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Caveolin-1 prevents palmitate-induced NF-κB signaling by inhibiting GPRC5B-phosphorylation

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ABSTRACT

Tyrosine phosphorylation of GPRC5B and phosphorylation-dependent recruitment of Fyn through the SH2 domain have been implicated in NF-κB activation and obesity-linked adipose inflammation. GPRC5B tightly associates with caveolin-1 (Cav1); however, the role of this interaction remains elusive. Here, we report that Cav1 reduces GPRC5B-mediated NF-κB signaling by blocking GPRC5B-phosphorylation. We demonstrate highly abundant tyrosine phosphorylation of GPRC5B is observed in Neuro2a cells lacking endogenous Cav1 expression. Reversely, exogenous expression of Cav1 in these cells inhibits GPRC5B-phosphorylation. Although GPRC5B lacks conventional caveolin-binding motif, cytoplasmic tail of GPRC5B directly interacts with the C-terminal domain of Cav1. The vacant scaffolding domain of Cav1 in the protein complex suggests a potential mechanism for blocking GPRC5B-phosphorylation by Cav1, because Fyn loses the activity by binding with Cav1-scaffolding domain. Enhanced GPRC5B-mediated NF-κB signaling in Cav1-deficient cells were observed under palmitate-induced metabolic stress. These results support Cav1 functions as a negative modulator for GPRC5B action.

1. Introduction

GPRC5B was initially identified as a retinoic acid-induced gene product [1]. It belongs to GPRC5 family that is comprised of GPRC5A, GPRC5B, GPRC5C and GPRC5D in mammals, and its amino acid sequence is similar to G protein-coupled receptors (GPCR) in the class C family, such as metabotropic glutamate receptors, γ-aminobutyric acid receptors, and taste receptors. Although it has a similar sequence signature for GPCR, its endogenous agonist and G protein-related signaling are completely unknown.

A genome-wide sequence analysis revealed a strong correlation between body mass index and the presence of a 21-kb copy number variation upstream of the human *GPRC5B* gene suggesting its crucial role in metabolic regulation [2]. And the *GPRC5B* gene is evolutionarily conserved in *Drosophila*, and its orthologue in fly is called *BOSS*. *BOSS*-deficient fly shows abnormal energy metabolism, insulin signaling, change of feeding behavior, and shortened lifespan [3–5]. *GPRC5B* deficiency in mice [6] protects from diet-induced obesity and insulin resistance the underlying mechanism by which GPRC5B recruits Fyn involved in NF-κB activation implicated in chronic inflammation in adipose tissues [7]. These suggest that GPRC5B plays a significant biological role in the metabolic regulation of insulin-sensitive organs, including the central nervous system, muscles, and adipose tissues.

Cav1 is an integral membrane protein that has multi-functional features, such as organization of membrane rafts/caveolae and scaffolding various factors to regulate cellular signaling cascades [8]. Our previous study showed GPRC5B was localized in membrane rafts, and tightly associated with Cav1 [7]. However, the role of Cav1 affects to GPRC5B has not been investigated. Therefore, we focus on the potential role of Cav1 in GPRC5B-derived signaling, in particular, metabolic stress-induced NF-κB activation.

2. Material and methods

2.1. Antibodies and plasmids

Anti-GPRC5B rabbit polyclonal antibody and anti-phosphosite-specific antibodies were described previously [7]. Anti-phosphotyrosine (P-Tyr-1000), anti-caveolin-1, anti-IκBα (L35A5), anti-a-tubulin (DM1A) and anti-flotillin-1 antibodies were purchased from Cell Signaling Technology. Anti-transferrin receptor antibody was purchased from Thermo Fisher Scientific. Anti-GFP

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antibody (GF200) was purchased from Nacalai tesque. Expression plasmids encoding GPRC5B-3 × Flag and GPRC5B-AcGFP were described previously [7]. Coding regions for human Cav1 and AcGFP was amplified by PCR and inserted into pcDNA5/FRT plasmids (Invitrogen). This plasmid expressed C-terminally AcGFP tagged human Cav1 in cultured cells. PCR-amplified cDNA fragments for full-length Cav1 or its subdomains were inserted into pGEX4T-3 (GE healthcare). PCR-amplified cDNA fragment for C-terminal domain of Cav1 (135–178) was inserted into pEGFP-C1 (Clontech). All plasmids were verified by nucleotide sequencing analysis.

2.2. Cell culture and transfection

HEK293 cells and mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All culture incubations were performed in a humidified incubator at 37 °C and 5% CO_2. Spontaneously immortalized Cav1 MEFs were kindly provided by Dr. Jin-ichi Inoguchi. GPRC5B MEFs were described previously [7]. Transfection plasmid DNA into HEK293 cells was performed using TurboFect transfection reagent (Thermo Fisher Scientific) according to manufacturer's protocol. MEFs cells were transfected by electroporation under a pre-optimized square pulse condition (1400 V, 20 ms, 1 pulse) with the NEON transfection system (Invitrogen).

2.3. Confocal microscopy

GPRC5B-AcGFP transfected HEK293 cells were fixed in 3.3% paraformaldehyde for 15 min at room temperature, and then permeabilized with 0.1% saponin. Endogenous Cav1 was labeled with anti-Cav1 antibody and Alexa 546-labeled secondary antibody. The single confocal image was obtained using FLUOVIEW FV1000 confocal laser scanning microscope (Olympus).

2.4. Protein-protein interaction assay

Immunoprecipitation and GST pulldown assays were performed to determine protein-protein interactions. Cell lysates were prepared in TNE buffer (20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM EDTA, protease inhibitor and phosphatase inhibitor cocktails (Roche)) supplemented with various detergent contents (1% Triton X-100, 1% NP-40, 1% Triton X-100 plus 2% octylglucoside, or 1% Triton X-100 plus 0.1% SDS, each) with brief sonication. For immunoprecipitation, the lysates were incubated at 4 °C for 1 h with anti-Flag tagged antibody coupled magnetic beads (Wako). For the GST-pulldown assay, 1 μg of GST fusion protein–coupled glutathione-Sepharose beads (GE Healthcare) were incubated with 200 μg of cell lysates at 4 °C for 1 h. After incubation, the mixtures were extensively washed with lysis buffer. The proteins were then eluted with Laemmli sample buffer, resolved by SDS-PAGE, and detected by Western blotting. Western blotting images were analyzed using ImageJ software [9].

2.5. Membrane rafts isolation

Membrane rafts were isolated using sucrose density centrifugation as described elsewhere [10].

2.6. Luciferase reporter assay

Cells were co-transfected with p NF-κB -Luc and pGL4.75[hRLuc/CMV] plasmids (Promega). After 24 h of transfection, cells were stimulated with either 0.5 mM palmitate-BSA complex or BSA alone as a control for 8 h. Luciferase activity was measured using the Dual-Glo luciferase assay system (Promega).

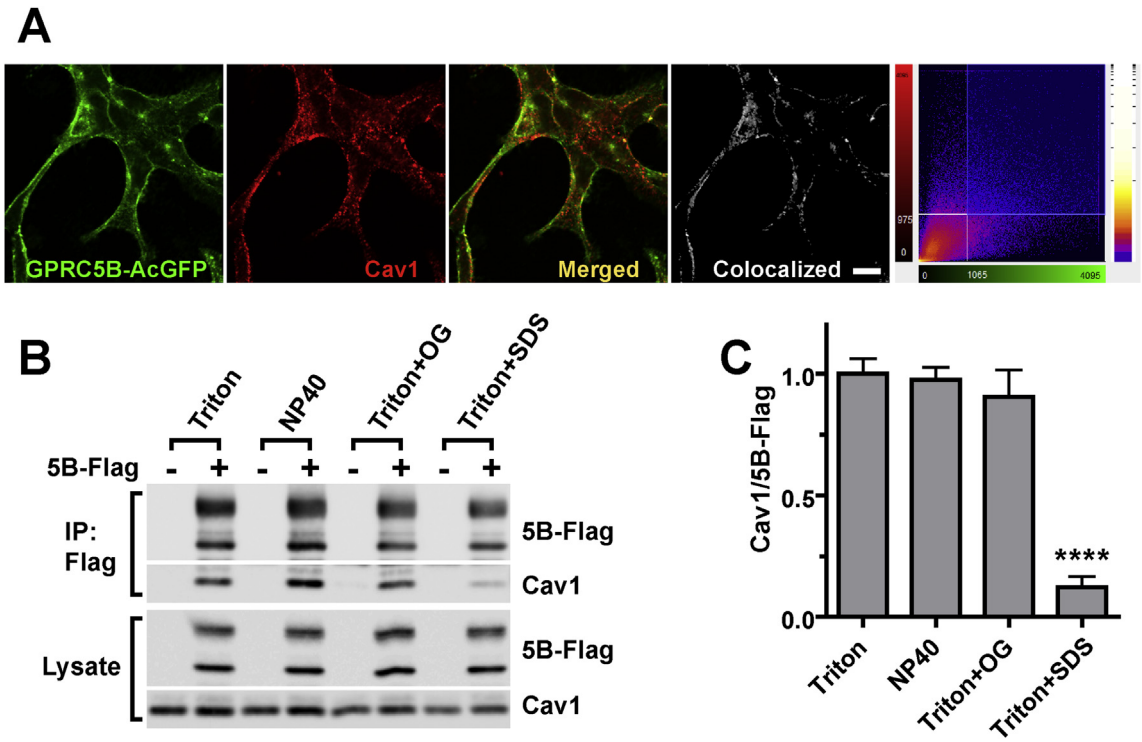

Fig. 1. GPRC5B interacts with Cav1. (A) GPRC5B is colocalized with Cav1 in the plasma membrane. Confocal images were obtained from HEK293 cells transfected with GPRC5B-AcGFP. Endogenous Cav1 was labelled using anti-Cav1 antibody and Alexa 546-labeled secondary antibody. (B) Effect of the indicated detergents on the interaction of GPRC5B and Cav1. Immunoprecipitation in lysis buffers, each containing 1% Triton X-100, NP-40, 1% Triton X100 plus 2% octylglucoside (OG), and 1% Triton X-100 plus 0.1% SDS, were examined for protein interactions between GPRC5B and Cav1. (C) Quantification of GPRC5B-associated Cav1. Data are means ± SEM (n = 3; ANOVA, $^{**}P < 0.01$).

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