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Fatty acylated caveolin-2 is a substrate of insulin receptor tyrosine kinase for insulin receptor substrate-1-directed signaling activation

Hayeong Kwon [1], Jaewoong Lee [1], Kyuho Jeong [1], Donghwan Jang, Yunbae Pak *

Department of Biochemistry, Division of Applied Life Science (BK21 Plus Program), PMBBRC, Gyeongsang National University, Jinju 660-701, Republic of Korea

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ABSTRACT

Here, we demonstrate that insulin receptor (IR) tyrosine kinase catalyzes Tyr-19 and Tyr-27 phosphorylation of caveolin-2 (cav-2), leading to stimulation of signaling proteins downstream of IR, and that the catalysis is dependent on fatty acylation status of cav-2, promoting its interaction with IR. Cav-2 is myristoylated at Gly-2 and palmitoylated at Cys-109, Cys-122, and Cys-145. The fatty acylation deficient mutants are unable to localize in the plasma membrane and not phosphorylated by IR tyrosine kinase. IR interacts with the C-terminal domain of cav-2 containing the cysteines for palmitoylation. IR mutants, Y999F and K1057A, but not W1220S, fail interaction with cav-2. Insulin receptor substrate-1 (IRS-1) is recruited to interact with the IR-catalyzed phosphotyrosine cav-2, which facilitates IRS-1 association with and activation by IR to initiate IRS-1-mediated downstream signaling. Cav-2 fatty acylation and tyrosine phosphorylation are necessary for the IRS-1-dependent PI3K-Akt and ERK activations responsible for glucose uptake and cell survival and proliferation. In conclusion, fatty acylated cav-2 is a new substrate of IR tyrosine kinase, and the fatty acylation and phosphorylation of cav-2 present novel mechanisms by which insulin signaling is activated.

1. Introduction

Insulin receptor (IR) signaling pathways regulating metabolic and mitogenic functions have been well documented [1]. IR signaling diverges into different pathways by activating multiple substrates. Caveolins, lipid rafts-associated integral membrane proteins, are required for caveolar biogenesis and caveolin-1 (cav-1) in caveolae is known to regulate IR signaling pathway [2,3]. IR was reported to phosphorylate cav-1 at Tyr-14 via binding of scaffolding domain of cav-1 (residues 82-101) to a specific motif (residues 1220-1227; *WSF*GVV*LW*) [2,4] within kinase domain of IR [2,5,6]. Caveolin-3 (cav-3) was also reported to associate directly with IR and act as an enhancer of insulin signaling in skeletal muscle [3,7]. However, recent structural and bioinformatic analyses argue against such direct physical interactions [8,9].

In a series of investigations, we have demonstrated that caveolin-2 (cav-2) interacts with IR and is phosphorylated at Tyr-19 and Tyr-27 in response to insulin, and that the phospho-tyrosine cav-2 functions as a mitogenic activator for insulin signaling in fibroblasts [10–15]. Recently, we have shown various intracellular distribution of cav-2 [15,16] and demonstrated that cav-2 forms homooligomeric cav-2 noncaveolar microdomain in the plasma membrane (PM), in which cav-2 facilitates IR recruiting and regulates activation of insulin signaling cascade by direct interaction with IR [16]. However, the underlying molecular mechanisms of their association and the consequent activation of downstream signaling have not been thoroughly investigated.

Fatty acylation of proteins is involved in the regulation of numerous cellular processes including membrane targeting and subcellular trafficking of protein, vesicular biogenesis, receptor signaling, and protein stability [17]. Multiple studies have described that both myristoylation and palmitoylation of proteins play a role in their localization to lipid rafts. Many signaling proteins residing inner side of the PM such as Lck, Fyn, and flotillins are both myristoylated and palmitoylated and the fatty acylation mediates their raft membrane association in a regulated fashion [18–20]. Cav-1 was shown to be palmitoylated at Cys-133, Cys-143, and Cys-156. The palmitoylation was not required for its localization to caveolae [21] and c-Src-mediated Tyr-14 phosphorylation of cav-1 was dependent on the palmitoylation at Cys-156 [22]. Cav-2 has putative cysteine residues at the C-terminal domain and a glycine residue at the N-terminus, but actual palmitoylation on the cysteine residues and myristoylation of the glycine are not demonstrated. Thus, the functional consequence of fatty acylation of cav-2 is not known.

Abbreviations: IR, insulin receptor; Cav-2, caveolin-2; Cav-1, caveolin-1; IRS-1, insulin receptor substrate-1; PI3K, phosphoinositide 3-kinase; PM, plasma membrane; Hirc-B, human insulin receptor-overexpressed rat 1 fibroblast; 2-BP, 2-bromopalmitate; MβCD, methyl-β-cyclodextrin; CHX, cycloheximide; MBP, maltose binding protein; IP, immunoprecipitation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; FAE, fatty acyl biotin exchange; 2-NBDG, 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose; CBM, caveolin-binding motif; CSD, caveolin scaffolding domain

* Corresponding author. Tel.: +82 55 772 1354; fax: +82 55 759 9363.
E-mail address: ybpak@gnu.ac.kr (Y. Pak).

[1] These authors contributed equally to this work.

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Data are presented to show that (i) cav-2 is myristoylated at Gly-2 and palmitoylated at Cys-109, Cys-122, and Cys-145; (ii) the fatty acylation is indispensable for the PM localization of cav-2 to interact with IR; (iii) IR catalyzes Tyr-19 and Tyr-27 phosphorylation of the fatty acylated cav-2; and (iv) IRS-1 binding to the phospho-tyrosine cav-2 facilitates IRS-1 association with and tyrosine phosphorylation by IR for IRS-1-directed metabolic and mitogenic insulin signaling activation.

2. Materials and methods

2.1. Materials

Antibodies and reagents used were purchased as follows: anti-cav-2 (BD 610685), anti-cav-1 (BD 610406), anti-phospho-tyrosine (PY20) (BD 610000), anti-ERK (BD 610124), anti-IRS-1 (BD 611395), and anti-E-cadherin (BD 610182) antibodies from BD Transduction Laboratories; anti-IR (sc-711), anti-pY1162/1163-IR (sc-25103), anti-GFP (sc-9096 AC), anti- maltose binding protein (MBP) (sc-73416), anti-α-tubulin (sc-5286), anti-calnexin (sc-11397), anti-Shc (sc-967), anti-p85α (sc-423), and anti-F-actin (sc-1616) antibodies and imatinib-mesylate (sc-202180) from Santa Cruz Biotechnology; anti-pT202/204-ERK (#9101) and anti-pS473-Akt (#9271) antibodies from Cell Signaling; anti-pY19-cav-2 (ab3417) and anti-phospho-serine (PS) (ab17465) antibodies from Abcam; anti-pY418-Src (44660G) antibody from Invitrogen; anti-FLAG® M2 (F 1804), FITC-conjugated anti-mouse (F 0257), anti-rabbit (A 6154) and anti-mouse IgG-peroxidase/HRP conjugate (A 4416) antibodies, and 2-bromopalmitate (2-BP) (21064), methyl-β-cyclodextrin (MβCD) (C4555), and cycloheximide (CHX) (C7698) from Sigma-Aldrich; PP2 (529573) from Calbiochem; human insulin from Eli Lilly; [9,10-^{3}H]Palmitic acid (30-60ci/mmol), [9,10-^{3}H]Myristic acid (10-60ci/mmol), and [γ-^{32}P]ATP (6000ci/mmol) from PerkinElmer Life Sciences.

2.2. Cell culture

Human insulin receptor-overexpressed rat 1 fibroblast (Hirc-B) cells [10,23] and cav-2 short-hairpin RNA (shRNA) stable Hirc-B cells established as described [16] were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5 mM D-glucose supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma), 100 nM methotrexate (Sigma), and 0.5% gentamycin (Gibco/BRL) in a 5% CO_2 incubator at 37 °C. HEK293T and Rat-1 cells were grown in DMEM containing 5 mM D-glucose supplemented with 10% (v/v) FBS and 0.5% penicillin/streptomycin (Sigma) in a 5% CO_2 incubator at 37 °C. 3T3L1 preadipocytes were grown in DMEM containing 25 mM D-glucose supplemented with 10% calf serum (Gibco/BRL). Confluent 3 T3-L1 preadipocytes were induced to differentiate into adipocytes as described [24].

2.3. Plasmid constructs, mutagenesis and transfection

A full-length cav-2 cDNA was subcloned into pcDNA3 vector (Invitrogen) [11]. Constructs encoding wild type (WT) cav-2 and point mutants including G2A, C109A, C122A, C145A, C109/122A, C122/145A, C109/145A, C109/122/145A (3CA), Y19A, Y27A, Y19/27A, and Y19/27E were generated by PCR mutagenesis using mutated oligonucleotides. Cav-2 domain truncation mutants including Δ1-13, Δ1-46, Δ1-70, Δ47-86 and Δ120-162 were generated by using the cav-2-GFP (WT, residues 1-162) as template via EZchange site-directed mutagenesis kit (Enzynomics, Daejeon, Korea). The resulting entry vectors of WT and mutants were converted into self-constructed FLAG tagging destination expression vector (pDS_FLAG-XB vector), GFP tagging destination expression vector (pEGFP-N1 vector, Clontech Laboratories), or MBP tagging destination expression vector (pMGWA vector). IR (NM_10051.1) plasmid (Gateway PLUS shuttle clone for INSR, GC-Y2826-CF) was obtained from Genecopoeia. For construction of a vector expressing FLAG-tagged IR, IR was moved into the pDS_FLAG-XB vector using Gateway™ Technology (Invitrogen). A construct encoding Y999F, W1220S, and K1057A mutants were generated by using the FLAG-IR (WT) as template via EZchange site-directed mutagenesis kit. All expression vectors were verified by sequencing. The pECFP-Golgi and pECFP-endoplasmic reticulum (ER) plasmids were obtained from Clontech Laboratories. For transient expression of the plasmid constructs, HEK293T cells were transfected using a modified calcium phosphate precipitation protocol as described [10,14]. 3 T3-L1 adipocytes were transfected by electroporation as described [25,26].

2.4. Silencing of IRS-1, cav-1 and cav-2 by RNA interference

Small interfering RNA (siRNA) targets of IRS-1 and cav-1 were purchased from Bioneer Corp. (Daejon, Korea) and cav-2 and scramble control from Dharmacon. The siRNA oligonucleotides were synthesized to the following target sequences: IRS-1; sense (5′-CGGUAUCGUUUCGCAUGGA-3′) and anti-sense (5′-UCCAUGCGAAACGAUACCG-3′), cav-1; sense (5′-CAGUUGUACCAUGCAUUAA-3′) and anti-sense (5′-UUAAUGCAUGGUACAACUG-3′), cav-2; sense (5′-GUAAAGACCUGCCUAAUGGUU-3′) and anti-sense (5′-PCCAUUAGGCAGGUCUUUACUU-3′), scramble control; 5′-GGAAAGACUGUUCCAAAAA-3′. Transfection of the siRNA duplexes was carried out using DharmaFECT Transfection Reagents (Dharmacon) for 48 h.

2.5. Biochemical methods

Immunoblotting, immunoprecipitation (IP), densitometry, and quantification of cholesterol were performed as described [15,16].

2.6. In vitro kinase assay

Hirc-B cells were treated with or without 100 nM insulin for 10 min and lysed with IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA) (pH 8.0), 0.2 mM sodium ortho-vanadate, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Nonidet P-40, and 60 mM n-octylglucoside). Cell lysates were immunoprecipitated with anti-IR antibody and the immunoprecipitates were washed two times in kinase assay buffer (20 mM Hepes, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 10 μM ATP, 1 mM EDTA, 1 mM sodium ortho-vanadate, and 0.4 mM PMSF). The kinase assay was conducted at 30 °C for 30 min in kinase assay buffer containing 5 μCi of [γ-^{32}P]ATP and 20 μg of MBP-cav-2 (WT), MBP-Y19A, MBP-Y27A, or MBP-Y19/27A. The reaction products were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane for autoradiography or immunoblot analysis.

2.7. In vitro binding assay

HEK293T cells were transfected with FLAG vector, FLAG-IR, FLAG-Y999F, FLAG-W1220S, or FLAG-K1057A for 48 h followed by incubation with 100 nM insulin for 10 min and lysed with IP buffer. Cell lysates (1 mg proteins) were incubated with purified MBP-cav-2, MBP-Δ1-13, MBP-Δ1-46, MBP-Δ1-70, MBP-Δ47-86, or MBP-Δ120-162 (100 μg) prebound to amylose resin at 4 °C for 4 h. Amylose resins were washed four times with IP buffer. Bound proteins were analyzed by immunoblotting.

2.8. Metabolic labeling

Hirc-B and cav-2-transfected HEK293T cells were washed with phosphate-buffered saline (PBS) and incubated in the DMEM containing 10 mg/ml fatty acid-free bovine serum albumin (BSA) for 1 h at 37 °C before incubation with 0.5 mCi/ml [9,10-^{3}H]Palmitic acid or