Insulin stimulates atypical protein kinase C-mediated phosphorylation of the neuronal adaptor FE65 to potentiate neurite outgrowth by activating ARF6-Rac1 signaling

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Abstract

Neurite outgrowth is a fundamental process in neurons that produces extensions and, consequently, neural connectivity. Neurite damage and atrophy are observed in various brain injuries and disorders. Understanding the intrinsic pathways of neurite outgrowth is essential for developing strategies to stimulate neurite regeneration. Insulin is a pivotal hormone in the regulation of glucose homeostasis. There is increasing evidence for the neurotrophic functions of insulin, including the induction of neurite outgrowth. However, the associated mechanism remains elusive. Here, we demonstrate that insulin potentiates neurite outgrowth mediated by the small GTPases ADP-ribosylation factor 6 (ARF6) and Ras-related C3 botulinum toxin substrate 1 (Rac1) through the neuronal adaptor FE65. Moreover, insulin enhances atypical protein kinase Cι/λ (PKCι/λ) activation and FE65 phosphorylation at serine 459 (S459) in neurons and mouse brains. In vitro and cellular assays show that PKCι/λ phosphorylated FE65 at S459. Consistently, insulin potentiates FE65 S459 phosphorylation only in the presence of PKCι/λ. Phosphomimetic studies show that an FE65 S459E mutant potently activates ARF6, Rac1, and neurite outgrowth. Notably, this phosphomimetic mutation enhances the FE65–ARF6 interaction, a process that promotes ARF6-Rac1-mediated neurite outgrowth. Likewise, insulin treatment and PKCι/λ overexpression potentiate the FE65–ARF6 interaction. Conversely, PKCι/λ...
INTRODUCTION

Insulin is an anabolic hormone that regulates carbohydrate metabolism in various tissues, but especially the liver, muscle, and adipose tissue. There is increasing evidence for the importance of insulin in the central nervous system. For example, it has been shown to function as a neurotrophic factor and has been implicated in neuronal survival and neurite outgrowth through the insulin receptor/phosphoinositol 3-kinase (InsR/PI3K) pathway. However, the exact downstream cascades triggered by the InsR/PI3K pathway in these processes remain obscure. Nevertheless, insulin has been shown to activate the ADP-ribosylation factor (ARF) family of small GTPases to stimulate phospholipase D, but not its nonfunctional counterpart, in fibroblasts overexpressing insulin receptors. Moreover, ARF6 is involved in insulin-stimulated adipisin secretion by 3T3-L1 adipocytes. The inactivation of ARF6 by myristoylated-ARF6 peptide results in the inhibition of insulin-stimulated glucose transporter type 4 trafficking to the plasma membrane in adipocytes. Perturbation of the ARF6 guanine-nucleotide exchange factor (GEF), steppke, in Drosophila blocks insulin receptor signaling, leading to larval growth defects. These findings underscore the functional association of insulin and ARFs.

FE65, also known as amyloid-β precursor protein-binding family B member 1, is a brain-enriched adaptor protein with multiple protein-binding domains, including a tryptophan-tryptophan domain and two phosphotyrosine-binding domains (PTB1 and PTB2). Initially, FE65 was found to interact with APP and thus, alter APP processing, which is a crucial event in the pathogenesis of Alzheimer’s disease (AD). Subsequent studies showed that FE65 participates in other neuronal processes by forming complexes with other molecules. By the PTB1 domain, FE65 interacts with ARF6, and co-expression of ARF6 and FE65, but not the binding defective mutant of FE65, markedly potentiates the neurite outgrowth in primary neurons. Of note, aberrant neurite outgrowth contributes to developmental and neurological disorders, and neurite atrophy is always observed in brain injuries. Understanding how this process occurs may allow the development of therapies to promote neurite outgrowth to facilitate the re-wiring of injured nerve cells.

As ARF6 may function downstream of insulin and the FE65-ARF6 interaction induces neurite outgrowth, it is possible that insulin promotes neurite extension by regulating this interaction. Dynamic protein phosphorylation is a key mechanism used to control intracellular signal transduction by different means, such as modulating protein–protein interactions. Of note, FE65 is a highly phosphorylated protein and some of the phosphorylated residues are reported to alter the interactions of FE65 with its interactors. Hence, phosphorylation of FE65 may play role in regulating FE65-ARF6 interaction. In this study, we show that insulin induces the PKCι/λ-mediated phosphorylation of FE65 at serine 459 (S459) and potentiates the FE65-ARF6 interaction, and consequently, neurite outgrowth.

MATERIALS AND METHODS

Cell culture and transfection

Chinese hamster ovary (CHO), human embryonic kidney 293 (HEK293) cells, and E18 rat primary cortical neurons were cultured as previously described. Cells were...
transfected either by X-tremeGene HP (Roche) or poly-ethylenimine (Polysciences, Warrington, PA, USA). FE65 KO HEK293 cells were generated from a previous study as described. Cortical neurons were transfected with Endofectin Max (GeneCopoeia, Rockville, MD, USA).

2.2 | Generation of knockout cells

PKC\(\iota/\lambda\) knockout (KO) HEK293 cells were generated as described. In brief, the single guide RNAs (sgRNAs) targeting PRKCI gene exon 1 were designed using CHOPCHOP. The sequences of the oligos were as follows: PRKCI-sgRNA1-F (5’ CACCGCGCGGTAG TGGTTCACC 3’), PRKCI-sgRNA1-R (5’ AAACGGTGGCTACTACCGCGC 3’), PRKCI-sgRNA2-F (5’ CACCGACTACCGCGGGTGAGTGTCC 3’) and PRKCI-sgRNA2-R (5’ AAACGGACACTCACCCCGGGTAGTC 3’). The designed oligos were inserted into BbsI digested pSpCas9(BB)-2A-Puro (PX459). The cells were co-transfected with the sgRNAs for 48 h and selected with 3 \(\mu\)g/ml puromycin (Invivogen) for 36 h. Successful PKC\(\iota/\lambda\) gene KO was confirmed by DNA sequencing and western blot analysis.

2.3 | Gene knockdown

Knockdown (KD) of specific gene expression in HEK293 cells and primary neurons were achieved by transfecting corresponding ON-TARGET plus siRNA and Accell siRNA (Horizon Discovery), respectively. ON-TARGET plus non-targeting control siRNAs or Accell control siRNA (Horizon Discovery) were used to control KD.

2.4 | Neurite length measurement

Measurement of neurite outgrowth was performed as described. In brief, DIV 1 E18 rat cortical neurons were co-transfected with EGFP (as morphology marker) and indicated constructs. Twenty-four hours after transfection, cells were fixed with 4% (w/v) paraformaldehyde in PBS. Fluorescent images of neurons were taken by Nikon Eclipse Ni-U upright microscope with a Nikon DS-Qi2 camera. The length of the longest neurite of each neuron was measured by ImageJ (NIH) software with the NeuronJ plugin. Neurite length was determined from soma to growth cone. For each assay, experiments were repeated three times with at least 40 neurons analyzed in each experiment. Statistical analyses were performed using one-way ANOVA (Graphpad InStat).

2.5 | Retinal explant culture

Single explants were placed on 13mm coverslips in a 24-well plate. Coverslips were coated with poly-D-lysine overnight, and cover with laminin (20\(\mu\)g/ml) for 2 h. The coverslips were washed with ultrapure water prior to use. Explants were maintained in neurobasal supplemented with B27. Insulin (10 nM) was administrated on DIV 5. Twenty-four hours after incubation, and explants were fixed with 4\% (w/v) paraformaldehyde in PBS. For immunofluorescence staining, \(\beta\)-III-tubulin was labeled using a mouse anti-\(\beta\)-III-tubulin antibody (Promega) and phosphorylated-FE65 S459 by the rat anti-pS459 anti-serum described above. Primary antibodies were detected using AlexaFluor-coupled secondary Igs and nuclei labeled with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescent images of neurons were taken by Nikon Eclipse Ni-U upright microscope with a Nikon DS-Qi2 camera. Data were obtained from at least 60 neurites (maximum 10 longest neurites from a single explant) and the experiments were repeated three times.

2.6 | Protein–protein interaction assays

2.6.1 | GST pull-down assay

GST and GST-ARF6 were expressed in Escherichia coli strain Rosetta 2 and conjugated on glutathione resin (Genscript). CHO cells transfected with indicated constructs were harvested in ice-cold lysis buffer composed of 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1\% (v/v) Triton X-100, pH 7.6 with protease inhibitor cocktail (Pierce) as described. Transfected cell lysates were incubated with GST-proteins conjugated resins at 4°C for 16 h. After incubation, unbound proteins in lysates were removed by washing the resins three times with ice-cold lysis buffer. Proteins in pulldowns were eluted in an SDS sample buffer and analyzed by western blotting. The levels of GST-baits were determined by Coomassie blue staining.

2.6.2 | Immunoprecipitation

Transfected HEK cells transfected with indicated constructs were harvested in ice-cold lysis buffer composed of 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1\% (v/v) Triton X-100, pH 7.6 with protease inhibitor cocktail (Pierce) as described. Transfected cell lysates were incubated with indicated antibodies at 4°C for 16 h. Immunoprecipitates were captured by protein A-agarose (Santa Cruz) at 4°C for 2 h. Unbound proteins
were then removed by washing the resins three times with ice-cold lysis buffer. Immunoprecipitates were resuspended in the SDS sample buffer and analyzed by western blotting.

### 2.6.3 Proximity ligation assays

Proximity ligation assay (PLA) was performed using a Duolink In Situ Fluorescence kit (Sigma). In brief, transfected HEK 293 cells on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton 24 h post-transfection. After blocking with 5% FBS in PBS at 37°C for 1 h, the cells were then incubated with goat anti-FE65 (Santa Cruz) and mouse anti-ARF6 3A-1 (Santa Cruz) for 1 h at room temperature. The cells were then washed three times with 1X Wash buffer A, followed by incubation with Duolink In Situ PLA probe anti-mouse PLUS and anti-goat MINUS at 37°C for 1 h in a humid incubator. After incubation, the cells were washed three times with 1X Wash buffer A and diluted ligase at 37°C for 30 min, and then followed by two washes with 1X Wash buffer A. Ligation was performed by adding 1X Ligation stock and diluted ligase at 37°C for 30 min, and then followed by two washes with 1X Wash buffer A. Amplification was carried out in a darkened humid incubator by incubating the cells with 1X amplification stock and diluted polymerase at 37°C for 100 min. Then the cells were washed two times with 1X Wash buffer B and then one time with 0.01X Wash buffer B. The coverslips were mounted with Duolink In Situ mounting medium with DAPI. Images were captured using an Olympus IX71 fluorescence microscope with an UPlanSAPo 60X water immersion objective. Fluorescence images were captured by a Nikon DS-Qi2 camera, and the fluorescence signals were quantified by the Object Count tool in Nikon NIS Elements. Cells were also stained with anti-β-tubulin as the morphology marker.

### 2.7 ARF6 activation assays

Rat primary embryonic cortical neurons or HEK 293 cells were lysed in ice-cold ARF6 activation buffer composed of 25 mM Tris–HCl, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 2% (v/v) glycerol, 1% (v/v) Triton-X-100, and pH 7.6. Bacterially expressed GST-tagged p21-activated kinase 1 (PAK1) p21-binding domain (PBD) was conjugated on glutathione resin (Genscript) and used as bait to pulldown active Rac1 (Rac1-GTP) in cell lysates. Cell lysates were incubated with GST- PAK1 PBD resins at 4°C for 1 h. After incubation, resins were washed with the same buffer three times. The protein that remained on resins was eluted in the SDS sample buffer. The levels of total Rac1 and Rac1-GTP were determined by western blotting.

### 2.8 Rac1 activation assays

Rat primary embryonic cortical neurons or HEK 293 cells were lysed in ice-cold Rac1 activation buffer composed of 25 mM HEPES-NaOH, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 2% (v/v) glycerol, 1% (v/v) NP40, and pH 7.5. Bacterially expressed GST-tagged p21-activated kinase 1 (PAK1) p21-binding domain (PBD) was conjugated on glutathione resin (Genscript) and used as bait to pulldown active Rac1 (Rac1-GTP) in cell lysates. Cell lysates were incubated with GST- PAK1 PBD resins at 4°C for 1 h. After incubation, resins were washed with the same buffer three times. The protein that remained on resins was eluted in the SDS sample buffer. The levels of total Rac1 and Rac1-GTP were determined by western blotting.

### 2.9 In vitro kinase assay

PKCι/λ in transfected cell lysate was immunoprecipitated and immobilized on protein A-agarose (Sigma). The immunoprecipitate was incubated with protein substrates in 1X kinase buffer (60 mM HEPES-NaOH pH 7.5, 3 mM MgCl2, 3 mM MnCl2, 1.2 mM DTT, 3 μM sodium orthovanadate). The reaction mixtures were incubated at 30°C for 5 min. The reactions were stopped by the addition of an SDS sample buffer. The mixtures were resolved on SDS-PAGE.

### 2.10 Generation of FE65 S459 phospho-specific antibody

The phospho-specific antibody against phosphorylated FE65 at S459 (pS459) was generated by immunizing rats with a synthetic FE65 phosphopeptide containing the phosphorylated S459 (amino acid 454–465; [GVGRD[pS]GRERDFC]) (Genscript). A cysteine (C) was introduced to the C-terminus to allow carrier protein conjugation and coupling to the purification column. Carrier protein conjugation was performed using the Imject Maleimide-Activated mcKLH Spin kit (ThermoFisher). The immunized rat sera were purified by sequentially passing through the non-phosphopeptide (GVGRD[SJRERDFC]) and the phosphopeptide (GVGRD[pS]GRERDFC) coupled columns prepared with the SulfoLink Immobilization kit for
peptides (ThermoFisher). The purified antibody was dialyzed against PBS/0.05% sodium azide and then concentrated with Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore).

2.11 Animal

All animal procedures were conducted with the approval of the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

2.12 Stereotaxic surgery

For stereotaxic injection, 6-month-old male mice were anesthetized with inhalation of isoflurane (VWR). Injections of FITC-insulin into the lateral ventricles were performed by removing the scalp and drilling holes 1 mm lateral and 0.5 mm posterior to the bregma. Animals received an injection of FITC-insulin (15 mU in 2 μl) or control saline into the left and right lateral ventricle, respectively, at 2.5 mm deep to the skull surface using a 26 g Hamilton syringe. The syringe was left in place for 10 min before the withdrawal. Four hours after injection, mice were perfused with PBS, and brains were fixed with 4% paraformaldehyde. Consecutive coronal brain sections were prepared by Leica CM 1950 cryostat for immunohistochemistry studies.

2.13 Plasma membrane isolation

Plasma membrane (PM) was isolated from CHO cells using a Plasma Membrane Protein Extraction Kit (Abcam) as described. Thirty percent of the elution was used for immunoblotting and 1% of total lysate was loaded as a size control. The purity of the plasma membrane preparations was validated by probing the samples with corresponding subcellular compartment marker antibodies.

2.14 Quantification and statistical analyses

All experiments were repeated at least three times. Statistical analyses were performed using one-way ANOVA with Bonferroni post hoc test or unpaired t test. Significance is indicated between different treatments as ***p < .001; **p < .01; *p < .05; ns, not significant, p > .05. Error bars shown are either SD or SEM.

2.14.1 Analysis of western blots

Chemiluminescent western blots were acquired by ChemiDoc Touch Imaging System (Bio-Rad). Densitometric analysis was performed by Image Lab Software (Bio-Rad). Data were obtained from at least three independent experiments.

3 RESULTS

3.1 Insulin potentiates neurite outgrowth by activating ARF6-Rac1 signaling via FE65

To explore the role of insulin in neurite outgrowth in an ex vivo setting, retinal explants from embryonic day 20 rats were treated with 10 nM insulin, a concentration that has been shown to markedly enhance neurite outgrowth in rat cortical neurons (Figure S1A). As shown in Figure 1A, neurite outgrowth increased significantly in retinal explants treated with insulin compared with untreated explants. Insulin has been reported to induce actin cytoskeleton remodeling through the activation of Ras-related C3 botulinum toxin substrate 1 (Rac1), a small GTPase implicated in regulating neurite outgrowth. Therefore, we sought to determine whether insulin activates neuronal Rac1. Rac1 activation was markedly enhanced in neurons treated with insulin, compared with untreated neurons (Figure 1B). Likewise, the activity of the small GTPase ARF6, an upstream regulator of Rac1, was significantly increased in insulin-treated neurons (Figure 1C).

Previously, we showed that the neuronal adaptor FE65 potentiates ARF6-Rac1-dependent neurite outgrowth. However, the signal(s) upstream of FE65 remains unknown. As our results suggested that insulin promoted neurite extension through the ARF6-Rac1 pathway, we postulated that insulin acts through FE65 to trigger this process. To test this hypothesis, neurite outgrowth was analyzed in FE65 knockdown (KD) neurons with or without insulin treatment. As previously reported, FE65 KD (FE65 siRNA1) reduced neurite outgrowth markedly in the absence of insulin (Figure 1D). Likewise, the stimulatory effect of insulin on neurite outgrowth was significantly attenuated in FE65 KD neurons (Figure 1D). The above FE65 KD effect on neurite outgrowth was confirmed by another FE65 siRNA (FE65 siRNA2) (Figure S1B).

We then used ARF6 KD neurons to investigate whether ARF6 was required for the combined effect of insulin and FE65. The overexpression of FE65 significantly promoted neurite extension (Figure 1E). Neurite outgrowth was...
further stimulated in FE65-transfected neurons treated with insulin (Figure 1E). Conversely, KD of ARF6 suppressed neurite extension, in the presence and absence of insulin (Figure 1E). Of note, the stimulatory effect of FE65 on neurite outgrowth was markedly decreased in ARF6 KD neurons, either with or without insulin treatment (Figure 1E).

To further examine the role of FE65 in transducing insulin signaling, ARF6 and Rac1 activation assays were performed in FE65 knockout (KO) cells. ARF6 was potently activated upon insulin treatment of wild-type cells, but not FE65 KO cells (Figure 1F). Similarly, insulin only stimulated Rac1 in wild-type cells and not in FE65 KO cells (Figure 1G). These results suggest a functional association between insulin and FE65 in modulating ARF6-Rac1-dependent neurite extension.

### 3.2 Insulin stimulates PKCι/λ-mediated phosphorylation of FE65 S459

FE65 is a highly phosphorylated protein with several reported phosphoresidues. Some of these residues have been shown to differentially regulate the functions of FE65. Insulin is known to regulate the activity of kinases that phosphorylate their downstream targets for
FIGURE 1  Insulin stimulates neurite outgrowth via FE65-ARF6-Rac1 signaling. (A) Insulin potentiates neurite outgrowth in retinal explant cultures. Rat E20 retinae were isolated and stimulated with 10 nM insulin on DIV 5. Explants were fixed 24 h after insulin treatment and immunostained with mouse anti-βIII-tubulin antibody. Data were obtained from at least 60 neurites (maximum 10 longest neurites from a single explant) and the experiments were repeated three times. ***p < .001. Scale bar = 50 μm. (B) Rat E18 cortical neurons were incubated with (+) or without (−) 10 nM insulin. GST-PAK1 PBD was used as bait to pull down Rac1-GTP from cell lysates. Rac1 was detected with anti-Rac1 23A8. α-tubulin was detected with anti-α-tubulin DM1A as a loading control. The relative level of Rac1-GTP was expressed as a densitometric ratio of Rac1-GTP/Rac1. **p < .01. (C) Rat E18 cortical neurons were incubated with (+) or without (−) 10 nM insulin. GST-GGA3 VHS-GAT was used as bait to pull down ARF6-GTP from cell lysates. ARF6 was detected with anti-ARF6 3A1. α-tubulin was detected with anti-α-tubulin DM1A as a loading control. The relative level of ARF6-GTP was expressed as a densitometric ratio of ARF6-GTP/ARF6. **p < .01. (D and E) Rat embryonic E18 cortical neurons were isolated and transfected with EGFP as a cell morphology marker, together with different combinations of plasmid DNAs and/or siRNAs as indicated. All transfections received the same amounts of DNA. The length of the longest neurite was measured 24 h post-transfection. Data were obtained from at least 40 cells per transfection and the experiments were repeated three times. Statistical analyses were performed by one-way ANOVA with the Bonferroni post hoc test. ***p < .001. Error bars are SEM. (F) Wild-type (WT) and FE65 knockout (KO) HEK293 cells were incubated with (+) or without (−) 10 nM insulin. GST-GGA3 VHS-GAT was used as bait to pull down ARF6-GTP from cell lysates. ARF6 and FE65 were detected with anti-ARF6 3A1 and anti-FE65 E20, respectively. α-tubulin was detected with anti-α-tubulin DM1A as a loading control. The relative level of ARF6-GTP was expressed as a densitometric ratio of ARF6-GTP/ARF6. ***p < .001. (G) Wild-type (WT) and FE65 knockout (KO) HEK293 cells were incubated with (+) or without (−) 10 nM insulin. GST-PAK1 PBD was used as bait to pull down Rac1-GTP from cell lysates. Rac1 and FE65 were detected with anti-Rac1 23A8 and anti-FE65 E20, respectively. α-tubulin was detected with anti-α-tubulin DM1A as a loading control. The relative level of Rac1-GTP was expressed as a densitometric ratio of Rac1-GTP/Rac1. ***p < .001.

signal transduction. 27,28 Thus, we anticipated that insulin would induce the phosphorylation of FE65. Western blotting analyses showed that insulin increased the levels of higher molecular weight FE65 species, which have been reported to be phosphorylated forms of FE65, 17,25 in both neurons (Figure 2A) and HEK293 cells (Figure S2A). Moreover, the intensities of the higher molecular weight species were reduced markedly after the treatment with λ protein phosphatase which suggests that they are phospho-FE65 (Figure 2A). These observations provide the first evidence that insulin triggers the phosphorylation of FE65.

Next, we attempted to identify the kinase(s) associated with FE65- and insulin-mediated neurite outgrowth. A family of insulin-activated kinases, namely aPKCs, is of interest as they have been implicated in neurite outgrowth. 7–9 Immunoblot analyses revealed that PKCλ was the major aPKC in the embryonic brain and neurons (Figure S2B), suggesting a role for this isoform in the developing brain. As previously reported, insulin treatment increased the activity of PKCλ, 29,30 as indicated by increased levels of PKCλ phosphorylation at threonine 412 (T412) (Figure S2C). Given that insulin promotes FE65 phosphorylation and PKCλ activation, we hypothesized that PKCλ phosphorylates FE65. To test this, PKCλ and two reported FE65 kinases, glycogen synthase kinase3β (GSK3β) 17 and serum/glucocorticoid regulated kinase 1 (SGK1) 16 which are known to phosphorylate FE65 PTB2, were immunoprecipitated from transfected cells and incubated with a recombinant glutathione S-transferase (GST)-fused FE65 PTB1 protein in an in vitro kinase assay. We found that only PKCλ could phosphorylate the recombinant FE65PTB1 but not the other two kinases (Figure S2D).

Among the phosphorylated residues identified in previous studies, including mass spectrometric analyses, S459 in the PTB1 domain of FE65 was predicted to be a putative target residue for aPKCs using Kinexus. Notably, this residue is found to be highly conserved among mammals (Figure S2E). To validate this prediction, in vitro kinase assays were performed. PKCλ was immunoprecipitated from transfected cells and incubated with a recombinant glutathione S-transferase (GST)-fused wild-type FE65 protein fragment (GST-FE65 450–473 ) and a mutant protein fragment with a serine to alanine mutation at residue 459 (GST-FE65 450–473S459A). As shown in Figure 2B, PKCλ robustly phosphorylated GST-FE65 450–473, but not its mutant counterpart. As the S459A mutation precludes phosphorylation of the residue, our observation suggests that FE65 S459 is a target residue for PKCλ in vitro.

To determine whether PKCλ phosphorylates FE65 S459 in cells, a phospho-specific antibody against phosphorylated FE65 S459 (pS459-FE65) was generated. We found that the overexpression of PKCλ increased the level of pS459-FE65 in cells transfected with wild-type FE65, but not in cells transfected with the S459A mutant (Figure 2C). This was consistent with the results of the in vitro kinase assay. pS459-FE65 was also detected in immunoprecipitants pulled down by an anti-FE65 antibody from embryonic rat brains (Figure 2D), suggesting that FE65 S459 phosphorylation occurs in vivo.

To examine whether insulin triggers PKCλ-mediated phosphorylation of endogenous FE65 at S459, wild-type and PKCλ KO cells were treated with insulin. As shown in Figure 2E, insulin treatment increased the level of pS459-FE65 in wild-type, but not PKCλ KO cells. Additionally, PKCλ isolated from insulin-treated cells...
was more potent at phosphorylating GST-FE65<sup>450-473</sup> in vitro than the kinase isolated from untreated cells (Figure S2F). Likewise, the pS459-FE65 levels increased in insulin-treated primary neurons (Figure 2F). We also found that pS459-FE65 staining and neurite extension were markedly enhanced in insulin-treated retinal explants,
FIGURE 2  Insulin stimulates PKCι/λ and potentiates FE65 S459 phosphorylation. (A) Rat E18 cortical neurons were incubated with (+) or without (−) 10 nM insulin. The insulin-treated neuron lysate was incubated with (+) or without (−) λ protein phosphatase (λPPase) for 1 h. Phosphorylated FE65 in cell lysates were separated with 8% SDS-PAGE and total FE65 levels were determined with 12% SDS-PAGE. Immunoblotting of FE65 was performed with an anti-FE65 E20 antibody. α-tubulin was detected with anti-α-tubulin DM1A as a loading control. The relative level of phospho-FE65 was expressed as a densitometric ratio of phosphorylated FE65 to total FE65. ***p < .001. (B) Bacterially expressed GST-FE65450-473 and GST-FE65450-473 S459A were incubated with PKCι/λ immunoprecipitated from transfected cell lysate together with (γ-[32P])-ATP for 5 min at 30°C. RM is the reaction mix only without substrate. Upper panel: autoradiograph; lower panel: Coomassie Blue staining. (C) HEK293 cells were transfected with FE65, FE65 + PKCι/λ S459E, and FE65 S459A + PKCι/λ. The amount of phosphorylated FE65 S459 (pS459), FE65, and PKCι/λ were analyzed by immunoblotting with anti-phospho-FE65 pS459, anti-FE65 E20, anti-PKCι/λ C83H11. α-tubulin was detected with anti-α-tubulin DM1A as a loading control. ***p < .001. ns, p > .05. (D) FE65 was immunoprecipitated from rat brain homogenate using anti-FE65 E20. FE65 in lysate and immunoprecipitate was probed with anti-FE65 E20, Phosphorylated FE65 S459 (pS459) in the immunoprecipitate was probed with anti-phospho-FE65 pS459, anti-FE65 E20, anti-PKCι/λ C83H11. α-tubulin was detected with anti-α-tubulin DM1A as a loading control. ***p < .001. ns, p > .05. (E) Serum-starved HEK293 cells were incubated in culture media containing 0 (−) or 10 nM (+) insulin. Phosphorylated FE65 S459 (pS459), total FE65, and α-tubulin were detected by a rat anti-phospho-S459 Fe65, goat anti-FE65 E20, and anti-α-tubulin DM1A antibody, respectively. Bar chart shows densitometric ratio of phosphorylated FE65 S459/ total FE65 (n = 4). ***p < .001. (G) Rat E20 retinas were isolated and stimulated with 10 nM insulin on DIV5. Explants were fixed 24 h after insulin administration and were co-immunostained with rat-anti-phospho-FE65 S459 and mouse anti-βIII-tubulin antibody. Data were obtained from at least 60 neurites (maximum 10 longest neurites from a single explant) and the experiments were repeated three times. ***p < .001. Scale bar = 50 μm. (H) Coronal sections of FITC-insulin injected mice brains were immunostained with rat-anti-phospho-FE65 S459 and goat anti-FE65 E20. Zoomed area of boxes at the injected sites are shown. Scale bar = 50 μm.

compared with untreated control explants (Figure 2G). We further tested whether insulin stimulates FE65 S459 phosphorylation in vivo. Mouse cerebellar cortices were stereotaxically injected with fluorescein isothiocyanate (FITC)-labeled insulin or saline as a control. The injection of FITC-insulin, compared with the injection of saline, markedly increased the staining of pS459-FE65, which largely overlapped with FE65-labeled cells (Figure 2H). Moreover, intense staining of pS459-FE65 was observed in neurons which were illustrated by β-Tubulin III (TuJ1), a neuronal marker, staining in the insulin-injected cortex (Figure S3A). Increased staining of active PKCι/λ (i.e. PKCι/λ T412) was also observed in the FITC-insulin-injected region of the cortex (Figure S3B). Together, these findings suggest that insulin stimulates the activation of PKCι/λ, and consequently, phosphorylation of FE65 at S459.

3.3  FE65 S459 phosphorylation stimulates ARF6-Rac1 signaling by potentiating FE65-ARF6 interaction

To precisely determine the effect of FE65 S459 phosphorylation, we constructed a phosphomimetic mutant of FE65 S459 (i.e. FE65 S459E). As previously reported, both ARF6 and Rac1 activities increased significantly in the presence of FE65 (Figure 3A,B).11 Intriguingly, further ARF6 and Rac1 activation were observed in transfected cells (Figure 3A,B). Moreover, FE65 S459E was found to be more potent than its wild-type counterpart at stimulating neurite outgrowth in primary embryonic neurons (Figure 3C). These stimulatory effects of FE65 and the S459E mutant were completely abolished in ARF6 KD neurons (Figure 3C). This observation is consistent with our previous finding that ARF6 is essential for the stimulatory effect of FE65 on neurite extension.11

FE65 is a molecular adaptor that functions in orchestrating protein complex formation.10,32 FE65 phosphorylation has been shown to modulate the function of FE65 by altering binding to its interactors.15–17 As ARF6 is an FE65 PTB1 interactor and the binding of ARF6 to FE65 triggers neurite outgrowth,11 we postulated that FE65 S459 phosphorylation alters the FE65-ARF6 interaction. In a co-immunoprecipitation assay, the FE65–ARF6 interaction was markedly enhanced by the S459E phosphomimetic mutation (Figure 3D). A similar result was observed in GST-pulldown assays, in which a recombinant GST-FE65 S459E bait pulled down more ARF6 than the wild-type protein (Figure 3E). We further performed proximity ligation assays (PLAs) in which increased PLA signal counts were observed in cells co-transfected with ARF6 + FE65 S459E compared with those transfected with ARF6 + FE65 (Figure 3F).

3.4  Insulin stimulates neurite outgrowth by PKCι/λ-mediated phosphorylation of FE65 S459

Our phosphomimetic study showed that FE65 S459 phosphorylation potentiates the FE65-ARF6 interaction and


FIGURE 3  FE65 S459 phosphomimetic mutation potentiates FE65-mediated neurite outgrowth and ARF6-Rac1 activation by enhancing FE65-ARF6 interaction. (A) HEK293 cells were transfected with FE65 or FE65 S459E. GST-GGA3 VHS was used as bait to pull down ARF6-GTP from cell lysates. ARF6 and FE65 were detected with anti-ARF6 3A1 and anti-FE65 E20, respectively. α-tubulin was detected with anti-α-tubulin DM1A as a loading control. The relative level of ARF6-GTP was expressed as a densitometric ratio of ARF6-GTP/ARF6. ***p < .001. **p < .01. (B) HEK293 cells were transfected with FE65 or FE65 S459E. GST-PAK1 PBD was used as bait to pull down Rac1-GTP from cell lysates. Rac1 and FE65 were detected with anti-Rac1 23A8 and anti-FE65 E20, respectively. α-tubulin was detected with anti-α-tubulin DM1A as a loading control. The relative level of Rac1-GTP was expressed as a densitometric ratio of Rac1-GTP/Rac1. ***p < .001. **p < .01. (C) Rat embryonic E18 cortical neurons were isolated and transfected with EGFP as a cell morphology marker, together with different combinations of plasmid DNAs and/or siRNAs as indicated. All transfections received the same amounts of DNA. The length of the longest neurite was measured 24 h post-transfection. Data were obtained from at least 40 cells per transfection and the experiments were repeated three times. Statistical analyses were performed by one-way ANOVA with Bonferroni post hoc test. ***p < .001. *p < .05. Error bars are SEM, scale bar = 10 μm. (D) Cells were transfected with ARF6-myc + FE65 and ARF6-myc + FE65 S459E. ARF6 was immunoprecipitated by an anti-myc 9B11 antibody. Immunoprecipitated FE65 and ARF6 were detected with anti-FE65 E20 and anti-myc 16286-1-AP. (E) Bacterially expressed GST, GST-FE65 PTB1, and GST-FE65 PTB1 S459E were used as baits to pull down ARF6 from transfected cell lysate. The amount of ARF6 was analyzed with immunoblotting with an anti-ARF6 3A1 antibody. ***p < .001. (F) HEK293 cells were transfected with FE65 + ARF6 or FE65 S459E + ARF6. β-Tubulin and DAPI were used as morphology and nucleus markers, respectively. Representative images are shown. The bar chart shows the relative PLA signal (fold to FE65 + ARF6). Data were obtained from at least 60 cells per transfection and the experiments were repeated 3 times. Error bars are SEM. ***p < .001. scale bar = 10 μm.
thereby ARF6-Rac1 signaling. As FE65 S459 is a target of PKCι/λ, we investigated the effect of the kinase on FE65-mediated neurite outgrowth. Overexpression of either PKCι/λ, FE65, or the FE65 S459A mutant stimulated neurite extension (Figure 4A). The effect of FE65, but not FE65 S459A, on neurite outgrowth was further potentiated when co-transfected with PKCι/λ (Figure 4A). Similarly, activation of ARF6 and Rac1 were observed in cells singly transfected with either PKCι/λ, FE65 or FE65 S459A (Figure 4B,C). The stimulatory effects of FE65, but not FE65 S459A, on ARF6 and Rac1 activation, were further potentiated by PKCι/λ (Figure 4B,C). In contrast, KD of PKCι/λ significantly reduced neurite outgrowth (Figure 4D). Notably, the effect of FE65 on neurite outgrowth was markedly attenuated in PKCι/λ KD neurons (Figure 4D). Likewise, marked reductions in ARF6 and Rac1 activation were observed in PKCι/λ KD cells. The stimulatory effects of FE65 on ARF6 and Rac1 activation were also significantly decreased in PKCι/λ KD cells compared with wild-type cells (Figure 4E,F). We also found that the effect of insulin on neurite outgrowth was markedly reduced in PKCι/λ KD neurons, compared with wild-type neurons, in the presence and absence of FE65 (Figure 4G). Similarly, the stimulatory effect of FE65 on neurite outgrowth, either in the presence or absence of insulin, was decreased by the atypical PKC inhibitor 2-acetyl-1,3-cyclopentanedione (ACPD)33,34 (Figure S4A).

As the phosphomimetic studies described above showed that FE65 S459 phosphorylation altered the FE65-ARF6 interaction and that S459 was a target of PKCι/λ, we sought to determine whether insulin affected the FE65-ARF6 interaction. FE65 + ARF6 and FE65 S459A + ARF6 co-transfected cells were treated with or without insulin. ARF6 was immunoprecipitated from the cell lysates using an anti-ARF6 antibody. We found that an increased amount of FE65 was co-immunoprecipitated with ARF6 in cells treated with insulin (Figure 4H, left panel). In contrast, insulin did not affect the interaction between FE65 S459A and ARF6 (Figure 4H, right panel). Similarly, more FE65 was co-immunoprecipitated with ARF6 when co-transfected with PKCι/λ (Figure 4I). PKCι/λ had no noticeable effect on the interaction between FE65 S459A and ARF6 in the co-immunoprecipitation assays (Figure 4I). ARF6 is a crucial regulator of endocytic trafficking between cytoplasm and plasma membrane (PM).35 PM is a major cellular compartment that ARF6-Rac1-mediated neurite outgrowth occurs. Since FE65 has been reported to potentiate the trafficking of a multimeric complex that contains ARF6 and FE65,12 we, therefore, tested the effect of insulin on their PM trafficking. As shown in Figure S4B, the amounts of FE65 and ARF6 in the PM fraction were increased markedly upon insulin treatment. Hence, insulin promotes neurite outgrowth, at least in part, by inducing the trafficking of FE65 and ARF6.

In conclusion, our findings suggest that insulin stimulates the PKCι/λ-mediated phosphorylation of FE65 S459, consequently enhancing the interaction between FE65 and ARF6 and leading to neurite outgrowth (Figure 5).

4 DISCUSSION

In addition to its importance in regulating blood glucose homeostasis, increasing evidence suggests that insulin has roles in the nervous system. As insulin and its receptors are expressed abundantly in the developing brain,36,37 the role of insulin signaling in the development of the central nervous system has also been proposed. Indeed, the neuron-specific knockout of insulin receptors affects hypothalamic circuit formation during mouse development, resulting in increased appetite and obesity.38 Similarly, the suppression of insulin receptor expression in the rat hypothalamus triggers hyperphagia.39 The disruption of brain insulin signaling by knocking out insulin and insulin-like growth factor 1 receptors results in cognitive impairments in mice.40 Insulin has been found to regulate synaptic density and promote dendrite and synapse regeneration, thereby restoring neuronal circuit function and plasticity.41,42 Furthermore, several studies have demonstrated the significance of insulin in regulating neurite outgrowth. Insulin has been reported to stabilize the mRNA of tubulin, an important component of neurites.43 Moreover, insulin has been proposed to stimulate neurite outgrowth by activating the PI3K-Akt pathway, as several downstream effectors of this pathway have been implicated in neurite outgrowth.44–46 These findings demonstrate the significance of insulin in neurodevelopment and maintenance. However, the exact mechanism(s) by which insulin potentiates neurite outgrowth remains obscure. Our results revealed a novel mechanism whereby insulin induces neurite outgrowth by activating the PKCι/λ-mediated phosphorylation of FE65 at S459. Of note, FE65 also binds to ELMO1, the regulatory subunit of the ELMO1-Dock1/2 bipartite Rac1 GEF complex.18 Intriguingly, FE65 has been found to mediate the formation of a multimeric complex containing ARF6 and ELMO1 and to promote the trafficking of the complex to the PM where ELMO1-Dock1/2 GEF activates Rac1.12 As ARF6 is an important regulator of endocytic trafficking between cytoplasm and PM,35 it is possible that insulin triggers neurite outgrowth by inducing PKCι/λ-mediated phosphorylation of FE65 S459 which potentiates FE65-ARF6 interaction and ARF6 activation, consequently the ARF6-directed endocytic
trafficking of the ARF6-FE65-ELMO1-Dock1/2 complex to the PM to stimulate Rac1.

The small GTPase Rac1 is a reported effector of the PI3K-Akt cascade. Insulin has been shown to activate Rac1 through the cascade to regulate some cellular processes including glucose uptake and cell migration. However, there is also evidence to suggest that the effect of insulin on Rac1 activation is independent of Akt. For example, Rac1, but not Akt, is required for actin polymerization during insulin-stimulated glucose uptake. Hence, there
is another pathway(s) that transduces the signal from insulin to Rac1. Of note, PI3K also activates phosphoinositide-dependent protein kinase 1 (PDK1), which is a kinase that phosphorylates and activates PKCι/λ. Here, we show that insulin potentiates PKCι/λ-mediated phosphorylation of FE65 S459, consequently the activation of ARF6-Rac1 signaling. As both PKCι/λ and FE65 are highly expressed in the brain, it is possible that the insulin-PI3K-PDK1 cascade plays a significant role in Rac1-mediated neurite outgrowth via PKCι/λ and FE65.

In addition to its normal neuronal roles, impaired insulin signaling has been implicated in several neurodegenerative disorders, including AD and Parkinson's disease (PD). Decreased insulin and insulin receptor expression levels have been observed with aging and in patients with AD. Type 2 diabetes (T2D) is reported to be a
risk factor for AD. Of note, intranasal insulin treatment has been shown to improve cognitive performance and/or motor function in animal models and clinical trials of AD and PD. However, the mechanism by which insulin exerts these positive effects remains elusive. As neurite degeneration and atrophy are commonly observed in neurodegenerative disorders, it has been suggested that insulin stimulates neurite regeneration. Our findings support this notion, as insulin markedly stimulated neurite outgrowth through the activation of PKCζ/λ. There is mounting evidence revealing the importance of aPKCs in the nervous system. For example, the downregulation of aPKC has been reported in AD. Moreover, PKCζ/λ has also been found to be associated with tauopathies and α-synucleinopathies, which have been suggested to impair the functions of the kinase. In contrast, in mice, the activation of aPKCs improves spatial reversal learning, which has been found to be associated with the enhanced transition of neural precursors to neurons. Taken together with our findings, these data suggest that insulin may exert its neuroprotective/regenerative role, at least in part, through the activation of PKCζ/λ.

Phosphorylation is a dynamic and reversible mechanism to regulate protein function, including altering the strength of protein–protein interaction. The functions of FE65 can be regulated by phosphorylation. The phosphorylation of FE65 at tyrosine 547 promotes its interaction with the Ras-family small GTPase Dextras1 and suppresses FE65-APP-mediated transcription. Moreover, the binding of FE65 and APP is differentially regulated by phosphorylation of FE65 at serine 610 and threonine 579, which differentially alter the effect of FE65 on APP processing. Notably, phosphorylation has been shown to alter small GTPase signaling by regulating small GTPase interactions with adaptor proteins. The phosphorylation of cell division control protein 42 (CDC42)-interacting protein 4 by protein kinase A facilitates the interaction between CDC42-interacting protein 4 and CDC42 itself, which promotes the formation of invadopodia in cancer cells. Epidermal growth factor stimulates the extracellular signal-regulated kinase-dependent phosphorylation of the adaptor SH3 protein interacting with Nck, 90 kDa (SPIN90), which is essential for SPIN90 to form a scaffold between Rab5 and the GEF Gapex5. Consistent with the above findings, the current data support the functional role of FE65 phosphorylation in modulating ARF6-Rac1-mediated neurite outgrowth. There is also evidence suggesting that phosphorylation is essential for neurite outgrowth, as pharmacological agents that alter protein phosphorylation modulate neurite outgrowth. Moreover, genome-wide KD has revealed the importance of kinases, including aPKCs, in neurite development and extension. Indeed, suppressing the expression of PKCζ, another aPKC isoform, results in a reduction in neurite outgrowth in PC12 cells. Conversely, overexpression of either PKCζ or PKCζ/λ potentiate neurite outgrowth in nerve growth factor-treated PC12 cells. Additionally, PKCζ/λ is implicated in Wnt3a-dependent neurite outgrowth through an association with Dvl2. Thus, our findings provide new insights into the mechanism by which PKCζ/λ triggers neurite outgrowth.

Although the exact mechanisms remain elusive, several kinases have been shown to activate Rac1. For example, cyclic-GMP-dependent protein kinase indirectly stimulates Rac1. Furthermore, retinoic acid-induced Rac1 activation is mediated by PI3K. Our data suggest that PKCζ/λ also acts upstream of Rac1, as PKCζ/λ overexpression potentiates Rac1 activation, whereas PKCζ/λ knockdown reduced Rac1 activation. Likewise, PKCζ/λ has been shown to activate Rac1 to increase matrix metalloproteinase-10 expression levels, which then promotes the transformation of non-small cell lung cancer cells. Notably, constitutively active Rac1 has been reported to stimulate the activity of aPKCs in prostate cancer cells, which suggests an upstream role of Rac1 in regulating aPKCs. This discrepancy may be explained by the fact that Rac1 has different functions in different cell types. For example, dominant-negative Rac1 induces the death of glioma cells but acts as a pro-survival factor in sympathetic neurons. On the contrary, the Rac1 effector p21-activated kinase, together with the actin-binding protein coronin 1A, the Rac1 GEF ArhGEF7 and the Rac1 inhibitor RhoGDIA, have been found to participate in a feed-back loop to activate Rac1. Hence, it is also possible that PKCζ/λ and Rac1 form a positive feedback loop to stimulate each other. The above findings highlight the complexity of the regulation of Rac1 by different pathways. Our study provides a detailed mechanism by which PKCζ/λ activates Rac1. However, further studies are required to delineate how Rac1 feeds back to stimulate PKCζ/λ.

**AUTHOR CONTRIBUTIONS**

Dennis Dik-Long Chau, Wen Li, Wai Wa Ray Chan, Jacquelyne Ka-Li Sun, and Yuqi Zhai performed the experiments. Dennis Dik-Long Chau, Wen Li, Wai Wa Ray Chan, Jacquelyne Ka-Li Sun, Yuqi Zhai, Hei-Man Chow, and Kwok-Fai Lau conceived the study, designed the experiments, analyzed the data, and wrote the paper. All authors read and approved the manuscript.

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