Biochemical and Biophysical Research Communications 421 (2012) 651–657

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Rab21 attenuates EGF-mediated MAPK signaling through enhancing EGFR internalization and degradation

Xi Yang, Yanquan Zhang, Shan Li, Chunxiao Liu, Zhe Jin, Yinyin Wang, Fangli Ren, Zhijie Chang *

State Key Laboratory of Biomembrane and Membrane Biotechnology, School of Medicine, National Engineering Laboratory for Anti-Tumor Therapeutics, Tsinghua University, Beijing 100084, China

ARTICLE INFO

Article history:
Received 6 April 2012
Available online 14 April 2012

Keywords:
Rab21
EGFR
Degradation
Internalization
MAPK signaling

ABSTRACT

Epidermal growth factor (EGF) receptor (EGFR) signal transduction is regulated by endocytosis where many Rab proteins play an important role in the determination of the receptor recycle or degradation. In an effort to better understand how EGF signaling is regulated, we examined the role of Rab21 in regulation of the degradation and signal transduction of the EGFR. Using a transient expression protocol in HEK293T and HeLa cells, we found that Rab21 enhanced the degradation of EGFR through accelerating its internalization in both EGF-independent and EGF-dependent manners. We further demonstrated that Rab21 interacted with EGFR by immunoprecipitation experiments. Interestingly, we observed that overexpression of Rab21 attenuated EGF-mediated mitogen-activated protein kinase (MAPK) signaling by inducing EGFR degradation. Taken together, these data suggest that Rab21 plays a negative role in the EGF-mediated MAPK signaling pathway.

1. Introduction

Epidermal growth factor (EGF) receptor (EGFR) is a member of EGF receptor family that controls the proliferation, differentiation, cell survival, migration and adhesion of cells [1,2]. In the absence of ligand binding, EGFR exists on cell membrane as a monomeric, single membrane spanning protein. Binding of EGF to the EGFR induces dimerization of the receptor and subsequent trans-autophosphorylation of tyrosine residues in the cytoplasmic domain of EGFR. These tyrosine residues serve as docking sites for Src-homology-2 (SH2)- or phosphotyrosine binding (PTB)-domain-containing downstream effector proteins. The activated effectors, alone or in concert with other effectors, initiate multiple intracellular signaling pathways [3,4].

In addition to the signal events initiated by ligand binding, the EGFR activation also triggers the internalization of ligand–receptor complexes which are primarily transferred to the early endosomes [5]. The receptor complexes then can either be recycled back to the plasma membrane through recycling endosomes or be directed to multivesicular bodies (MVBs) and subsequently sorted to late endosomes/lysosomes for degradation [6].

The magnitude and duration of signal transduction through EGFR are regulated by ligand-mediated endocytosis and postendocytic trafficking of activated receptors [7]. A wealth of evidence, stemming from the observation that blockade of EGFR internalization decreases EGF-induced mitogen-activated protein kinase (MAPK) pathways, has suggested that endocytic trafficking of activated EGFR plays a critical role in establishing and controlling EGFR signaling pathways [8–10]. It has been demonstrated that the internalized EGF–EGFR complex maintains an ability for EFGR to generate cell signaling from endosomes [11]. However, when EGFR is sorted into the luminal vesicles of MVBs, the acidic compartments cause the dissociation of the EGF–EGFR complex, resulting in EGF signal termination [12]. Therefore, either abrogation of ubiquitylation mediated EGFR endocytosis or interruption of EGFR localization into the late endosomes causes a delay in EGFR degradation and a sustained MAPK signaling [13,14].

Rab GTPases constitute the largest family of small GTPases, which control membrane identity and vesicle budding, uncoating, motility and fusion through the recruitment of effector proteins. To date, over 60 different Rab proteins have been identified and each is believed to be specifically associated with a particular organelle or an endocytic pathway [15,16]. One of the important pathways regulated by many Rabs has been implicated in EGF signaling where EGFR undergoes endocytic trafficking. In particular, Rab5, a well-characterized Rab mediating entry of the receptor into the early endosomes, plays positive roles in MAPK signal transduction and EGF-induced cell proliferation [17], while, Rab7, a regulator of the distal stages of the endocytic pathway, enhances EGFR degradation and attenuates EGF induced MAPK signaling [18]. Many studies have suggested that Rab21 functions similarly with its close homologue Rab5 [19], however, the role of Rab21 in regulation of EGF signaling remains elusive.

* Corresponding author. Fax: +86 10 62773624.
E-mail address: zhijiec@tsinghua.edu.cn (Z. Chang).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.04.049

Rab21, a 25-kDa protein, was originally identified in a canine MDCKII cell library [20] and reported to be ubiquitously expressed [19]. Although Rab21 was reported to play important roles in phagocytosis, macropinocytosis and tubular endosome genesis [21–23], most studies revealed its function in the early endocytic pathway due to its predominant localization in early endosomes. These reports proposed that Rab21 regulates the dynamic process of sorting cargoes from early endosomes to late endosomes/lysosomes. A recent study suggested that Rab21 regulates cell adhesion through controlling endosomal traffic of β1-integrins [24]. Interestingly, it was reported that cells overexpressing wild-type and mutant Rab21 had numerous endocytic defects, including an inability to efficiently deliver the EGF–EGFR complexes from early endosomes to late endosomes/lysosomes [25]. However, the exact mechanism of how Rab21 influences the EGFR stability and signal transduction remains to be determined. In this report, we present evidence that Rab21 interacts and co-localizes with EGFR. We find that Rab21 enhances EGFR degradation and thereby attenuates EGF-mediated MAPK signaling.

2. Materials and methods

2.1. Antibodies and other reagents

Anti-Myc (9E10), anti-GFP (FL), anti-phospho-Erk1/2 (E-4) and anti-EGFR (1005) were purchased from Santa Cruz Biotechnology. Anti-β-actin (AC-15) and anti-Flag (M2) antibodies were from Sigma–Aldrich. Fluorescent secondary antibodies (goat anti-rabbit IgG) were purchased from Jackson ImmunoResearch Laboratories. The HRP-conjugated secondary antibodies for ECL were from Pierce. Human EGF was purchased from Merck. Cyclohexamide (CHX) was from Amresco.

2.2. Plasmid construction

pEGFP-C1/EGFP-Rab21 was a gift from Dr. Arwyn T. Jones (Cardiff University, Cardiff, UK). pEFNeo/Rab21-Myc plasmid was constructed using PCR based strategy from pEGFP-C1/EGFP-Rab21. The Elk-1 luciferase reporter plasmid and the GFP-Erk2 construct were kindly provided by Dr. Akihiko Yoshimura (Kyushu University, Fukuoka, Japan).

2.3. Reverse transcription-PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen). Total RNA (2 μg) for each sample was used as template for one-step reverse transcription-PCR analysis (Takara Biotechnology). Primers used for amplification of EGFR were 5′-GAGAGGAGAACTGCCAGAA-3′ (forward) and 5′-GTAGCATTTATGGAGAGTG-3′ (reverse). The sequences of GAPDH primers are 5′-ACCCAGAAGACTGTGGATGG-3′ (forward) and 5′-AGGGGTCTACATGGCAACTG-3′ (reverse). Twenty-five cycles of the PCR reactions were performed at the condition: denature, 94 °C, 30 s; annealing, 55 °C, 30 s; elongation, 72 °C, The PCR products were resolved on a 1% agarose gel stained with ethidium bromide. GAPDH was amplified as an internal control.

2.4. Cell culture and transfection

HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco), 100 mg/ml penicillin and 100 mg/ml streptomycin. All the cells were kept at 37 °C in a 5% CO_2-containing atmosphere. Cells were transfected with indicated plasmids using VigoFect (Vigorous).

2.5. Immunoprecipitation assay and immunoblotting

HEK293T cells cultured in 60-mm dishes were transfected with the indicated plasmids. For cross-linking, cells were fixed at 37 °C for 30 min with 1% formaldehyde. The fixation reaction was quenched with 1 ml ice-cold 1.25 M glycine/PBS. Cells were lysed in 800 μl cell lysis buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 10 mM $MgCl_2$, 0.5% NP40, 10% glycerol, 1 mM DTT, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstantin) and sonicated at 4 °C for 30 s at level 2 (Ultrasonic Processor, Sonics). Immunoprecipitation was done in 750 μl whole-cell lysates incubated with 2 μg of indicated antibody and 30 μl protein G Sepharose beads (Santa Cruz Biotechnology) at 4 °C for 8 h. The beads were washed 4 times with cell lysis buffer and precipitates were eluted with 2× SDS–PAGE sample buffer and analyzed by Western Blotting.

2.6. EGFR degradation assay

HEK293T or HeLa cells cultured in 12-well plates were transfected with the indicated plasmids. Control or experimental cells were serum-starved and then treated with 100 ng/ml EGF or 50 μg/ml cyclohexamide for different times. At the end of each time point, the cells were washed with PBS and then lysed in the lysis buffer as indicated above. The lysates were subjected to SDS–PAGE and immunoblotting with appropriate antibodies.

2.7. Immunostaining and confocal microscopy

HeLa cells were seeded on cover slips in 6-well plates the day before transfection. Two micrograms of pEGFP-C1/EGFP-Rab21 or pEGFP-C1 were transfected per well. Twenty-four hours after transfection, cells were washed with PBS, fixed for 20 min at room temperature with 4% paraformaldehyde in PBS, and perforated for 10 min with 0.3% Triton X-100 in PBS. Cells were blocked with 10% fetal bovine serum (Gibco) for 1 h at room temperature. Followed by incubation with rabbit anti-EGFR antibodies at 37 °C for 1 h, cells were incubated with anti-rabbit antibodies conjugated with TRITC at 37 °C for 1 h. Cover slips were mounted in a glycerol-based anti-fade mounting medium. Images were obtained with a confocal laser scanning microscope (OLYMPUS BX61).

2.8. Luciferase assay

HEK293T cells were plated in 24-well plates the day before transfection. Fifty nanograms of reporter plasmid PFA-Elk-1 and PFR-luciferase together with 5 ng of the internal control plasmid pRL-TK were transfected per well. 0.5 μg of plasmids for the expression of pEFneo/Myc or pEFneo/Rab21-Myc were co-transfected as indicated per well. Twenty-four hours after transfection, cells were serum-starved overnight. Following the starvation, cells were stimulated by EGF (100 ng/ml) while the control cells were cultured in the DMEM in the absence of serum for 8 h. The Elk-1 luciferase activity was assayed using the Dual Luciferase Assay System (E1910; Promega). The results were expressed as mean ± SD from three independent experiments.

3. Results

3.1. Rab21 interacts with EGFR

To test whether Rab21 regulates EGF signaling, we first examined the possibility that Rab21 may interact with EGFR. For this purpose, we co-expressed Myc-Rab21 and Flag-EGFR in HEK293T cells followed by an immunoprecipitation assay. A Western Blot

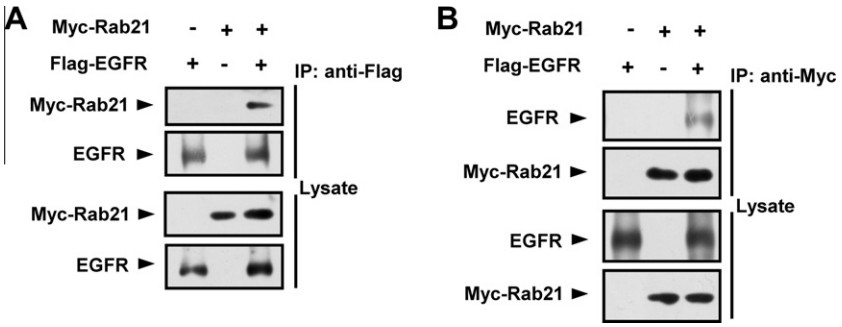

Fig. 1. Rab21 interacts with EGFR. (A) Myc-Rab21 is co-immunoprecipitated with Flag-EGFR. HEK293T cells were co-transfected with Flag-EGFR and Myc-Rab21. Immunoprecipitation was performed using an anti-Flag antibody and the precipitants were detected by a Western Blot with indicated antibodies. (B) Flag-EGFR is co-immunoprecipitated with Myc-Rab21. HEK293T cells were co-transfected with Flag-EGFR and Myc-Rab21. Immunoprecipitation was performed using an anti-Myc antibody and the precipitants were detected.

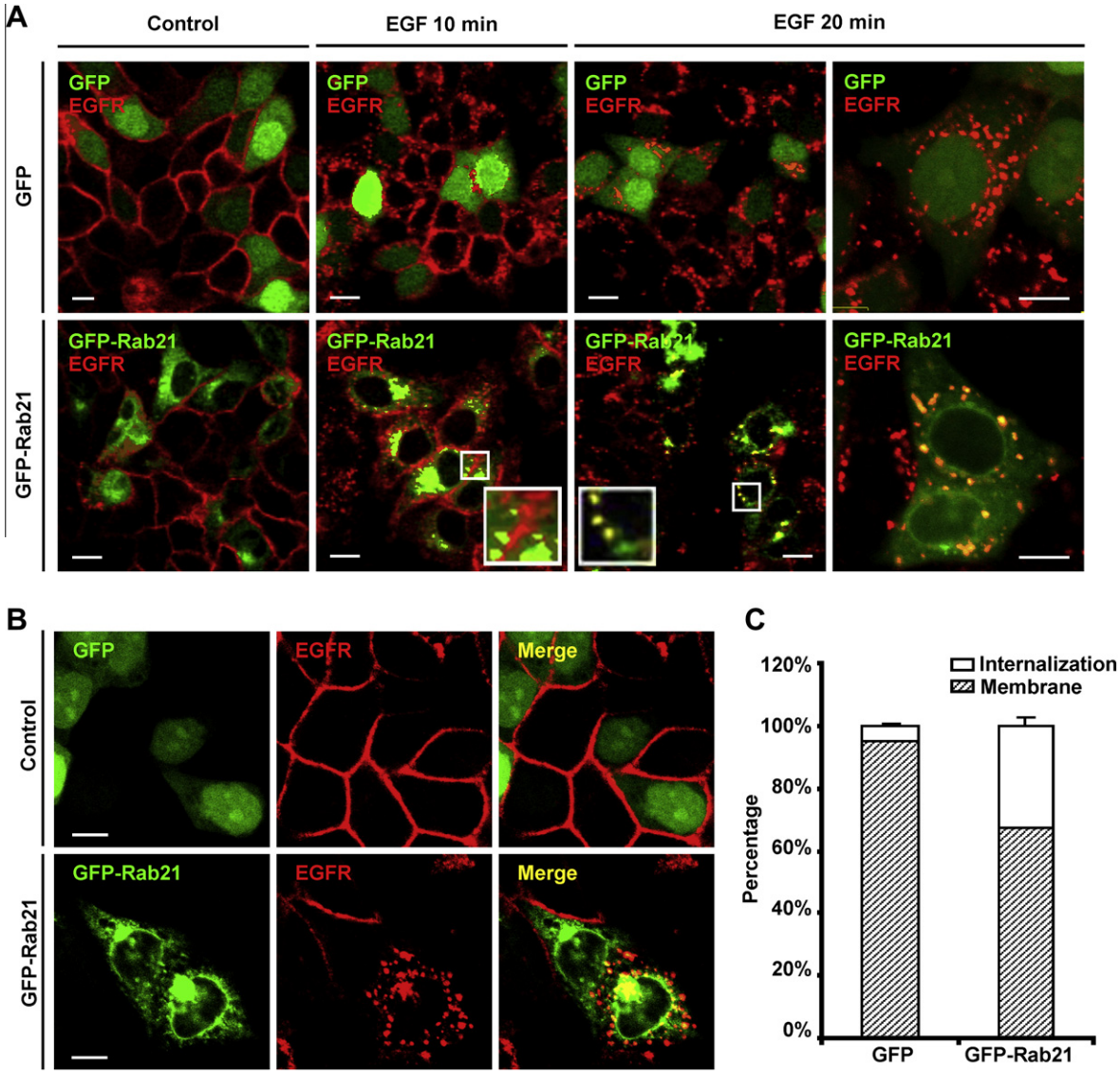

Fig. 2. Rab21 co-localizes with EGFR and enhances EGFR internalization. (A) Rab21 co-localizes with EGFR. GFP or GFP-Rab21 was expressed in HeLa cells. Twenty-four hours later, cells were treated with EGF (100 ng/ml) for 0, 10 and 20 min. The cells were stained with a rabbit polyclonal anti-EGFR antibody (red). The co-localization of GFP-Rab21 and EGFR in the vesicle structures is shown as yellow color. Bar 10 μm. (B) Rab21 changes EGFR distribution. HeLa cells transfected with GFP or GFP-Rab21 were stained with a rabbit polyclonal anti-EGFR antibody (red). Bar 10 μm. (C) Rab21 enhances EGFR internalization. The graph shows a quantitative analysis of the percentage of the cells with EGFR internalized based on cells with green fluorescence. Error bars represent standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analysis indicated that Myc-Rab21 was co-immunoprecipitated with Flag-EGFR by using an anti-Flag antibody when both Myc-Rab21 and Flag-EGFR were co-expressed while no specific band was observed in the Myc-Rab21 or Flag-EGFR individually expressed cells (Fig. 1A), suggesting that Myc-Rab21 interacts with Flag-EGFR. In a reciprocal co-immunoprecipitation experiment, we observed that the Flag-EGFR was precipitated by Myc-Rab21 protein (Fig. 1B). These results suggest that Rab21 interacts with EGFR in the cells.

3.2. Rab21 co-localizes with EGFR and enhances EGFR internalization

To investigate whether the interaction of Rab21 and EGFR occurs physiologically in the intact cells, we performed an immunostaining assay to observe the co-localization of GFP-Rab21 with endogenous EGFR. The results showed that most EGFR distributed on the cell membrane (Fig. 2A, first panel, red) and GFP-Rab21 was localized in the cytoplasm in the absence of EGF (Fig. 2A, first panel, green). However, after EGF treatment for 10 min, EGFR underwent a process of endocytosis and distributed into vesicles where GFP-Rab21 was located (Fig. 2A, second panel). Furthermore, when the cells were treated with EGF for 20 min, we observed more clear co-localization of GFP-Rab21 with endogenous EGFR in vesicle structures (Fig. 2A, third panel, see the enlarged box), which were in distinct perinuclear structures close to the nucleus (Fig. 2A, last panel). These results suggest that Rab21 and EGFR co-localize in the internalized vesicles induced by EGF.

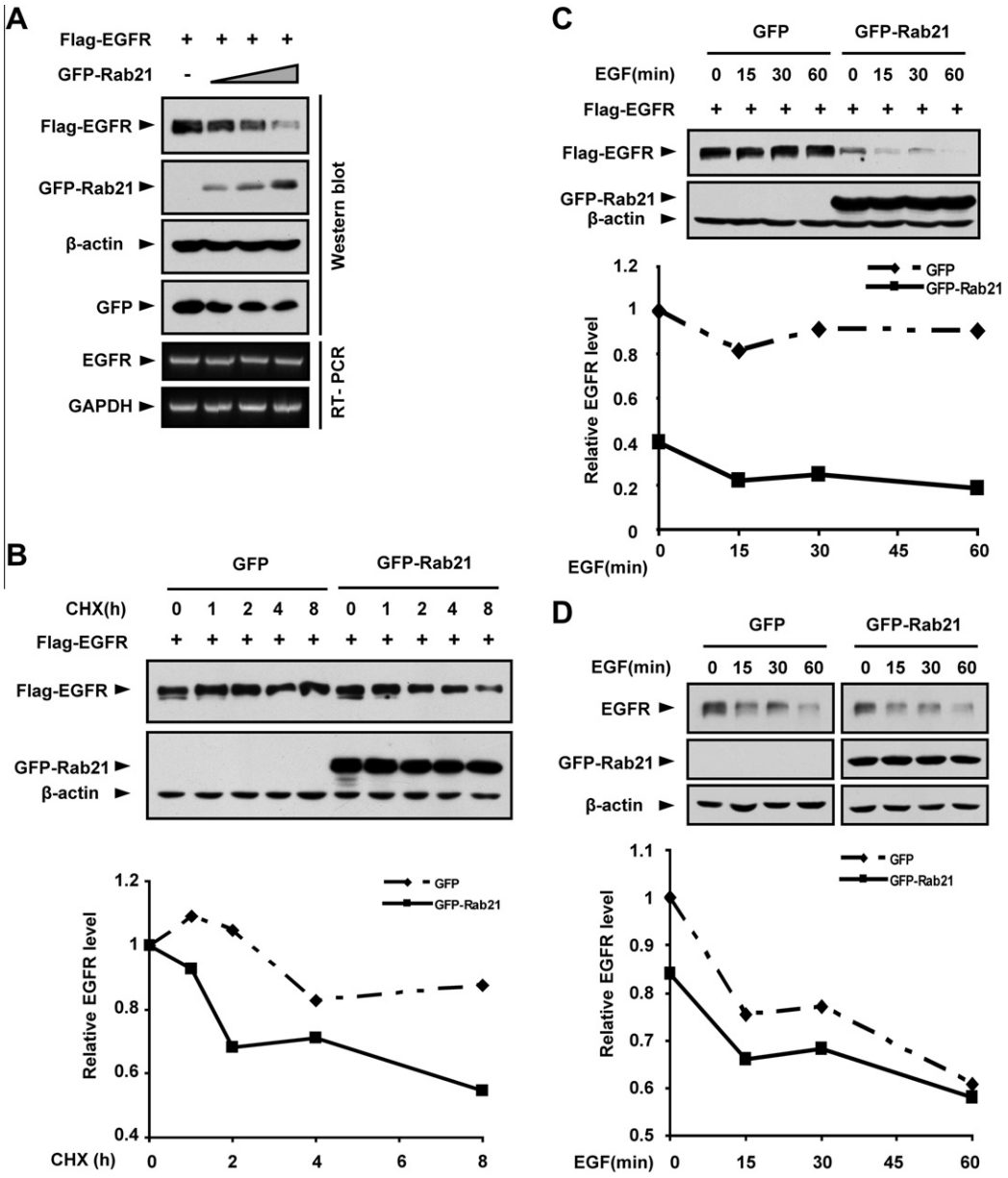

Fig. 3. Rab21 accelerates EGFR degradation. (A) Rab21 degrades EGFR in HEK293T cells. An increased amount of GFP-Rab21 was co-transfected with Flag-EGFR in HEK293T cells, and Flag-EGFR protein level was analyzed by Western Blotting. The mRNA level of EGFR was analyzed by RT-PCR. (B) Degradation of EGFR is accelerated by Rab21. One microgram of Flag-EGFR was transfected with 0.1 μg of GFP or GFP-Rab21 in HEK293T. Twenty-four hours later, cells were treated with CHX (50 μg/ml) for 0, 1, 2, 4 and 8 h. The bottom panel depicts the relative intensity of the EGFR bands. (C) Rab21accelerates EGF-dependent EGFR degradation in HEK293T cells. 1 μg of Flag-EGFR was transfected with 0.5 μg of GFP or GFP-Rab21 in HEK293T. Twenty-four hours later, cells were treated with EGF (100 ng/ml) for 0, 15, 30, 60 min. The bottom panel depicts the relative intensity of the EGFR bands. (D) Rab21accelerates EGF-dependent EGFR degradation in HeLa cells. GFP or GFP-Rab21 was transfected in HeLa cells. 24 h later, cells were treated with EGF (100 ng/ml) for 0, 15, 30, 60 min. The bottom panel depicts the relative intensity of the EGFR bands.

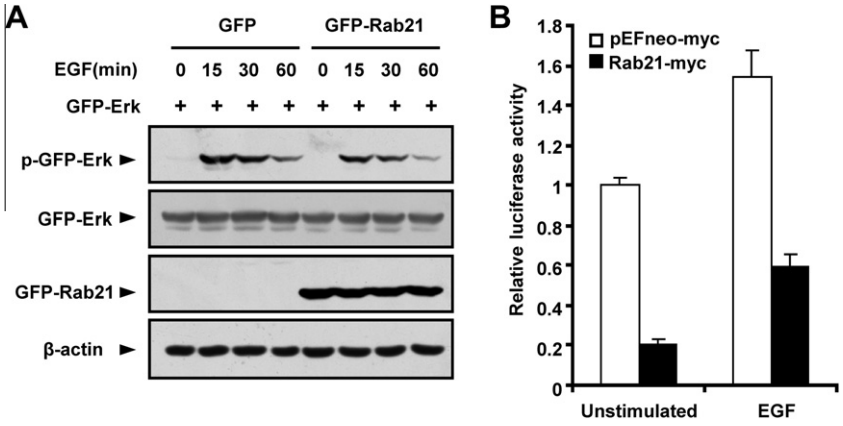

Fig. 4. Rab21 inhibits EGF-mediated MAPK signaling. (A) Rab21 inhibits EGF-activated Erk phosphorylation. GFP-Erk was transfected with GFP or GFP-Rab21 in HEK293T. Cells were treated with EGF (100 ng/ml) for indicated times. Protein levels of GFP-Erk and phosphorylated GFP-Erk were analyzed by Western Blotting. (B) Rab21 inhibits EGF-induced Elk transcriptional activity. Luciferase assays were performed using HEK293T cells with transient expression of Myc-Rab21 or pEFneo/Myc, under transfection of Elk-1 responsive reporter plasmid PFA-Elk-1, PFR-Luciferase and pRL-TK (as an internal control). After serum-starvation for overnight, cells were treated with or without EGF (100 ng/ml) for 8 h. Results presented are from one experiment assayed in triplicate.

To study whether Rab21 affects the internalization of EGFR, we observed the endogenous EGFR distribution in Hela cells under overexpression of GFP-Rab21. A confocal microscopy observation showed that EGFRs were mainly localized to the cell surface in the control cells (Fig. 2B, upper panel), however, a fraction of EGFRs internalized into the vesicles to be localized with GFP-Rab21 when GFP-Rab21 was overexpressed, (Fig. 2B, lower panel). In order to quantify the change of EGFR distribution pattern, we counted the number of EGFR-internalized cells based on cells with green fluorescence. A statistics analysis revealed that 5.16% of the cells underwent internalization of EGFR in GFP expressing cells, but 32.55% of cells showed internalized EGFR in GFP-Rab21 expressing cells (Fig. 2C). These data suggest that Rab21 enhances the internalization of EGFR.

3.3. Rab21 promotes EGFR degradation

The association between Rab21 and EGFR in the internalized EGFR vesicles promoted us to speculate that Rab21 might affect the stability of EGFR. To address this hypothesis, Flag-EGFR and GFP-Rab21 were co-transfected into HEK293T cells, and the protein levels were analyzed by a Western Blot analysis. The result showed that an increasing amount of overexpressed Myc-Rab21 correlated to a decreasing level of Flag-EGFR protein in the HEK293T cells (Fig. 3A). In contrast, the mRNA levels of EGFR in these cells showed no significant change, suggesting that the negative correlation of the two protein levels is due to a protein degradation rather than a transcriptional mechanism (Fig. 3A). To further confirm the role of Rab21 on the degradation of EGFR, we examined the half-life of Flag-EGFR in the absence or presence of GFP-Rab21 by blocking protein synthesis with cyclohexamide (CHX). The result showed that overexpression of GFP-Rab21 significantly shortened the half-life of Flag-EGFR (Fig. 3B). These data suggest that Rab21 is a potent factor enhancing EGFR degradation. Since our study was performed under a condition that EGFR was not stimulated by its ligand, EGF, we concluded that Rab21 enhances degradation of unliganded EGFR.

As EGFR undergoes degradation after EGF stimulation, we further measured the protein levels of Flag-EGFR in the HEK293T cells transfected with GFP or GFP-Rab21 after different times of EGF stimulation. Our results indicated that GFP-Rab21 obviously accelerated the EGF-induced degradation of Flag-EGFR (Fig. 3C). It appeared that overexpression of GFP-Rab21 significantly reduced the basal levels of Flag-EGFR (Fig. 3C, bottom panel). To confirm the above results, we transiently transfected GFP or GFP-Rab21 into HeLa cells and treated them with EGF for varying periods of time (0–60 min), and then examined the levels of the endogenous EGFR protein. We found that overexpression of GFP-Rab21 down-regulated the endogenous EGFR protein level (Fig. 3D, top panel). The quantification analysis determined by densitometry clearly indicated that overexpression of GFP-Rab21 resulted in an increased rate of EGFR degradation (Fig. 3D, bottom panel). Taken together, these data suggest that Rab21 accelerates EGF-stimulated EGFR degradation.

3.4. Rab21 attenuates EGF-stimulated MAPK signaling

Based on the observations that Rab21 interacted and co-localized with EGFR and enhanced the degradation of the receptor, we sought to determine whether Rab21 affects the downstream signaling pathway mediated by EGFR. To this end, we transfected GFP-Rab21 together with GFP-Erk2 into HEK293T cells and tested the ligand-induced activation of the MAPK pathway using an antibody specifically against the phosphorylated Erk proteins. We found that the activation of Erk by EGF stimulation was attenuated in the cells under overexpression of GFP-Rab21 (Fig. 4A), suggesting that Rab21 reduces the duration of EGFR activation indicated by Erk phosphorylation.

Activation of EGF signaling can be demonstrated by Elk-1 activity. To measure the affect of Rab21 on EGFR signaling, we finally used an Elk-1 luciferase reporter, which responses to EGF stimulation in activation of Elk-1. Our data showed that EGF stimulated the activation of the reporter in HEK293T cells with transfection of control vector, however, EGF almost totally lost its activity on the luciferase reporter in the presence of Myc-Rab21, (Fig. 4B). These data, consistent with the Erk phophorylation results, confirm that Rab21 plays a negative role in EGF mediated MAPK signaling.

4. Discussion

The internalization and degradation of the EGFR are important for the regulation of EGFR signal transduction. Although many Rabs were identified as critical proteins that regulate trafficking during various endocytic stages, it remains unclear whether Rab21 functions in the process. In this report, we found the small GTPase Rab21 regulates EGFR trafficking. We have provided a set of evidence that Rab21 interacts with EGFR and promotes its degradation by accelerating the internalization process. Consequently, we

observed that Rab21 attenuates EGF mediated MAPK signaling. Our study provided novel evidence that Rab family proteins are critical for the maintenance of EGFR stability through an entocytosis-induced degradation mechanism.

Several studies indicated that the endocytosis of EGFR occurs in either a way dependent of ligand stimulation (known as trafficking of liganded EGFRs) or a way without ligand stimulation (known as trafficking of unliganded EGFRs) [26,27]. In most cells, EGFR can be constitutively internalized in the absence of EGF. After internalization, receptors are mainly recycled back to cell surface. Because the recycling rate is several times higher than the internalization rate, the bulk of EGFRs are present at the cell surface. Rab5, the best-studied Rab protein regulating EGFR trafficking, was reported to attenuate EGFR presentation on cell surface without EGF stimulation. Expression of activating mutants of Rab5 (Rab5Q78L) leads to a redistribution of unliganded EGFR from the plasma membrane to an endosomal compartment [28]. In this study, we demonstrated that Rab21, which is more closely homologous to Rab5 than other Rabs, plays a similar role in regulation of unliganded EGFR degradation. Like Rab5 (Q78L), overexpression of Rab21 shunts endosomal EGFR toward a degradation pathway in an EGF-independent manner. Consistently, we observed that the interaction of Rab21 with EGFR occurred in the absence of EGF, indicating that Rab21 is associated with unliganded EGFR. We speculate that Rab21 disrupts the balance between the recycling and the internalization of receptors, and results in the un-stabilization of unliganded EGFR. Interestingly, our data demonstrated that Rab21 also promoted the internalization of liganded EGFR. It appeared that Rab21 may regulate the degradation of EGFR in both the EGF dependent and independent manners. In this case, we speculate that Rab21 also regulates the basal levels of the EGFR.

A subset of Rab proteins, including Rab4, Rab5, Rab11, Rab22b and Rab7, have been shown to regulate EGFR endocytic trafficking and thereafter the EGFR signaling [29]. Due to their differential localizations to intracellular membranes, different Rabs function on distinct process of EGFR trafficking. For example, expression of inhibitory form of Rab5 blocks MAPK signal activated by EGF activation and thereafter inhibits EGF induced cyclinD1 transcription, suggesting that Rab5 promotes EGFR activation. In contrast, inhibition of active Rab7 results in a sustained MAPK signaling, suggesting that Rab7 inhibits EGF signaling [13,17]. Since both Rab5 is located in the early endosomes and Rab7 is located in the late endosomes/lysosomes, these observations implies that regulation of MAPK signaling induced by EGF occurs in both early and late endosomes. A previous study indicated that Rab21 is predominantly localized to the early endocytic pathway and functions in early endosomes dynamics [25]. Therefore, we proposed that the regulation of EGFR by Rab21 occurs in the early endosomes. However, it appears that Rab21 has an opposite role in regulation of EGFR compared with Rab5. Our data indicated that Rab21 inhibits EGF-mediated MAPK signaling while others reported that Rab5 enhances the signaling. We speculate that Rab21 may be responsible for sorting the EGF–EGFR complex from early endosomes to late endosomes/lysosomes, while Rab5 is responsible for entry of EGF–EGFR complex to early endosomes. Detailed function of Rab21 in endocytic trafficking of the EGF–EGFR complex requires further analyses with endosome makers tracing the receptor trafficking routes after EGF stimulation.

Another question for the role of Rab21 in regulation of EGFR stability is the specificity. A previous report indicated that Rab21 also regulates trafficking and signaling of integrins [24]. In this study, we found that Rab21 regulates EGFR trafficking through an interaction of EGFR with Rab21. However, it is unclear how the regulation is specified in a particular bioprocess. We envision that a specific adaptor protein may determine the specificity of the interaction of Rab21 with EGFR, thereafter to prefer the regulation of EGF signaling to the other signalings. It is possible that a guanine nucleotide exchange factor or GTPase-activating protein functions as such an adaptor [30]. More evidence should be provided to define the specificity in our future studies.

In conclusion, we have provided a set of evidence that Rab21 plays an important role in negatively regulating EGF signaling by mediating the internalization and degradation of EGFR. Our study expanded our knowledge for understanding the regulation of endocytosis of EGFR by different Rab proteins.

Acknowledgments

We thank Dr. Akihiko Yoshimura (Kyushu University, Fukuoka, Japan) for Elk-1 luciferase reporter plasmids and GFP-Erk2 constructs, and Dr. Arwyn T. Jones (Cardiff University, Cardiff, UK.) for EGFP-Rab21 constructs. This work was supported by grants from 973 Project of China (2011CB910502), the NSFC (31071225), National Key Project (2012AA021703), and the Tsinghua Internal Research Funds (2011THZ02-20, 2011Z01011).

References

[1] R.N. Jorissen, F. Walker, N. Pouliot, T.P.J. Garrett, C.W. Ward, A.W. Burgess, Epidermal growth factor receptor: mechanisms of activation and signalling, Experimental Cell Research 284 (2003) 31–53.
[2] J. Mendelsohn, J. Baselga, The EGF receptor family as targets for cancer therapy, Oncogene 19 (2000) 6550–6565.
[3] N. Normanno, A. De Luca, C. Bianco, L. Strizzi, M. Mancino, M.R. Maiello, A. Carotenuto, G. De Feo, F. Caponigro, D.S. Salomon, Epidermal growth factor receptor (EGFR) signaling in cancer, Gene 366 (2006) 2–16.
[4] Y. Yarden, M.X. Sliwkowski, Untangling the ErbB signalling network, Nature Reviews Molecular Cell Biology 2 (2001) 127–137.
[5] P. Gorden, J.L. Carpentier, S. Cohen, L. Orci, Epidermal growth-factor–morphological demonstration of binding internalization, and lysosomal association in human fibroblasts, Proceedings of the National Academy of Sciences of the United States of America 75 (1978) 5025–5029.
[6] C. Le Roy, J.L. Wrana, Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling, Nature Reviews Molecular Cell Biology 6 (2005) 112–126.
[7] F.T. Huang, D. Kirkpatrick, X.J. Jiang, S. Gygi, A. Sorkin, Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain, Molecular Cell 21 (2006) 737–748.
[8] S. Sigismund, E. Argenzio, D. Tosoni, E. Cavallaro, S. Polo, P.P. Di Fiore, Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation, Developmental Cell 15 (2008) 209–219.
[9] A.V. Vieira, C. Lamaze, S.L. Schmid, Control of EGF receptor signaling by clathrin-mediated endocytosis, Science 274 (1996) 2086–2089.
[10] E.M. van Dam, W. Stoorvogel, Dynamin-dependent transferrin receptor recycling by endosome-derived clathrin-coated vesicles, Molecular Biology of the Cell 13 (2002) 169–182.
[11] Y. Wang, S. Pennock, X.M. Chen, Z.X. Wang, Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival, Molecular and Cellular Biology 22 (2002) 7279–7290.
[12] D.J. Katzmann, G. Odorizzi, S.D. Emr, Receptor downregulation and multivesicular-body sorting, Nature Reviews Molecular Cell Biology 3 (2002) 893–905.
[13] N. Taub, D. Teis, H.L. Ebner, M.W. Hess, L.A. Huber, Late endosomal traffic of the epidermal growth factor receptor ensures spatial and temporal fidelity of mitogen-activated protein kinase signalling, Molecular Biology of the Cell 18 (2007) 4698–4710.
[14] E.S.M. Wong, C.W. Fong, J. Lim, P. Yusoff, B.C. Low, W.Y. Langdon, G.R. Guy, Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling, Embo Journal 21 (2002) 4796–4808.
[15] H. Stenmark, Rab GTPases as coordinators of vesicle traffic, Nature Reviews Molecular Cell Biology 10 (2009) 513–525.
[16] J.S. Rodman, A. Wandinger-Ness, Rab GTPases coordinate endocytosis – Commentary, Journal of Cell Science 113 (2000) 183–192.
[17] M.A. Barbieri, S. Fernandez-Pol, C. Hunker, B.H. Horazdovsky, P.D. Stahl, Role of Rab5 in EGF receptor-mediated signal transduction, European Journal of Cell Biology 83 (2004) 305–314.
[18] B.P. Ceresa, S.J. Bahr, Rab7 activity affects epidermal growth factor: epidermal growth factor receptor degradation by regulating endocytic trafficking from the late endosome, Journal of Biological Chemistry 281 (2006) 1099–1106.
[19] F.J.M. Opdam, G. Kamps, H. Croes, H. van Bokhoven, L.A. Ginsel, J.A.M. Fransen, Expression of Rab small GTPases in epithelial Caco-2 cells: Rab21 is an apically located GTP-binding protein in polarised intestinal epithelial cells, European Journal of Cell Biology 79 (2000) 308–316.
[20] M. Zerial, L.A. Huber, Guidebook to the Small GTPases, Oxford University Press, Oxford, 1995.

[21] Y. Egami, N. Araki, Characterization of Rab21-positive tubular endosomes induced by PI3K inhibitors, Experimental Cell Research 314 (2008) 729–737.
[22] Y. Egami, N. Araki, Dynamic changes in the spatiotemporal localization of Rab21 in live RAW264 cells during macropinocytosis, Plos One 4 (2009).
[23] T. Khurana, J.A. Brzostowski, A.R. Kimmel, A Rab21/LIM-only/CH-LIM complex regulates phagocytosis via both activating and inhibitory mechanisms, Embo Journal 24 (2005) 2254–2264.
[24] T. Pellinen, A. Arjonen, K. Vuoriluoto, K. Kallio, J.A.M. Fransen, J. Ivaska, Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta 1-integrins, Journal of Cell Biology 173 (2006) 767–780.
[25] J.C. Simpson, G. Griffiths, M. Wessling-Resnick, J.A.M. Fransen, H. Bennett, A.T. Jones, A role for the small GTPase Rab21 in the early endocytic pathway, Journal of Cell Science 117 (2004) 6297–6311.
[26] A. Sorkin, Internalization and Degradation of the EGF Receptor, Humana Press, New York, 2008.
[27] A. Sorkin, L.K. Goh, Endocytosis and intracellular trafficking of ErbBs, Experimental Cell Research 314 (2008) 3093–3106.
[28] J.L. Dinneen, B.P. Ceresa, Continual expression of Rab5(Q79L) causes a ligand-independent EGFR internalization and diminishes EGFR activity, Traffic 5 (2004) 606–615.
[29] B.P. Ceresa, Regulation of EGFR endocytic trafficking by rab proteins, Histology and Histopathology 21 (2006) 987–993.
[30] X.J. Zhang, X. He, X.Y. Fu, Z.J. Chang, Varp is a Rab21 guanine nucleotide exchange factor and regulates endosome dynamics, Journal of Cell Science 119 (2006) 1053–1062.