

## Regulation Fe65 localization to the nucleus by SGK1 phosphorylation of its Ser566 residue

Eun Jeoung Lee<sup>1</sup>, Jaesun Chun<sup>2</sup>, Sunghee Hyun<sup>3</sup>, Hye Rim Ahn<sup>1</sup>, Jae Myung Jeong<sup>2</sup>, Soon-Kwang Hong<sup>4</sup>, Jin Tae Hong<sup>5</sup>, In Kyeong Chang<sup>1</sup>, Hye Yeon Jeon<sup>1</sup>, Yeon Soo Han<sup>6</sup>, Chung-Kyoon Auh<sup>7</sup>, Jae In Park<sup>8</sup> & Sang Sun Kang<sup>1,\*</sup>

<sup>1</sup>School of Science Education, Chungbuk National University, Cheongju, <sup>2</sup>Department of Biological Education, Korea National University of Education, Cheongwon, <sup>3</sup>Department of Premedicine, Eulji University School of Medicine, Daejeon, <sup>4</sup>Division of Life Science, Myungji University, Yongin, <sup>5</sup>Department of Pharmacy, Chungbuk National University, Cheongju, <sup>6</sup>Department of Agricultural Biology, Chonnam National University, Gwangju, <sup>7</sup>Division of Applied Biotechnology, Mokpo National University, Muan, <sup>8</sup>Department of Forest Science, Chungbuk National University, Cheongju, Korea

Fe65 is characterized as an adaptor precursor (APP) through its PID2 element, as well as with the other members of the APP protein family. With the serum- and glucocorticoid-induced kinase 1 (SGK1) substrate specificity information, we found that the putative site of phosphorylation in Fe65 by SGK1 is present on its Ser<sup>566</sup> residue in <sup>560</sup>CRVRFLSFLA<sup>569</sup>(X60469). Thus, we demonstrated that Fe65 and the fluorescein-labeled Fe65 peptide FITC-<sup>560</sup>CRVRFLSFLA<sup>569</sup> are phosphorylated *in vitro* by SGK1. Phosphorylation of the Ser<sup>566</sup> residue was also demonstrated using a Ser<sup>566</sup> phospho-specific antibody. The phospho Fe65 was found mainly in the nucleus, while Fe65 S556A mutant was localized primarily to the cytoplasm. Therefore, these data suggest that SGK1 phosphorylates the Ser<sup>566</sup> residue of Fe65 and that this phosphorylation promotes the migration of Fe65 to the nucleus of the cell. [BMB reports 2008; 41(1): 41-47]

### INTRODUCTION

Fe65 was originally identified as an expressed sequence tag (EST) corresponding to an mRNA that is expressed in large amounts in the rat brain. However, the cDNA of Fe65 encodes a protein consisting of 711 amino acids (1). A comparison of its amino acid sequence with sequences contained in data banks reveals that a region within this protein can be aligned with a region of the retroviral integrases, DNA binding proteins that catalyze the integration of proviral DNA into the host genome (2).

Serum- and glucocorticoid-induced kinase 1 (SGK1) is a Ser/Thr protein kinase that is transcriptionally regulated by se-

rum and/or glucocorticoids in mammary epithelial cells and by Rat-2 fibroblasts (3, 4). SGK1 is also transcriptionally regulated by cortico-steroids in several types of cells (5, 6). SGK1 has been identified as a protein that was designed to promote cell survival, it has the same function as Akt kinase (7-9). We found that SGK1 plays a role in the phosphorylation of MEKK3 and, thus, inhibits its kinase activity (10). The consensus SGK1 substrate site has been described by the structural formula R-X-R-X-X-(S/T)- $\phi$ , where  $\phi$  is any hydrophobic amino acid. The arginine residues are conserved at positions -5 and -3 relative to the positions of the Ser/Thr residues that are phosphorylated in the presence of SGK1 (11, 12). Using SGK1 substrate consensus sequence information, we noticed that the putative site of SGK1 phosphorylation site in Fe65 is Ser<sup>566</sup> residue of <sup>560</sup>CRVRFLSFLA<sup>569</sup> motif which is conserved in all Fe65 variants (1, 2). (We count the amino acid number, according to Fe65 variant X60469; NCBI Accession Number which was used in this study). We investigated this further to determine whether the phosphorylation of Ser<sup>566</sup> in Fe65 would affect the intrinsic kinase activity of Fe65 *in vivo* and *in vitro*, and found that SGK1 actually does induce phosphorylation of Ser<sup>566</sup> in Fe65. It was shown that Fe65 fusion protein and fluorescein-labeled Fe65 peptides (FITC-<sup>560</sup>CRVRFLSFLA<sup>569</sup>) are phosphorylated by SGK1 *in vitro*. Further, the phosphorylation of Ser<sup>566</sup> by SGK1 was demonstrated using a phospho-specific antibody. We also observed that a mutant Fe65 containing Ala instead of Ser at amino-acid 566 (Fe65<sup>S556A</sup>) is localized mainly in the cytoplasm, while the phosphorylated Fe65 is found mainly in the nucleus, with the phospho-specific antibody. These observations suggest that SGK1 phosphorylates the Ser<sup>566</sup> of Fe65 and that this phosphorylation promotes the localization of Fe65 to the nucleus of the cell.

### RESULTS AND DISCUSSION

#### Phosphorylation of the recombinant Fe65 proteins and the Fe65 peptides by SGK1

We observed the putative SGK1 phosphorylation site in Fe65

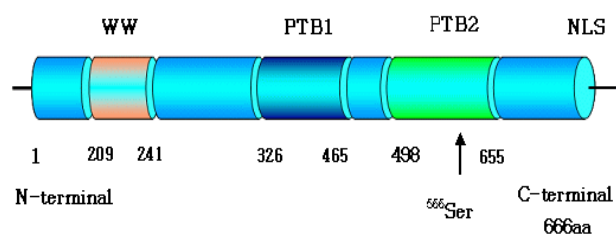
\*Corresponding Author. Tel: 82-43-261-3278; Fax: 82-43-271-0526; E-mail: jin95324@cnu.ac.kr

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(Fig. 1), Ser<sup>566</sup> in the <sup>561</sup>RvRfISF<sup>567</sup> of the Fe65 PTB2 domain, which is also responsible for the bond between Fe65 and APP (1, 13-16). We also notice that the putative nuclear export sequences (NES; characterized as L/MFX<sub>(2-3)</sub>, L/I/VX<sub>(2-3)</sub>, and LXL/I) seem to overlap with the SGK1 phosphorylation site (<sup>557</sup>LgecRVRfLSFLaV<sup>570</sup>; underline indicates the SGK1 phosphorylation site, is the Ser<sup>566</sup> residue) (17-21).

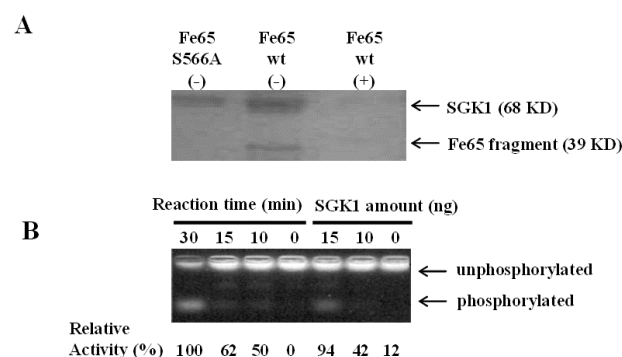
To determine whether SGK1 induces phosphorylation of Fe65, SGK1 was incubated in the presence of [ $\gamma$ -<sup>32</sup>P] ATP with a Fe65 fragment (370-666) and Fe65 mutant fragment (370-666, S566A) purified from recombinant *E. coli*. The recombinant wild-type Fe65 protein (352-666) was phosphorylated by SGK1, whereas the mutant Fe65 (370-666 S566A), in which Ser<sup>566</sup> was replaced with Ala, was not phosphorylated by SGK1 (Fig. 2A). Phosphorylation was verified by treating the reaction mixture with alkaline phosphatase and observing that



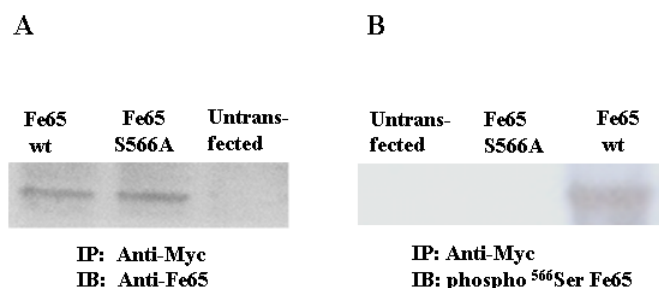
**Fig. 1.** Functional domains of Fe65 in the rat brain. The WW domain is located near the N-terminal of Fe65 and is followed by 2 phosphotyrosine-binding (PTB) domains—PTB1 and PTB2 (which is responsible for amyloid precursor protein cytoplasmic tail binding)—and the putative nuclear localization sequence (NLS) in Fe65. The SGK1 phosphorylation site, <sup>566</sup>Ser (<sup>561</sup>RvRfISF<sup>567</sup>), lies in the PTB2 domain, which is also responsible for the bond between Fe65 and APP. NLS and the SGK1 phosphorylation site are indicated. The SGK1 phosphorylation site, <sup>566</sup>Ser (<sup>561</sup>RvRfISF<sup>567</sup>), seems to overlap with the SGK1 phosphorylation site (see text for details). In this study, we used a Fe65 variant (X60469; NCBI Accession Number).

the phosphorylated protein bands disappeared (Fig. 2A, middle lane). These results suggest that SGK1 induces phosphorylation of the Ser<sup>566</sup> residue of Fe65.

To further determine whether the Ser<sup>566</sup> residue of Fe65 is phosphorylated by SGK1, the fluorescein-labeled Fe65 peptide FITC-<sup>560</sup>CRVRFLSFLA<sup>569</sup> was incubated with SGK1. The proportion of the Fe65 peptide that was phosphorylated increased with the duration of the incubation or the amount of SGK1 used (Fig. 2B). We did not observe any phosphorylation of the FITC-<sup>560</sup>CRVRFLAFLA<sup>569</sup> peptide (in which Ser was replaced with Ala), with or without SGK1 (data not shown).



**Fig. 2.** The phosphorylation of Fe65 by SGK1. (A) 100 ng of the purified Fe65 PTB2 fragment (39 kDa) or Fe65 PTB2 Ser<sup>566A</sup> was incubated with 10 ng of active SGK1 (Upstate Biotech) in the presence of 1 mCi [ $\gamma$ -<sup>32</sup>P]-ATP. Phosphorylation of the Fe65 fragment and SGK1 appears to have taken place. Alkaline phosphatase (AP) (+) was used to confirm that the phosphorylation took place. (B) Phosphorylation of the FITC-Fe65 peptide (<sup>559</sup>ecRvRfISF<sup>568</sup>) by SGK1. To determine whether SGK1 phosphorylates the <sup>566</sup>Ser moiety of Fe65, SGK was incubated with the FITC-Fe65 peptide (<sup>559</sup>ecRvRfISF<sup>568</sup>). The phosphorylated product migrated to the cathode (+) faster than the nonphosphorylated substrate did. The increase in FITC-Fe65 peptide phosphorylation is shown to occur along with increase in SGK1 concentration. The phosphorylated product and nonphosphorylated substrate are marked.



**Fig. 3.** The immunoblotting with the phosphorylated <sup>566</sup>Ser Fe65-specific antibody. COS-1 cells were transiently transfected with expression vectors containing cDNA for myc epitope-tagged Fe65 wt or myc epitope-tagged Fe65<sup>S566A</sup> mutant. The untransfected COS-1 represents the negative control of each panel. After immunoprecipitation with the myc antibody (mouse), a western blot analysis was performed using rabbit Fe65 Antibody (A) or the phospho <sup>566</sup>Ser Fe65 antibody (B). The phospho <sup>566</sup>Ser Fe65 antibody only recognized the wild type Fe65, not the Fe65<sup>S566A</sup> mutant.

### The phospho<sup>566</sup>Ser Fe65-specific antibody

We developed an antibody that recognizes the phosphorylated <sup>566</sup>Ser Fe65 with a <sup>560</sup>CRVRFLSFLA<sup>569</sup> peptide (see the Materials and Methods section). A western blot analysis was performed. COS-1 cells were transiently transfected with expression vectors containing the cDNA for myc epitope-tagged Fe65 or a myc epitope-tagged Fe65<sup>S566A</sup> mutant. The non-transfected COS-1 cell (Fig. 3A) was used as a negative control in each panel. After immunoprecipitation with the myc antibody (mouse), a western blot analysis was performed with rabbit Fe65 antibody (Fig. 3A) or with the phospho<sup>566</sup>Ser Fe65 specific antibody (Fig. 3B). These results showed that the phospho<sup>566</sup>Ser Fe65 specific antibody only recognized the wild-type Fe65 which is phosphorylated by SGK1, not the Fe65<sup>S566A</sup> mutant.

### Comparison of subcellular localization of wild-type Fe65 and Fe65<sup>S566A</sup> mutant

To determine the significance of phosphorylation of Fe65 Ser<sup>566</sup>, we compared the subcellular localization of wild-type Fe65 with that of the Fe65<sup>S566A</sup> mutant in COS-1 cells. The expression vectors containing cDNA for myc epitope-tagged Fe65 (Fig. 4A) or myc epitope-tagged Fe65<sup>S566A</sup> mutant (Fig. 4B) were transiently transfected in COS-1 cells. Myc-Fe65 was visualized by immunofluorescence in both fixed and permeabilized cells using monoclonal antibodies against myc and Alexa Fluor 568-conjugated donkey anti-mouse immunoglobulin G (red color). To define the cell boundary and cytoplasm, we also used an anti-caveolin-1 rabbit antibody and goat anti-mouse Alexa Fluor 488 (green). The wild-type Fe65 was detected both the nucleus and in the cytoplasm (Fig. 4A), while the Fe65<sup>S566A</sup> mutant was observed in the cytoplasm only (Fig. 4B). These results suggest that the Fe65<sup>S566A</sup> mutant is prohibited its nuclear localization.

### Confocal microscopy with the phospho-specific antibody against the phosphorylated<sup>566</sup>Ser Fe65

To confirm the observations summarized in Fig. 4 (that the phosphorylated wild-type Fe65 is transported into the nucleus and the Fe65<sup>S566A</sup> mutant is not in the nucleus), we monitored the phospho-specific antibody against the phosphorylated <sup>566</sup>Ser Fe65 in COS-1 cells, which were transfected with myc-Fe65 or myc-Fe65<sup>S566A</sup> mutant, using confocal microscopy. The confocal microscopic images show that the phosphorylated <sup>566</sup>Ser Fe65, which was detected with the phospho-specific antibody (green), localizes in the nucleus (Figs. 4C and 4D). By contrast, the localization of Fe65<sup>S566A</sup> was limited in the cytoplasm (Fig. 4D), while that of the Fe65 wild-type was not (Fig. 4C). The confocal fluorescence micrographs in Figure 4 show the endogenous phosphorylated <sup>566</sup>Ser Fe65 (green), the transfected myc epitope-tagged wild-type Fe65 (red), and the merge (yellow). Phosphorylated <sup>566</sup>Ser Fe65 was detected in the nucleus (green), while myc epitope-tagged Fe65<sup>S566A</sup>

was observed in the cytoplasm (red), consistent with the findings represented in Fig. 4. The merger (yellow) was detected in the nucleus when DNA for myc epitope-tagged wild-type Fe65 was transfected (Fig. 4C). However, the merger (yellow) was not detected in the nucleus when DNA for myc epitope-tagged Fe65<sup>S566A</sup> was transfected (Fig. 4D). These results suggest that the nuclear Fe65 is mainly the phosphorylated <sup>566</sup>Ser residue and that phosphorylation of Fe65 <sup>566</sup>Ser residue is required for translocation of Fe65 into the nucleus.

To test whether the translocation of Fe65 into the nucleus by phosphorylation of Fe65 <sup>566</sup>Ser residue depends on the cell line, we used HEK293 cell line instead of COS-1. With the phospho-specific antibody against the phosphorylated <sup>566</sup>Ser Fe65, the confocal microscopy was performed in HEK293 cells which were transfected with myc-Fe65 or myc-Fe65<sup>S566A</sup> mutant. And the similar Fe65 subcellular localization results were observed (Fig. 4E and F). The endogenous phosphorylated <sup>566</sup>Ser Fe65 (green), the transfected myc epitope-tagged wild-type Fe65 or Fe65<sup>S566A</sup> (red), and the merge (yellow) were detected with their specific antibody. Phosphorylated <sup>566</sup>Ser Fe65 was observed in the nucleus (green), however myc epitope-tagged Fe65<sup>S566A</sup> was shown in the cytoplasm (red), similar with the findings represented in Fig. 4C and D. While the merger (yellow) was shown in the nucleus when DNA for myc epitope-tagged wild-type Fe65 was transfected (Fig. 4E), the merger (yellow) was not detected in the nucleus when DNA for myc epitope-tagged Fe65<sup>S566A</sup> was transfected (Fig. 4F) in HEK293 cells. Together, these results suggest that phosphorylation of Fe65 <sup>566</sup>Ser residue is required for translocation of Fe65 into the nucleus, regardless of the transfected cell line specificity.

Further phosphorylation of the Fe65 Ser<sup>566</sup> residue by SGK1 seems to be required for its movement into the nucleus (Fig. 4).

The identification and characterization of SGK1 substrates may provide clues to our understanding of how SGK1 contributes to cell survival and death (3, 4, 8, 10, 11, 18). We demonstrated that Fe65 is a SGK1 substrate that contains the consensus motif RxRxxS/Tφ: where φ is a hydrophobic amino acid, which subsequently demonstrated that phosphorylation of Fe65 by SGK1 controls its subcellular localization (Figs. 2 and 3). Because of the similarity in substrates for Akt kinase and SGK1, we suspect that Akt kinase may also phosphorylate the <sup>566</sup>Ser moiety of Fe65 and contribute to the control of Fe65 subcellular localization. However, it remains to be determined whether Akt kinase also phosphorylates the Ser<sup>566</sup> moiety of Fe65.

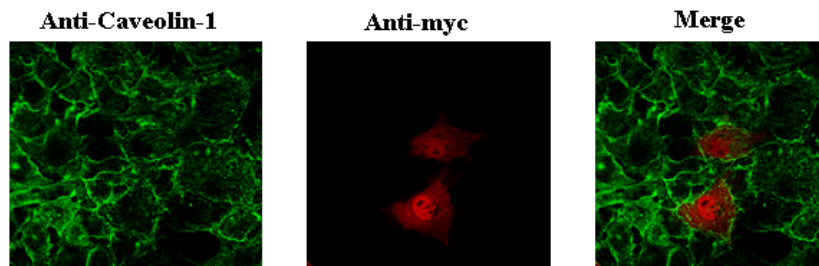
It is unknown whether phosphorylation of the <sup>566</sup>Ser moiety of Fe65 influences its ability to bind to APP. Thus, we speculated that its phosphorylation by SGK1 overtly influences APP/Fe65 binding, because the <sup>566</sup>Ser residue that is phosphorylated by SGK1 resides within the second PTB domain. However, it remains to be seen whether the precise mechanisms by which phosphorylation of Fe65 by SGK1 stimulate its

ability to migrate to the nucleus of the cell.

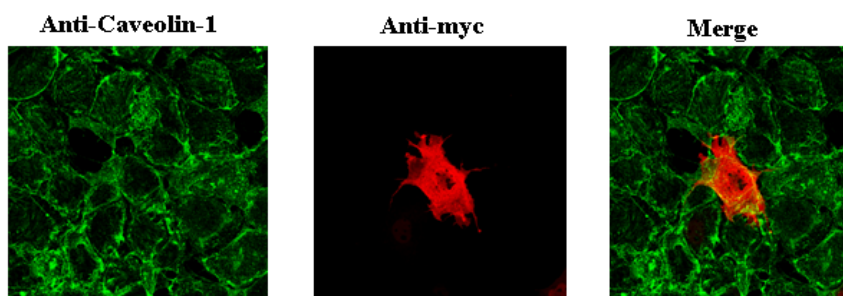
In conclusion, our results suggest that SGK1 phosphorylates the Ser<sup>566</sup> residue of Fe65 and that this phosphorylation is re-

quired for the nuclear localization of Fe65

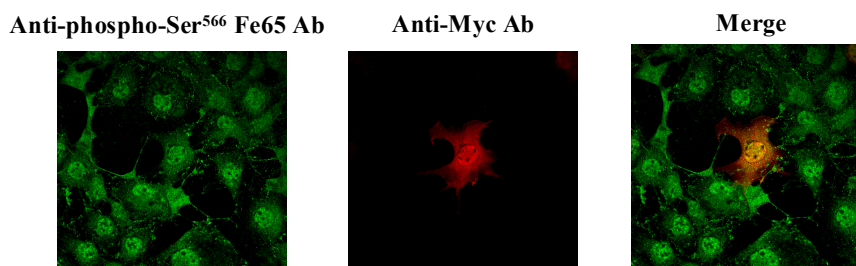
#### 4A: myc-Fe65 wt



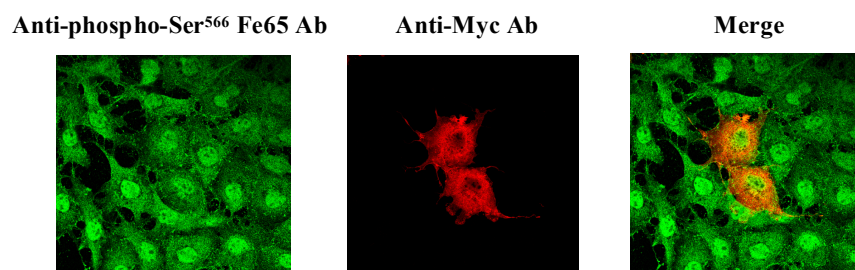
#### B: myc-Fe65<sup>S566A</sup>



#### C: myc-Fe65 wt

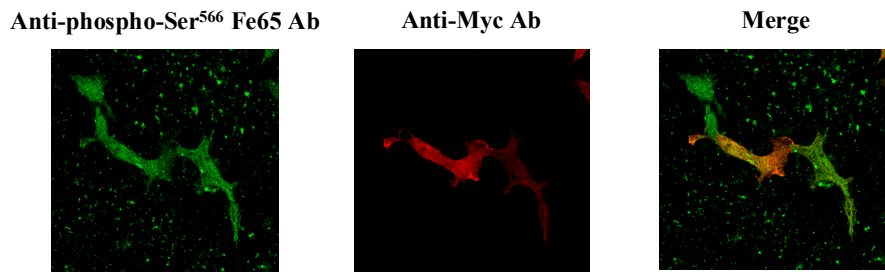


#### D: myc-Fe65<sup>S566A</sup>



**Fig. 4.** The subcellular localization of wild-type Fe65 and Fe65<sup>S566A</sup> mutant. COS-1 cells were transiently transfected with expression vectors containing cDNA for myc epitope-tagged Fe65 (A) or myc epitope-tagged Fe65<sup>S566A</sup> mutant (B). Myc-Fe65 was visualized by immunofluorescence in fixed and permeabilized cells using monoclonal antibodies against myc and Alexa Fluor 568-conjugated donkey anti-mouse immunoglobulin G. (red). To define the cell boundary and cytoplasm, we also used an anti-caveolin-1 rabbit antibody (green). The wild-type Fe65 was detected both the nucleus and cytoplasm (A), while the Fe65<sup>S566A</sup> mutant was observed in the cytoplasm only (B). COS-1 cells were transiently transfected with expression vectors containing the cDNA for myc epitope-tagged wild-type Fe65 (C) and for the cDNA for myc epitope-tagged S-A mutant Fe65 (D). HEK293 cells were transiently transfected with expression vectors containing the cDNA for myc epitope-tagged wild-type Fe65 (E) and for the cDNA for myc epitope-tagged S-A mutant Fe65 (F). Confocal fluorescence micrographs showing the endogenous phosphorylated Fe65 in COS-1 cells (left lane of C, D, E, or F), the transfected myc epitope tagged wild-type Fe65 or Fe65<sup>S566A</sup> mutant (middle lane of C, D, E, or F), and the merge (right lane of C, D, E, or F). The phosphorylated <sup>566</sup>Ser in Fe65 was detected in the nuclear region (green), while the myc epitope-tagged Fe65<sup>S566A</sup> was observed in the cytoplasm (red). The merger (yellow) was detected in the nuclear region when DNA for myc epitope-tagged wild-type Fe65 was transfected (C and E), but not when the Fe65<sup>S566A</sup> mutant DNA was transfected (C and F). The transfected DNA and the primary antibody are indicated above the picture.

## E: myc-Fe65 wt



## F: myc-Fe65<sup>S566A</sup>

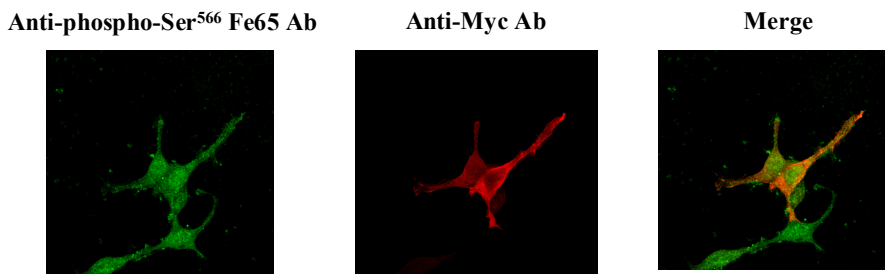


Fig. 4. Continued.

## MATERIALS AND METHODS

### Cell culture

COS-1 was purchased from ATCC. Media and supplements were obtained from GIBCO-BRL. The cell line was maintained in Dulbecco's Modified Essential Medium (DMEM) containing 10% fetal bovine serum (FBS), 10,000 U potassium penicillin/streptomycin/ml, 2 mM glutamine, and 20 mM sodium bicarbonate. The cells were incubated at 5% CO<sub>2</sub> with 95% humidity and in a 37°C chamber. The growth medium was changed every 3 days.

### Antibodies

The monoclonal antibody against the myc epitope was purchased from Cell Signaling Technology. Antibodies against caveolin-1 and Fe65 were purchased from Santa Cruz Biotechnology. An active SGK1 was purchased from Upstate Biotechnology.

### DNA constructs and site-directed mutagenesis

Fe65 (X60469; NCBI Accession Number, cloned in pRK5 Myc vector) was obtained from Dr. Margolis as a gift. To generate the mutant Fe65<sup>S566A</sup> construct, 2 mutagenic primers (up: 5'-GTGCGGTTCCCTCGCCTTCCTGGCTG-3'; and down: 5'-AGC-CAGGAAGGCG AGGAACCGCAC-3') and the QuikChange XL Mutagenesis Kit (Stratagene, TX, USA) were used according to the manufacturer's instructions. To express Fe65 fragment in *E. coli* Fe65 and mutant Fe65<sup>S566A</sup> were subcloned into prokaryotic expression vector pGEX-5X-1 with forward primer;

5'-CTACATGATCCCGATGTCTGGA-3' and reverse primer; 5'-CTCTGGGGTCT GGGATCCTAG-3'). Each DNA construct was confirmed by DNA sequencing.

### Transfection and immunoprecipitation

Wild-type and mutant myc-Fe65<sup>S566A</sup> constructs were transfected into the COS-1 or HEK293 cell using FuGENE6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cells ( $2 \times 10^7$ ) were lysed in 1 ml RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate) for 10 minutes at 4°C. Cell lysates were then centrifuged at 12,000 g for 15 minutes at 4°C, and the supernatant was precleaned using protein A agarose beads (Upstate Biochem). Anti-myc or anti-Fe65 antibodies were used to precipitate Fe65. The antibodies were incubated with the precleaned supernatant for 16 hours at 4°C and then precipitated using protein A agarose beads. Glutathione-Sepharose beads were used to purify the antibodies. The beads were washed 3 times with 1 ml of RIPA lysis buffer and then twice with phosphate-buffered saline (PBS). The final pellet was used for Fe65, western blot assay, as described.

### Phosphorylation of recombinant Fe65

GST-Fe65 (a peptide consisting of amino acids 370 through 666 of the Fe65 protein) or GST-Fe65<sup>S566A</sup> 370-666 proteins was expressed in *Escherichia coli* BL21 and purified using GST agarose beads according to the manufacturer's instructions. Purified proteins were used for the SGK1 kinase assay. Purified and preheated GST-Fe65 and GST-Fe65<sup>S566A</sup> 370-666 proteins

(30 pmol) were incubated with 0.5 µg of active SGK1 in 50 µl of protein kinase assay buffer (20 mM HEPES pH 7.2, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 1 mM DTT, 0.2 mM EGTA, 20 mM ATP, and 1 µCi [ $\gamma$ -<sup>32</sup>P]-ATP) for 1 hour at 30°C. To confirm that protein phosphorylation took place, the kinase reaction mixture was treated with 10ng of alkaline phosphatase for 1 hour at 30°C. The reaction mixture was loaded onto a 10% SDS-PAGE gel, and the dried gel was exposed on x-ray film.

#### Nonradioactive protein kinase assay of SGK1 with FITC-Fe65 peptide

An SGK1 assay was performed using fluorescein-conjugated Fe65 peptides (FITC-<sup>560</sup>CRVRFLSFLA<sup>569</sup>) and the control peptides (FITC bonded with <sup>560</sup>CRVRFLAFLA<sup>569</sup>), which were purchased from the Pepton Company (Daejeon, Korea). The fluorescein-labeled oligopeptide (0.8 µg) was incubated with 10 µl of differentially treated cell lysates and active SGK1 in 20 µl of protein kinase reaction buffer (20 mM Tris HCl pH 7.4, 10 mM MgCl<sub>2</sub>, and 1 mM ATP) at 30°C for 30 minutes. The reactions were stopped at 95°C for 10 minutes. The phosphorylated peptide was separated on an 0.8% agarose gel at 100 V for 15 minutes. The phosphorylated products gained one more negative charge and migrated to the anode. After electrophoresis, the gel was photographed on a transilluminator. The optical density of the phosphorylated product was measured by densitometry (10, 22).

#### Immunofluorescence microscopy

COS-1 cells were plated to about 30% of confluence on the microscope cover glass (Fisher) in 6-well plates (Corning). Wild-type myc-Fe65 and mutant myc-Fe65<sup>S566A</sup> constructs were transfected into COS-1 cells using FuGENE6 (Roche Molecular Biochemicals), according to the manufacturer's instructions. Cells were serum-starved for 36 hours and subsequently treated with 10% calf serum for 15 hours. Cell confluence did not exceed 60%. The cells were fixed, made permeable, and processed for the direct immuno-fluorescence microscopy, as described previously, with minor modifications (23). Cells were blocked in 5% BSA in PBS for 1 hour and incubated with a 1 : 1,000 dilution of a monoclonal anti-myc antibody (Cell Signaling) or a polyclonal anti-caveolin1 antibody (Santa Cruz Biotechnology), for 2 hours at room temperature. For indirect immunofluorescence microscopy, the washed slides were incubated for 1 hour at room temperature with a 1 : 200 dilution of goat anti-rabbit Alexa Fluor 568 or goat anti-mouse Alexa Fluor 488 (Molecular Probes, Inc., OR, USA). The slides were washed then mounted with Dako fluorescent mounting medium (Dako Co) and examined using the optics at Center for Experimental Research Facilities of Chungbuk National University (23).

#### Generation of anti-phospho-Ser<sup>566</sup> Fe65

The anti-phospho-Ser<sup>566</sup> Fe65 antibody was raised by immunizing New Zealand white rabbits with the CRVRFL(pS)FLA

VG peptide conjugated to keyhole limpet hemocyanin. The antibody was first-affinity purified with the immunizing peptide conjugated to Sepharose, then nonphospho-specific antibodies were removed by adsorption onto the non-phosphorylated peptide conjugated to Sepharose. The specificity of the affinity-purified antiphospho-Ser<sup>566</sup>-Fe65 antibody was verified by dot immuno-blot against the immunizing phosphorylated peptide (the corresponding nonphosphorylated peptide). The peptides were synthesized by Pepton. Ltd. (Daejeon, Korea).

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