Biochemical and Biophysical Research Communications 410 (2011) 29–33

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Role of a tyrosine phosphorylation of SMG-9 in binding of SMG-9 to IQGAP and the NMD complex

Saori Takeda [a], Ai Fujimoto [a], Emiko Yamauchi [b], Mineyoshi Hiyoshi [c], Hiroshi Kido [d], Takashi Watanabe [e], Kozo Kaibuchi [e], Takeshi Ohta [a], Hiroaki Konishi [a,*]

[a] Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Shobara, Hiroshima 727-0023, Japan
[b] Proteomics and Metabolomics, Biomarker and Personalized Medicine CFU, Eisai Co., Ltd., Tsukuba, Ibaraki 300-2635, Japan
[c] Department of Preventive Medicine, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan
[d] Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 770-8503, Japan
[e] Department of Cell Pharmacology, Nagoya University Graduate School of Medicine, Nagoya, Aichi 466-8550, Japan

ARTICLE INFO

Article history:
Received 14 May 2011
Available online 24 May 2011

Keywords:
mRNA surveillance
IQGAP
Tyrosine phosphorylation
EGF receptor

ABSTRACT

SMG-9 is a component of the NMD complex, a heterotetramer that also includes SMG-1 and SMG-8 in the complex. SMG-9 was also originally identified as a tyrosine-phosphorylated protein but the role of the phosphorylation is not yet known. In this study, we determined that IQGAP protein, an actin cytoskeleton modifier acts as a binding partner with SMG-9 and this binding is regulated by phosphorylation of SMG-9 at Tyr-41. SMG-9 is co-localized with IQGAP1 as a part of the process of actin enrichment in non-stimulated cells, but not in the EGF-stimulated cells. Furthermore, an increase in the ability of SMG-9 to bind to SMG-8 occurs in response to EGF stimulation. These results suggest that tyrosine phosphorylation of SMG-9 may play a role in the formation of the NMD complex in the cells stimulated by the growth factor.

1. Introduction

Phosphoproteomics has been investigated by many research groups, and has been one of the most popular efforts during the first decade of the post-genome era [1,2]. Through these analyses, many previously unknown proteins were revealed to contribute many signaling pathways of growth factors or cytokines in mammalian cells [3–5]. However, there are many uncharacterized proteins that have the potential to be phosphorylated as a result of various cellular stimuli.

SMG-9 has been recently characterized as a component of the nonsense-mediated mRNA decay (NMD) complex that contributes to a RNA surveillance by degradation of mRNAs containing premature translation termination codons [6,7]. The NMD complex contains many proteins such as SMG-1 to SMG-10 (SMG-2 to SMG-4 are also named as Upf1 to Upf3) [6–9]. SMG-1 is a large protein kinase that play a significant role by phosphorylating Upf1. Upf1 in turn interacts with eRF3 in the ribosomal complex and induces binding of the complex to the mutated mRNA [10]. Phosphorylation of Upf1 is essential for NMD, which contributes to the process of remodelling of the mRNA surveillance complex [11]. A heterodimer composed of SMG-8 and SMG-9 is bound to SMG-1, which regulates its protein kinase activity [7]. SMG-9 has been shown to be stoichiometric associated with SMG-8 and SMG-1, and the precise mechanism of each binding property is also characterized [8].

On the other hands, SMG-9 (previously named as FLJ12886) was originally identified by tyrosine phosphoproteomics, and the two different reports stated its tyrosine phosphorylation sites as Tyr-41 and Tyr-147 [12,13]. However, additional characteristics of these tyrosine phosphorylations have not been determined.

In this study, we identified IQGAP as a new SMG-9-binding protein. IQGAP is known to rearrange the cytoskeletal proteins via a mechanism mediated by the dependence of actin on growth factor stimulation [14–16]. We focus on the role of tyrosine phosphorylation of SMG-9 in the interaction between IQGAP1 and SMG-9, and determine its effect on the NMD complex in the EGF-stimulated cells.

2. Materials and methods

2.1. Cell culture, transfection and reagents

COS-7, human embryonic kidney (HEK) 293T, and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin, and 100 units/ml of penicillin. Transfection was carried out by electroporation using the Gene-Pulser system (Bio-Rad). EGF (100 ng/ml, Sigma) dissolved in serum-free medium was added.

* Corresponding author. Address: Faculty of Life and Environmental Science, Prefectural University of Hiroshima, 562 Nanatsuka, Shobara 727-0023, Japan. Fax: +81 82 474 1776.
E-mail address: hkonishi@pu-hiroshima.ac.jp (H. Konishi).

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doi:10.1016/j.bbrc.2011.05.099

2.2. cDNA cloning and vector constructions

SMG-9 (FLJ12886) cDNA, provided by the National Institute of Technology and Evaluation, Japan, was subcloned into pFLAG-CMV6a and pCMV-3Tag-2 vector (Stratagene) to be expressed as an N-terminal FLAG-tagged and 3× Myc-tagged protein, respectively. SMG-8 cDNAs were purchased from Open Biosystems, and inserted into a pFLAG-CMV6a vector and expressed as FLAG-tagged proteins at the N-terminus. Myc-tagged IQGAP1 was constructed as described previously [17].

2.3. Point mutations were introduced using the QuikChange Kit (Stratagene), and mutations were verified by DNA sequencing

2.3.1. Antibodies

An anti-SMG-9 rabbit polyclonal antibody was raised against peptides having an additional cysteine residue at the amino-terminal end of the SMG-9 amino acid sequence, RPTQPVYQIQNRG (amino acids 141–153). The peptides were coupled to keyhole limpet hemocyanin and used to immunize rabbits. The antibody was purified by using HiTrap *N*-hydroxysuccinimide-activated Sepharose columns (GE Healthcare) coupled with an immunizing antigen. Other antibodies were obtained commercially from various companies, as follows: anti-FLAG M2 (Sigma), anti-phosphotyrosine (4G10; Upstate Biotech Inc.), anti-IQGAP1 (Santa Cruz Biotechnology), and anti-Myc antibody (9E10; Roche).

2.3.2. Immunoprecipitation and immunoblot analysis

The following procedures were carried out at 0–4 °C. The transfected cells were lysed in a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, and a complete protease inhibitor mixture (Roche Applied Science) to produce a total cell lysate (TCL). For the immunoprecipitation experiments, the total cell lysate was centrifuged, and the supernatant was incubated for 2 h with either the primary antibody or an anti-FLAG affinity gel (Sigma). Protein G-Sepharose (GE Healthcare) was added, and the resulting mixture was rotated at 4 °C for 1 h. The beads were subsequently washed three times with the lysis buffer. The processed samples were performed as described previously.

2.3.3. GST pulldown assays

Various GST-IQGAP1 constructs were constructed as described previously [17]. Pulldown binding assays by using GST-IQGAP1 were performed as described previously [4].

2.3.4. Fluorescence microscopy analysis

Transfected cells were fixed and observed as described previously [4].

3. Results

3.1. Identification of IQGAP as a SMG-9-9-interacting protein

Although the SMG-9 protein functions as a significant component of the NMD machinery, it was originally identified as a tyrosine-phosphorylated protein in phosphoproteomic studies. Tyr-41 and Tyr-147 were identified as the phosphorylation sites of SMG-9 [12,13]. To gain insights into the functions of the tyrosine phosphorylation of SMG-9, we analyzed the proteins that were observed to co-precipitate with SMG-9 by performing immunoaffinity purification. HEK293T cells transfected with the expression plasmid for FLAG-tagged SMG-9 were lysed, and immunoprecipitation was carried out using an anti-FLAG affinity gel. The proteins that eluted along with the FLAG peptide were analyzed by tandem mass spectrometry (LC/MS/MS). The identified proteins were almost all related to RNA processing, and also included member of

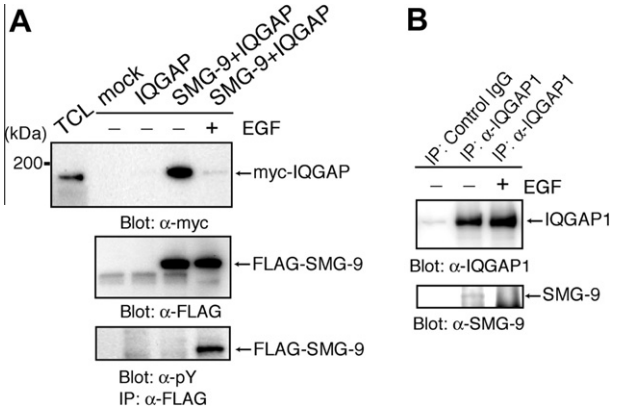

Fig. 1. Effects of tyrosine phosphorylation of SMG-9 on binding of SMG-9 to IQGAP1. (A) COS-7 cells were transfected with each of the indicated plasmids encoding the FLAG-tagged construct of SMG-9 and Myc-tagged IQGAP1. Cells were treated with (+) or without (−) EGF stimulation for 10 min and each FLAG-tagged molecule was immunoprecipitated with the respective anti-FLAG antibody. Immunoblot analysis was performed using anti-Myc (upper panel) anti-phosphotyrosine (middle panel) and anti-FLAG antibodies (lower panel). (B) EGF stimulation-dependent dissociation of endogenous SMG-9 and IQGAP1. Co-immunoprecipitation and immunoblot experiments were carried out using the indicated antibodies and HeLa cell lysates with (+) or without (−) EGF stimulation for 10 min.

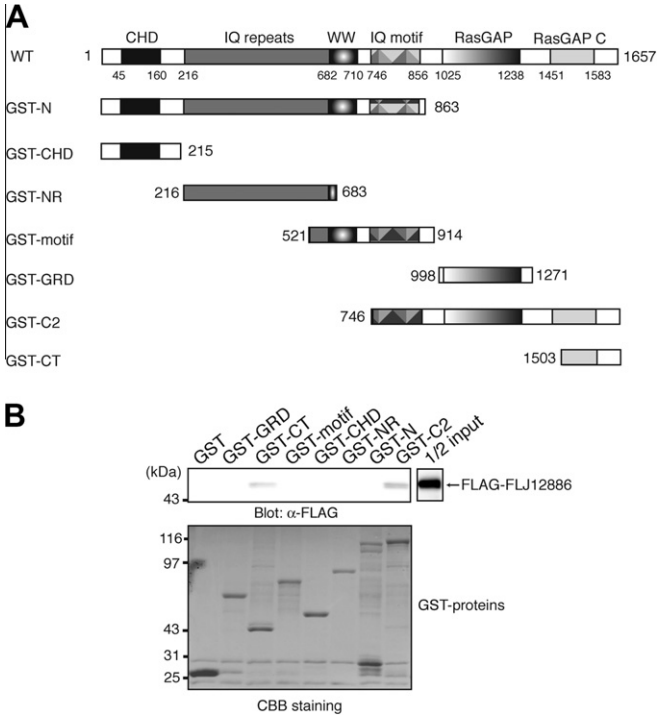

Fig. 2. Identification of the binding domain involved in the binding of IQGAP1 to SMG-9. The in vitro interaction between the COOH-terminal domain of IQGAP1 and SMG-9 was confirmed by GST pulldown assays. (A) Schematic representation of the constructs of GST-fused IQGAP1. Numbers indicate the amino acid residues. GST fusion proteins used in this assay were visualized by Coomassie Brilliant Blue (CBB) staining (lower panel). Immunoblotting was used to determine the amount of FLAG-SMG-9 (upper panel) bound to GST fusion proteins by using an anti-FLAG antibody. The total lysate (half of the total amount was used in this particular assay) from COS-7 cells expressing FLAG proteins is shown at the right side.

the IQGAP family. The proteins of this family are known to function as regulator proteins of cytoskeletal actin in the growth factor signaling pathway (data not shown).

We confirmed the existence of interactions between IQGAP1 and SMG-9. COS-7 cells co-expressing Myc-IQGAP1 and FLAG-SMG-9 were lysed, and the FLAG-SMG-9 protein was immunoprecipitated along with the FLAG antibody. IQGAP1 was found to be specifically bound to SMG-9 in non-stimulated COS-7 cells. Interestingly, this binding did not occur in the EGF-stimulated cells (Fig. 1A). Similar results for both endogenous proteins were obtained using HeLa cells (Fig. 1B). Thus, the interaction between SMG-9 and IQGAP1 may be regulated by post-translational modifications of these proteins with EGF stimulation of the cells.

3.2. SMG-9 binds to the COOH-terminal region of IQGAP1

Next, we proceeded to identify the SMG-9 binding site in IQGAP1. Bacterial produced GST-fused proteins containing various regions of IQGAP1 were prepared (Fig. 2A), and GST pulldown assays were performed with the total cell lysate of COS-7 cells expressing FLAG-SMG-9 protein. Consequently, only the proteins including the COOH-terminal region of IQGAP1 were found to be capable of binding to SMG-9 (Fig. 2B).

3.3. Binding of the amino-terminal region of SMG-9 to IQGAP1 is regulated by Tyr-41 phosphorylation of SMG-9

We attempted to identify the IQGAP1-binding site in SMG-9 by co-immunoprecipitation analysis. COS-7 cells co-expressing the Myc-IQGAP1 and FLAG-SMG-9 derivatives (Fig. 3A) were lysed, and the FLAG-proteins were immunoprecipitated along with the FLAG antibody. The results suggest that the 1–100 amino acid region of SMG-9 is necessary for binding of SMG-9 to IQGAP1 in non-stimulated cells (Fig. 3B). Furthermore, the point mutant Tyr-41 with Phe (Y41F) did not diminish the binding of SMG-9 to IQGAP1 in EGF-stimulated cells relative to the wild-type and relative to the another mutant, Y147F (Fig. 3C and D). Both mutants were subjected to a decrease in the phosphorylation level upon EGF stimulation. Therefore, Tyr-41 and Tyr-147 appear to be candidates of phosphorylation sites. Dephosphorylation of Tyr-41 is specifically required for binding of SMG-9 to IQGAP1. Furthermore, the interaction between IQGAP1 and SMG-9 might be inhibited to generate a heterodimer of SMG-8 and SMG-9. Release of IQGAP1 from SMG-9 was enhanced and led to the recruitment of SMG-9 into the NMD complex in the EGF-stimulated cells (Fig. 3E).

3.4. SMG-9 is co-localized with IQGAP1 in non-stimulated cells

Fluorescence microscopy analysis of COS-7 cells expressing FLAG-SMG-9 and Myc-IQGAP1 was performed. In non-stimulated cells, both proteins appeared to be co-localized (Fig. 4 right panel set). In contrast, a microvillus (an actin-enriched structure) was generated on the surface of the cells as a result of EGF stimulation. IQGAP1 was found to in the microvillus, but SMG-9 was not presented (Fig 4 right panels set). These results support our biochemical interaction analysis of both proteins in the presence or absence of EGF stimulation.

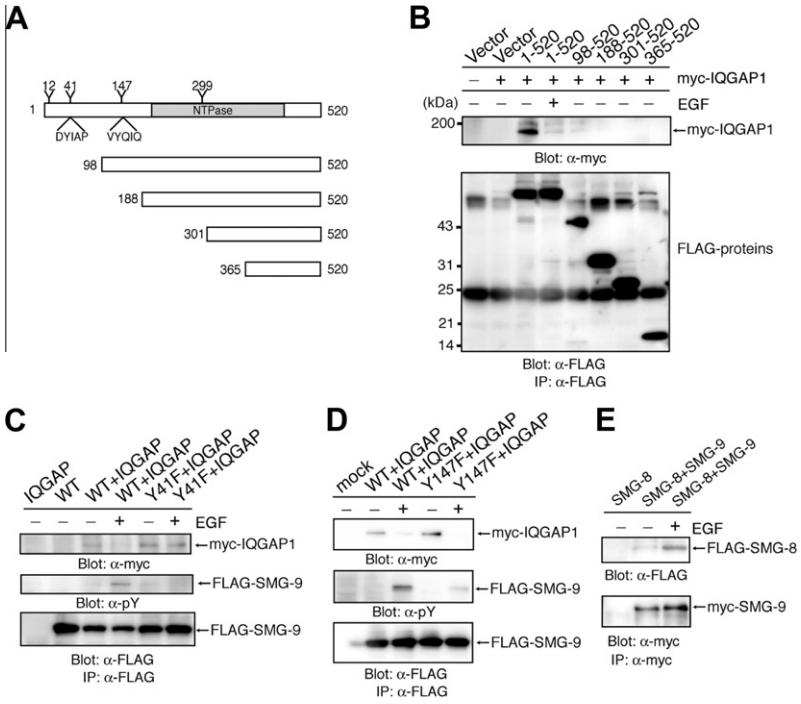

Fig. 3. Effect of tyrosine phosphorylation of SMG-9 on the binding of SMG-9 to IQGAP1 or SMG-8. (A) Schematic representation of SMG-9. The positions of the representative tyrosine residues in SMG-9 are indicated. The numbers indicate amino acid residues. (B) Detection of the IQGAP1-binding site in SMG-9. Co-immunoprecipitation studies were carried out using the cell lysates from COS-7 cells with (+) or without (−)-overexpressing Myc-IQGAP1 and each derivative molecule of FLAG-SMG-9. Each FLAG-tagged molecule was immunoprecipitated with an anti-FLAG antibody. Immunoblot analysis was carried out using an anti-Myc antibody (upper panel) or an anti-FLAG antibody (middle panel). (C and D) COS-7 cells were transfected with each of the indicated plasmids encoding the FLAG-tagged construct of SMG-9 derivatives in which tyrosine residues {Y} had been substituted with phenylalanine {F} and Myc-tagged IQGAP1. Cells were treated with (+) or without (−) EGF stimulation for 10 min and each FLAG-tagged molecule was immunoprecipitated with the respective anti-FLAG antibody. Immunoblot analysis was performed using anti-Myc (upper panel), anti-phosphotyrosine (middle panel), and anti-FLAG antibodies (lower panel). WT: wild-type SMG-9, Y41F: SMG-9 mutant with Tyr-41 replaced by Phe, Y147F: SMG-9 mutant with Tyr-147 replaced by Phe. (E) COS-7 cells were transfected with each of the indicated plasmids encoding the Myc-tagged construct of SMG-9 and FLAG-tagged SMG-8. Cells were treated with (+) or without (−) EGF stimulation for 10 min, and SMG-9 was immunoprecipitated with the respective anti-Myc antibody. Immunoblot analysis was performed using anti-FLAG (upper panel) and anti-Myc antibodies (lower panel).

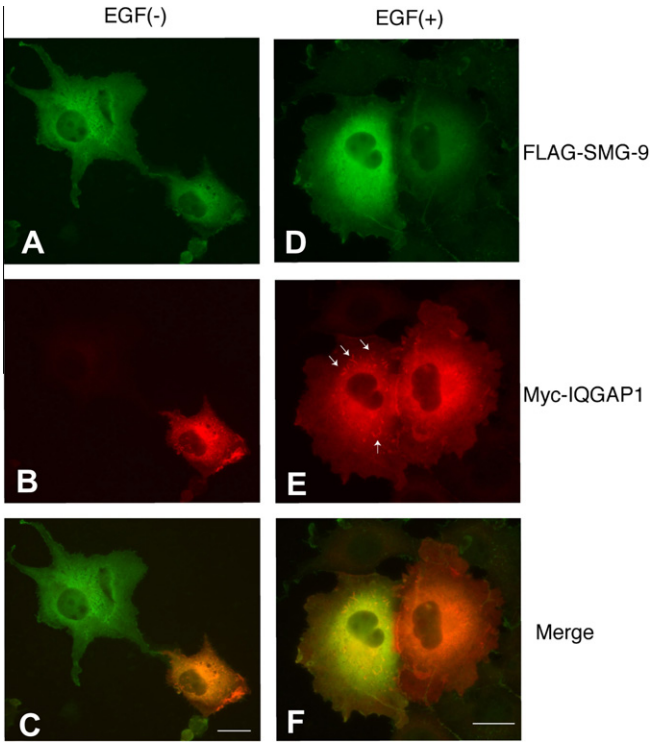

Fig. 4. Co-localization of SMG-9 with IQGAP-1 in non-stimulated COS-7 cells. (A) Subcellular localization of SMG-9 and IQGAP1. COS-7 cells expressing FLAG-SMG-9 and Myc-IQGAP1 with EGF treatment (D–F) and without EGF treatment (A–C) were processed for immunofluorescence staining using anti-FLAG (A and D) and anti-Myc (B and F) antibodies. Selected areas of accumulation of IQGAP1 in the microvillus are shown with arrows. The merged images are shown in the bottom panels. The scale bars represent 10 μm.

4. Discussion

The NMD complex is composed of many proteins. SMG-1 is a key protein kinase for Upf1, and SMG-8 and SMG-9 play critical roles as regulators of the kinase activity of SMG-1 [2]. Recently, a biochemical analysis of the ternary complex of SMG-1, SMG-8 and SMG-9 has been performed, and it was found that SMG-9 exists as a homodimer, as a heterodimer with either SMG-8 or SMG-1 and as a tight tetramer with SMG-8 and SMG-1 [18]. The COOH-terminal of SMG-9 binds to SMG-8, and the amino-terminal domain is an intrinsically disordered region that is essential for the maintenance of the structural integrity of the SMG-1:SMG-8:SMG-9 complex [19]. Although SMG-9 is known to be as a phosphoprotein, the role of its phosphorylation has not been defined.

In this study, we show that the tyrosine phosphorylation of SMG-9 evoked by EGF stimulation play a role in inducing the dissociation of SMG-9 from IQGAP1. This promotes the binding of SMG-9 to SMG-8. Fundamentally, SMG-9 may be involved in the NMD complex by associating with SMG-1 and SMG-8 in normal cells. This produces the functions of degradating nonsense mRNA to avoid the generation of abnormal proteins. We propose that the transcription activity may be upregulated in the growth factor-stimulated cells, and the possibility of generation of the mutated mRNA becomes higher in these cells than in normal cells. Under these situations, higher levels of the NMD complex may be necessary. Our microscopy data shows that SMG-9 is co-localized with the actin-binding protein IQGAP1, which functions as a regulator of actin cytoskeletal dynamics in non-stimulated cells. Because tyrosine phosphorylated SMG-9 dissociated from IQGAP1, a fraction of the population of SMG-9 is not localized in the actin-rich structure such as the microvillus in EGF-stimulated cells (Fig. 4). Although detailed biochemical analyses have revealed the molecular mechanism of the NMD complex and the functions of each of its components, there is a lack of agreement with regard to the subcellular localization of each molecule. SMG-1 was reported to be localized both in the cytoplasm and in the nucleus [20]. Immunoreactive protein bands of SMG-8 and SMG-9 were also detected both in the cytoplasmic and nuclear fractions [8]. However, the precise subcellular localization of cytoplasmic SMG-9 remains unknown. Unexpectedly, SMG-9 was found to be mainly co-localized with the cytoskeletal proteins. It is known that mRNA exists in the presence of many proteins for intron processing. These proteins assist in transporting the mRNA to its target position on the cytoskeletal filaments in the cells [21,22]. Nonsense mRNA might also behave in a similar way in the cytosol. It appears that the purpose of localization of SMG-9 at the cytoskeleton is to provide stabilization of the cytoplasmic NMD complex with respect to the aberrant mRNA molecules. Furthermore, tyrosine phosphorylation in the amino-terminal region of SMG-9 may regulate the amount of NMD complex. However, further study is needed to characterize the relationship between the tyrosine phosphorylation of SMG-9 and the efficiency of decay of the nonsense mRNA in the growth factor-stimulated cells.

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