starved of serum for 36 h, and then used for experiments.

2.3. Immunofluorescence microscopy

Parental control and transfected CHO cells cultured on glass coverslips were treated as described in the figure legends, fixed, permeabilized, and blocked as previously reported [19]. Cells were then stained for proteins to be visualized with appropriate antibodies and FITC- and/or Cy3-labeled secondary antibodies (Jackson ImmunoResearch Laboratories). F-actin was visualized with rhodamine phalloidin (Molecular Probes). The immunofluorescently stained cells were imaged using Zeiss Axiovert S100 (Plan Neofluar 40×/0.75 NA) equipped with a Yokogawa confocal scanner unit CSU21.

2.4. Western blotting

Western blotting analysis was performed as described previously

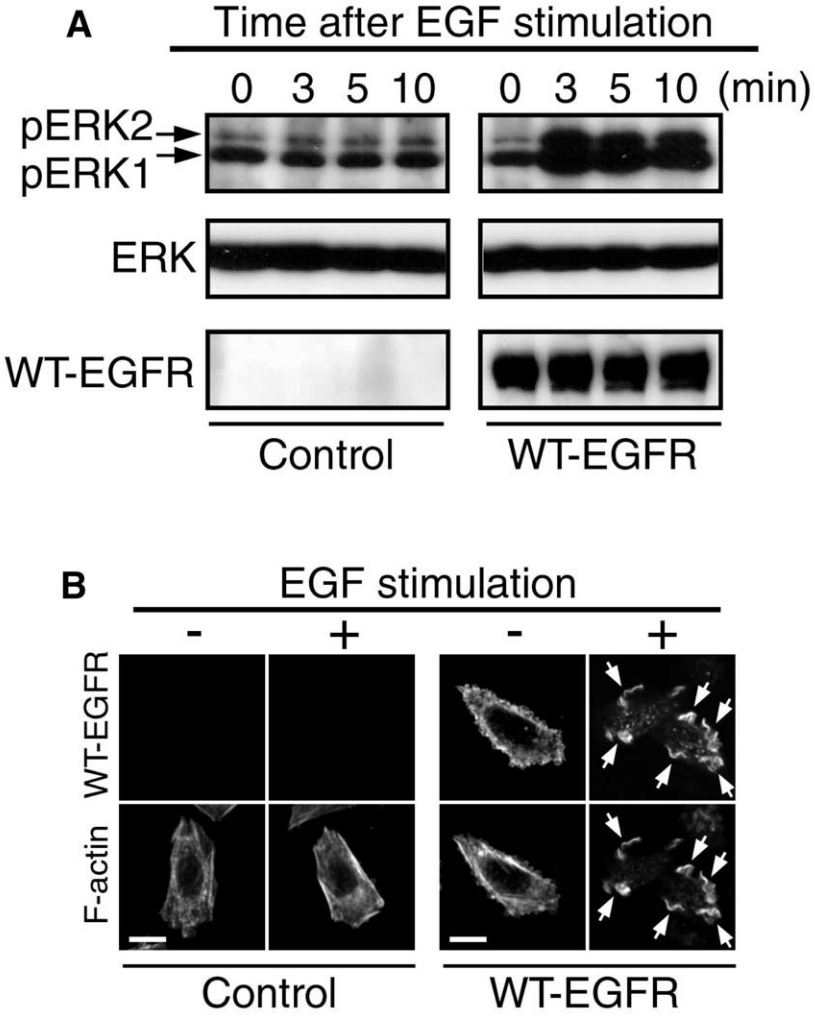

Fig. 1. Induction of EGF-dependent ERK activation and membrane ruffle formation by expression of WT-EGFR in CHO cells. Parental control and WT-EGFR-expressing cells were stimulated with 30 ng/ml of EGF at 37°C. After the indicated times of EGF stimulation, phosphorylation of ERK1/2 (pERK1/2), the ERK protein, and the expression level of WT-EGFR were detected by Western blotting probed with the anti-phospho ERK, anti-ERK, and anti-EGFR (6F1) antibodies, respectively (A). Cells transiently expressing WT-EGFR were also stimulated without or with 30 ng/ml of EGF at 37°C for 5 min, and stained for EGFR with the R1 antibody and for F-actin (B). Arrows in B indicate the membrane ruffles where WT-EGFR was colocalized with F-actin. Scale bars, 10 μm.

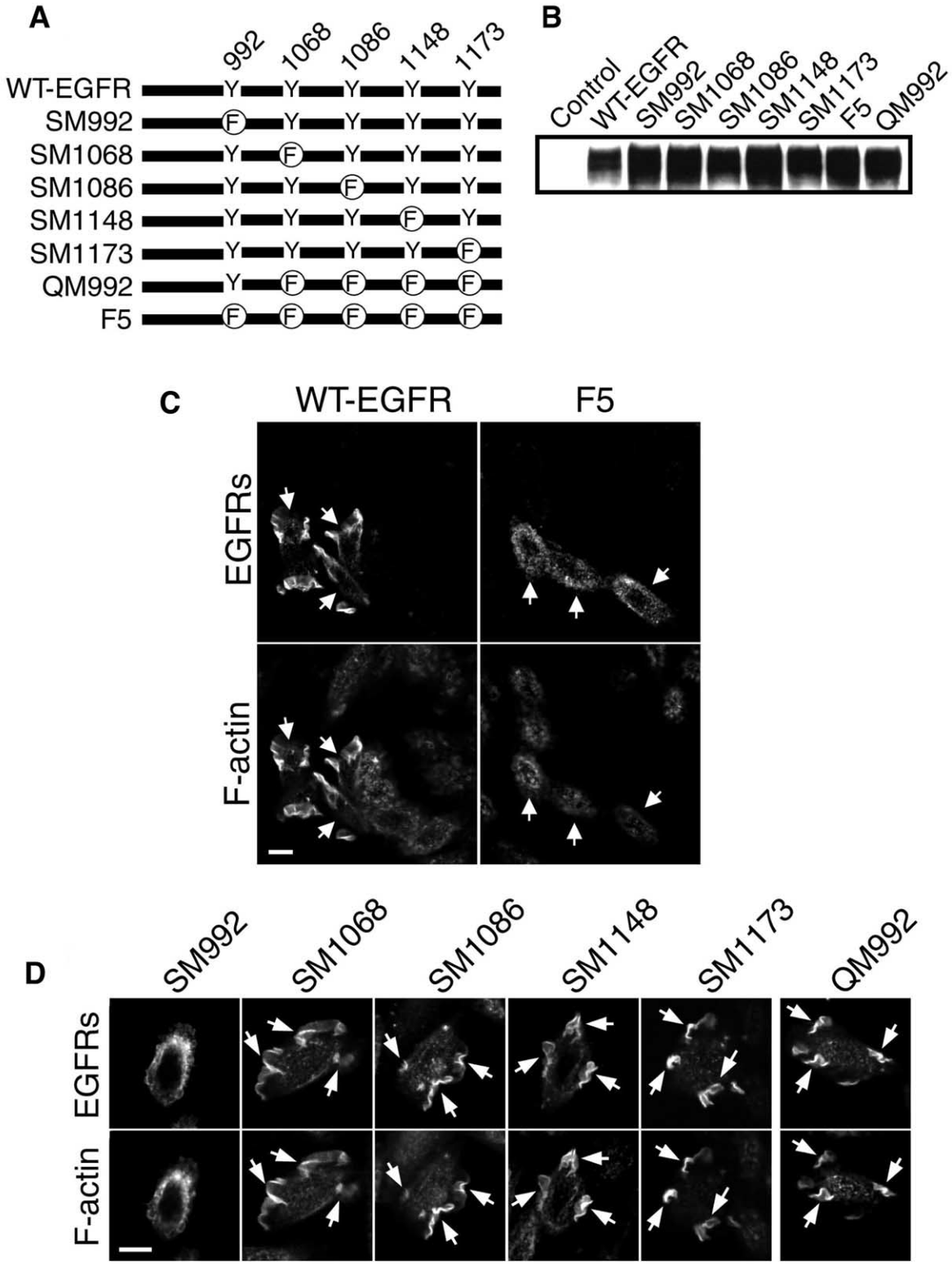

Fig. 2. Requirement of autophosphorylation of EGFR at tyrosine 992 for EGF-induced membrane ruffle formation. WT-EGFR and its mutants, in which phenylalanine (F) was substituted for tyrosine (Y), are schematically illustrated (A). These EGFRs were transiently expressed in CHO cells as described in Section 2, and their expression levels were determined by Western blotting probed with the anti-EGFR (6F1) antibody (B). Cells expressing WT-EGFR and mutated EGFRs were stimulated with EGF and stained for EGFRs and F-actin as described in Fig. 1B (C,D). Arrows in C and D indicate transfected cells and the membrane ruffles where EGFRs and F-actin were colocalized, respectively. Scale bars, 10 μm.

[22]. Briefly, proteins in cell lysates were separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with antibodies specific for each protein to be detected, and the reactive proteins were visualized by an ECL system (Amersham Pharmacia Biotech).

2.5. Assay of Rac1 activity

After CHO cells stably expressing QM992 were treated as described in the legend to Fig. 4C and lysed with lysis buffer consisting of 50 mM Tris, pH 7.5, 100 mM NaCl, 1% NP-40, 10 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 10 mg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride, activated Rac1 was precipitated with GST-CRIB and detected with the anti-Rac1 antibody as previously reported [23].

3. Results and discussion

CHO cells are very useful mammalian cells to investigate the functional domain of EGFR by means of techniques of molecular biology with EGFR mutants since they are devoid of endogenous EGFR. In this study, therefore, we employed these cells to investigate whether autophosphorylation of EGFR at specific tyrosine residues is required for EGF-induced membrane ruffle formation. When CHO cells transiently expressing WT-EGFR were stimulated with EGF, ERK1/2, which are normally activated through the autophosphorylated tyrosine 1068 of EGFR [15–17], were significantly activated as detected by their phosphorylation, while parental control cells did not respond to EGF (Fig. 1A), demonstrating that expressed EGFR is activated and autophosphorylated in an EGF-dependent manner. Similarly, EGF-dependent membrane ruffle formation was observed in CHO cells transiently expressing WT-EGFR, but not in parental control cells (Fig. 1B). Thus, CHO cells are useful to investigate the functional tyrosine residue(s) of EGFR for EGF-dependent membrane ruffle formation.

It has been reported that five major tyrosine residues at positions 992, 1068, 1086, 1148 and 1173 in the cytoplasmic region of EGFR are autophosphorylated when EGFR is activated by EGF stimulation [8–11]. To examine the requirement of EGFR autophosphorylation for EGF-induced membrane ruffle formation and to identify its site, various EGFR mutants illustrated in Fig. 2A, in which phenylalanine was substituted for tyrosine, were constructed and expressed in CHO cells. Expression levels of EGFR mutants were comparable to that of WT-EGFR (Fig. 2B). Although cells expressing WT-EGFR, but not surrounding non-transfected control cells, triggered EGF-induced membrane ruffle formation (Fig.

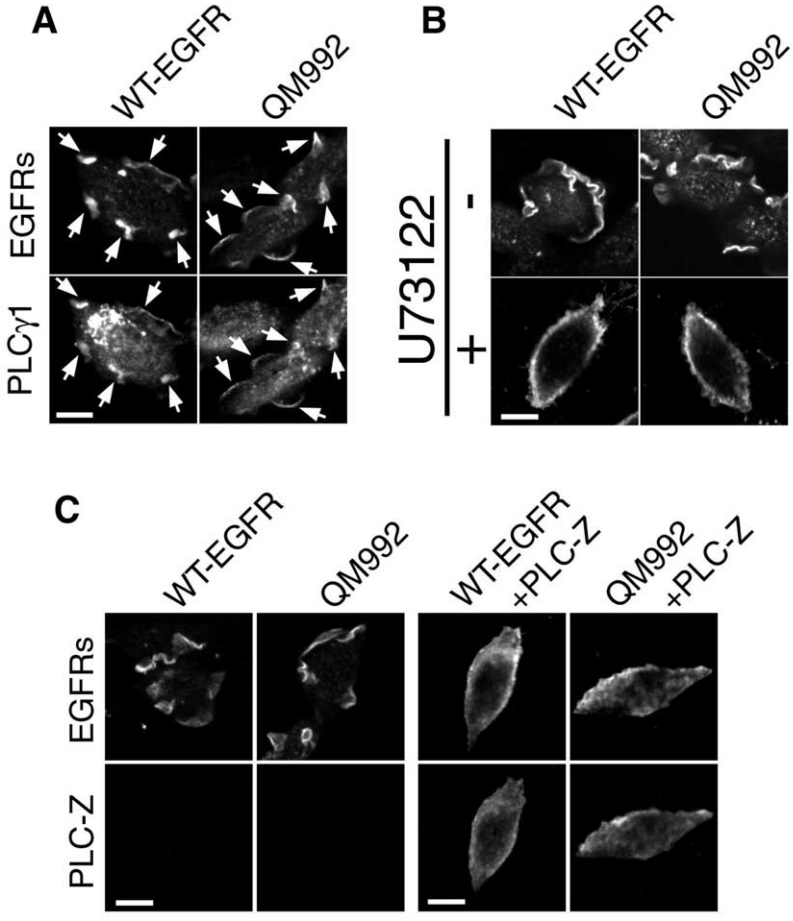

Fig. 3. Involvement of PLCγ1 in membrane ruffle formation mediated through the autophosphorylated tyrosine 992 of EGFR. CHO cells transiently expressing WT-EGFR or QM992 were stimulated with EGF as in Fig. 1B, and stained for expressed EGFRs and endogenous PLCγ1 with R1 and rabbit polyclonal anti-PLCγ1 antibodies, respectively (A). CHO cells expressing WT-EGFR or QM992 were treated with 0.5% DMSO (U73122−) or 5 μM U73122 in 0.5% DMSO (U73122+) at 37°C for 15 min, stimulated with EGF and then stained for EGFRs as in Fig. 1B (B). CHO cells coexpressing myc-tagged PLC-Z with WT-EGFR or QM992 were stimulated with EGF as in Fig. 1B and stained for EGFRs with the R1 antibody and for PLC-Z with the polyclonal anti-myc (A-14) antibody (C). Arrows in A indicate the membrane ruffles where EGFRs and PLCγ1 were colocalized. Scale bars, 10 μm.

2C), cells expressing F5, the EGFR mutant in which five tyrosine residues of major autophosphorylation sites were mutated, as well as surrounding non-transfected control cells, failed to do so (Fig. 2C), indicating that autophosphorylation of tyrosine residue(s) in EGFR is essential for this cellular response. In this experiment, about 70% of cells transfected with WT-EGFR responded to EGF stimulation, and more than 90% of cells transfected with F5 failed to do so (data not shown). Interestingly, cells expressing SM992, in which tyrosine 992 was mutated, did not induce membrane ruffles in response to EGF stimulation, whereas cells expressing the other four EGF mutants, SM1068, SM1086, SM1148 and SM1173, produced membrane ruffles (Fig. 2D). These results suggest that autophosphorylation of EGFR at tyrosine 992 is indispensable for EGF-induced membrane ruffle formation. Consistent with this idea, cells expressing QM992, in which four tyrosine residues other than tyrosine 992 were mutated, responded to EGF, resulting in the formation of membrane ruffles (Fig. 2D). Again, 65–80% of cells transfected with SM1068, SM1086, SM1173 and QM992 responded to EGF stimulation, and 90–95% of cells transfected with SM992 did not (data not shown). Furthermore, the EGF-induced membrane ruffles in these cells were inhibited by the specific EGFR kinase inhibitor AG1478 (data not shown), supporting the notion described above.

It has been reported that PLCγ1 binds to the autophosphorylated tyrosine 992 of EGFR through its SH2 domain [17,24], is activated by EGFR [25], and colocalizes with EGFR at membrane ruffles [26]. These reports led us to speculate that PLCγ1 is an EGFR-proximal signaling molecule involved in the signaling pathway of membrane ruffle formation. In support of this hypothesis, endogenous PLCγ1 colocalized with transiently expressed WT-EGFR and QM992 at membrane ruffles formed by EGF stimulation (Fig. 3A), and membrane ruffle formation in these cells was inhibited by the specific inhibitor of PLC, U73122 [27,28] (Fig. 3B). Furthermore, it was found that the dominant negative PLCγ1 fragment, PLC-Z [21], prevented EGF-induced membrane ruffle formation in cells expressing WT-EGFR and QM992 (Fig. 3C). These results, taken together, provide evidence that PLCγ1 functions as a downstream signaling molecule of the autophosphorylated tyrosine 992 of EGFR in the signaling pathway of EGF-induced membrane ruffle formation.

It is well known that the small GTP-binding protein Rac1 is the key molecule in EGF-induced membrane ruffle formation [18]. A question raised here is the relationship between PLCγ1

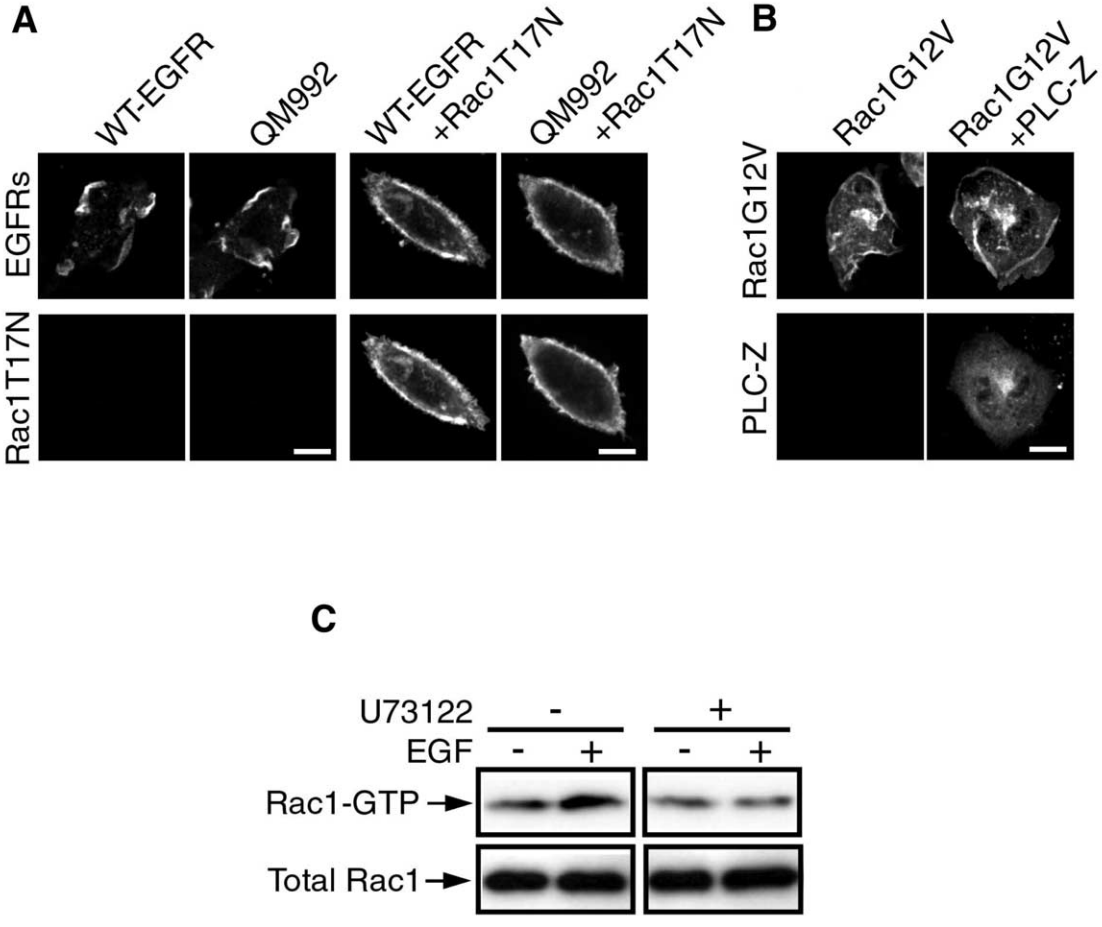

Fig. 4. PLCγ1 functions as an upstream signaling molecule of Rac1 in the signaling pathway of EGF-induced membrane ruffle formation. The dominant negative Rac1T17N tagged with HA was coexpressed with WT-EGFR or QM992, and cells were stimulated with EGF as in Fig. 1B and stained for EGFR with the R1 antibody and for HA-tagged Rac1T17N with the anti-HA antibody (A). The constitutively active mutant Rac1G12V tagged with HA was coexpressed with myc-tagged PLC-Z in CHO cells, and cells were stained for HA-tagged Rac1G12V and myc-tagged PLC-Z with anti-HA and anti-myc antibodies, respectively (B). Cells stably expressing QM992 were treated without or with 20 μM U73122 at 37°C for 15 min, stimulated without or with 30 ng/ml EGF at 37°C for 1 min, and then the active form of Rac1 was assessed as described in Section 2.

and Rac1 in the signaling pathway of membrane ruffle formation. To address this issue, we employed dominant negative and constitutively active Rac1 mutants, Rac1T17N and Rac1G12V, respectively. Rac1T17N inhibited EGF-induced membrane ruffle formation in cells expressing WT-EGFR and QM992, whereas PLC-Z failed to inhibit Rac1G12V-induced membrane ruffle formation, which was more intensive than that induced by EGF stimulation (Fig. 4A,B). Furthermore, it was found that the activation of Rac1 by EGF stimulation of cells stably expressing QM992 was inhibited by U73122. These results, taken together, provide evidence that PLCγ1 is the signaling molecule upstream of Rac1 in the signaling pathway of EGF-induced membrane ruffle formation.

Thus, we demonstrated that phosphotyrosine of EGFR at position 992 is indispensable for EGF-induced membrane ruffle formation and that PLCγ1 is the EGFR-proximal docking enzyme linking to Rac1 in this cellular response. Although, at present, the molecular mechanism by which PLCγ1 transduces the signal to Rac1 remains to be clarified, Rac1 activation may be mediated by protein kinase C (PKC) activation by the PLCγ1 product diacylglycerol (DAG). This idea is consistent with the recent report by Grimmer et al. that the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a potent activator of PKC, activates Rac1 and the TPA-induced membrane ruffles are inhibited by Rac1T17N in A431 cells [29].

We have recently reported that production of $PI(4,5)P_2$ by the action of PI(4)P 5-kinase is important for EGF-induced membrane ruffle formation, probably to reorganize the actin cytoskeleton at the cell periphery [19]. In the present study, the results provide evidence that activation of PLCγ1 that hydrolyzes $PI(4,5)P_2$ is also required for EGF-induced membrane ruffle formation. As was shown in Fig. 4, activation of PLCγ1 appears to be required for Rac1 activation, probably through PKC activation by the PLCγ1 product DAG as was discussed above. On the other hand, PI(4)P 5-kinase seems to function as a signaling molecule downstream of Rac1. These findings suggest that a temporary change in $PI(4,5)P_2$ level triggered by degradation by PLCγ1 and subsequent production by PI(4)P 5-kinase at the limited area of the plasma membrane is important for the signaling pathway of EGF-dependent membrane ruffle formation.

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