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A novel actin cytoskeleton-dependent noncaveolar microdomain composed of homo-oligomeric caveolin-2 for activation of insulin signaling

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ABSTRACT

The role of caveolin-2 (cav-2), independently of caveolin-1 (cav-1) and caveolae, has remained elusive. Our data show that cav-2 exists in the plasma membrane (PM) in cells lacking cav-1 and forms homo-oligomeric complexes. Cav-2 did not interact with cavin-1 and cavin-2 in the PM. Rab6-GTP was required for the microtubule-dependent exocytic transport of cav-2 from the Golgi to the PM independently of cav-1. The cav-2-oligomerized noncaveolar microdomain was unaffected by cholesterol depletion and protected from shearing of silica-coated PM. Activation of insulin receptor (IR) was processed in the microdomain. Actin depolymerization affected the formation and sustenance of cav-2-oligomerized noncaveolar microdomain and attenuated IR recruitment to the microdomain thereby inhibiting IR signaling activation. Cav-2 shRNA stable cells and the cells ectopically expressing an oligomerization domain truncation mutant, cav-$2_{\Delta 47-86}$ exhibited retardation of IR signaling activation via the noncaveolar microdomain. Elevation in status of cav-2 expression rendered the noncaveolar activation of IR signaling in cav-1 down-regulated or/and cholesterol-depleted cells. Our findings reveal a novel homo-oligomeric cav-2 microdomain responsible for regulating activation of IR signaling in the PM.

1. Introduction

Lipid rafts are fluctuating nanoscale domains assembled with cholesterol, sphingolipid, and proteins that can be stabilized into platforms and function in membrane signaling and trafficking [1–3]. Caveolae are cav-1-enriched invaginations of the PM that represent a subdomain of the lipid rafts [4]. Cholesterol is required for the caveolae formation and maintenance [5,6]. Caveolae localization of neutral glycolipids and sphingomyelin has been shown [7,8]. Ganglioside GM1 and GM3 were enriched at lesser extent in caveolae, but also present outside caveolae [9]. GM3-enriched microdomain had different lipid composition from caveolin-enriched microdomain and was not affected by cholesterol-sequestering drugs [9,10]. Thus, these reports suggest that caveolae and sphingolipid-enriched microdomain can exist independently and that specific sphingolipids and cholesterol dependency might partition differentially between caveolae and other noncaveolar microdomains.

Although cav-1 is an essential component of caveolae, cav-1 is expressed in neurons and leukocytes lacking caveolae, and caveolins can be found outside of caveolae even in cells with caveolae [11–13]. Cav-2 in the Golgi targets to caveolae upon formation of hetero-oligomers with cav-1 [14,15] but cav-2 is not essential for caveolae formation [16–18]. Homo-oligomeric cav-1 domain in the PM, distinct from caveolae has been reported [19]. Thus numerous studies have attempted to characterize heterogeneous distribution of lipids and proteins between caveolae and noncaveolar microdomains. Nevertheless, specificity for transmembrane signal activation through PM microdomains is restricted to caveolae because of lack of visibility as morphological structures and information for the dynamic formation of the noncaveolar microdomains.

Our investigation of cav-2 regulation of insulin signaling, independently of cav-1 and caveolae, in cells lacking cav-1 and cav-2-expressed cells having no endogenous caveolins showed that cav-2 interacts with IR and activates IR signaling [20–24]. Although cav-2 targeting to caveolae in PM was known to depend on cav-1 [14,15,18,25–27], our studies using subcellular fractionation and PM sheet assay indicated that cav-2 localizes in the PM in the absence of cav-1. The cav-2 localization was unaffected by cholesterol depletion and its level was not changed in response to insulin [28]. Further, our studies demonstrated that cav-2 but not cav-1 translocates to the nucleus specifically in response to insulin [20–24,28]. Our investigation on subcellular distribution of cav-2 and cav-2 pool responsible for the nuclear targeting showed that cav-2 in the Golgi, but not PM is transported to the inner nuclear membrane in response to insulin [28]. Thus the findings suggest that cav-2 pool in the PM is distinct from those in the Golgi and nucleus and might have different physiochemical property and function irrespectively of cav-1. However, even though cav-2 would presumably

Abbreviations: Cav-2, caveolin-2; Cav-1, caveolin-1; IR, insulin receptor; PM, plasma membrane; HLDM, heavy low-density insoluble membrane; LLDM, light low-density insoluble membrane; GPMV, giant plasma membrane vesicle; LDMV, low density membrane vesicle; CTB, cholera toxin B subunit; MβCD, methyl-β-cyclodextrin; CCD, cytochalasin D; LatB, latrunculin B; TfR, transferrin receptor

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interact with IR in the PM in the absence of cav-1, molecular mechanism of PM targeting of cav-2 and its functional significance in the PM have not been explored.

Here, we report that a novel actin cytoskeleton-dependent cav-2-enriched noncaveolar microdomain regulates IR signaling in the PM. Further, our data demonstrate that elevation of cav-2 expression status relative to cav-1 leads to reconstitution of the noncaveolar microdomain for activation of IR signaling in caveolae-depleted cells.

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-flotillin-1 (BD 610820), anti-ERK1 (BD 610031), anti-Akt (BD 610860), anti-phosphotyrosine-PY20 (BD 610000), and E-cadherin (BD 610182) antibodies from BD Transduction Laboratories; anti-GM130 (ab31561) antibody from Abcam; anti-calnexin (sc-11397), anti-IR (sc-711), anti-cav-2 (sc-7942), and anti-actin (sc-1616) antibodies from Santa Cruz Biotechnology; anti-GFP (#2555), anti-p-ERK (#9101), and anti-p-Akt (#9271) antibodies form Cell Signaling; anti-FLAG® M2 (F 1804), anti-rabbit IgG-peroxidase (A 6154), and anti-mouse IgG-peroxidase conjugate (A 4416) antibodies from Sigma-Aldrich; anti-transferrin receptor (TfR) (13-6800) antibody from Invitrogen; cholera toxin B subunit (CTB)-Alexa Fluor® 594 conjugates (C-34777) from Molecular Probes; human insulin from Eli Lilly; colchicine (C3915), methyl-β-cyclodextrin (MβCD) (C4555), cytochalasin D (CCD) (C8273), and 4′-6-Diamidino-2-phenylindole (DAPI) (D8417) from Sigma-Aldrich; U18666A (662015) and latrunculin B (LatB) (428020) from Calbiochem.

2.2. Cell culture

Hirc-B cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Grand Island, NY) containing 5 mM D-glucose supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma, St. Louis, MO), 100 nM methotrexate (Sigma, St. Louis, MO), and 0.5% gentamycin (Gibco/BRL, Grand Island, NY) in a 5% CO_2 incubator at 37 °C. HEK293T and 3T3L1 cells were grown in DMEM containing 5 mM D-glucose supplemented with 10% (v/v) FBS and 0.5% penicillin/streptomycin (Sigma, St. Louis, MO) in a 5% CO_2 incubator at 37 °C.

2.3. Plasmids

A full-length cav-2 (GenBank Accession No. NM_131914) [21–24,28], Rab6 (NM_002869) [28], cavin-1 (NM_012232.5), cavin-2 (NM_004657.5), or flotillin-1 (NM_005803) cDNA was subcloned into pcDNA3 vector (Invitrogen, Carlsbad, CA). The resulting entry vector of cav-2, cavin-1, cavin-2, or flotillin-1 was converted into self-constructed GFP tagging destination expression vector (pEGFP-N1 vector, Clontech Laboratories). IR (NM_10051.1) plasmid (Gateway PLUS shuttle clone for INSR, GC-Y2826-CF) was obtained from Genecopoeia. For construction of a vector expressing mCherry or FLAG-tagged cav-2 or GFP-tagged IR, cav-2 or IR was subcloned into the pDonor207 and then moved into the pDEST-N-RFP, pDS_FLAG-XB or pDS_GFP-XB vector using Gateway™ Technology (Invitrogen). DsRed-Monomer was replaced with mCherry in order to improve fluorescence intensity. FLAG-cav-$2_{\Delta 47-86}$ mutant was generated by using FLAG-cav-2 as template via EZchange site-directed mutagenesis kit (Enzynomics, Daejeon, Korea). A construct encoding T27N-Rab6 was generated by PCR mutagenesis using mutated oligonucleotides as described [28]. All expression vectors were verified by sequencing.

2.4. Reverse transcription-PCR

Total RNA was extracted from Hirc-B and HEK293T cells with TRIzole reagent (SolGent Co., Ltd.). cDNA was generated using reverse transcription kit (Bioneer). The cDNA was used as the template for the subsequent PCR amplification. PCR primers were for glyceraldehydes-3-phosphate dehydrogenase (GAPDH); 5′-ACCACCATGGAGAAGGCTGG-3′ and 5′-CTCAGTGTAGCCCAGGATGCC-3′, cav-2 (rat); 5′-ATGGGGCTGGAGACTGAGAAG-3′ and 5′-TCAGTCATGGCTCAGTTGCATG-3′, cav-2 (human); 5′-ATGGGGCTGGAGACGGAG-3′ and 5′-TCAATCCTGGCTCAGTTGCA-3′, cav-1 (rat); 5′-ATGTCTGGGGGTAAATACGTA-3′ and 5′-TCATATCTCTTTCTGCGTGCT-3′, cav-1 (human); 5′-ATGTCCACGGGCGGAGACTTC-3′ and 5′-TTATATTTCTTTCTGCAAGTTGATGCG-3′. PCR was performed using AccuPower PCR PreMix kit (Bioneer). The PCR fragments were separated by running on 1% agarose gels.

2.5. Ablation of endogenous cav-2 by shRNA

To establish stable cell lines that express a cav-2 short-hairpin RNA (shRNA), a cav-2 specific shRNA lentiviral plasmid set (MISSION®shRNA Bacterial Glycerol Stock) and the MISSION® Non-Target shRNA Control Vector (pLKO.1-puro) were purchased from Sigma. For stable transfection, Hirc-B cells were transfected with cav-2 shRNA expressing plasmid. After 48 h incubation, 1 μg/ml puromycin (Clontech, USA) was added to the cultures to select for puromycin-resistant clones. One week later, independent colonies were picked using cloning cylinder (Sigma, St. Louis, MO), subcultured, and tested for cav-2 expression by RT-PCR and immunoblot analysis. Stable cell lines that express a cav-2 shRNA were then selected.

2.6. Silencing of Rab6, cav-1 and cav-2 by siRNA

siRNA targets of Rab6, cav-1, cav-2, and scramble control were purchased from Bioneer Corp. (Daejon, Korea) and Dharmacon Research, Inc., respectively. The siRNA oligonucleotides were synthesized to the following target sequences: Rab6; sense (5′-AGGCAGAUCAGGUAAAGCA-3′) and antisense (5′-UGCUUUACCUGAUCUGCCU-3′), cav-1; sense (5′-CAGUUGUACCAUGCAUUAA-3′) and antisense (5′-UUAAUGCAUGGUACAACUG-3′), cav-2; sense (5′-GUAAAGACCUGCCUAAUGGUU-3′) and antisense (5′-PCCAUUAGGCAGGUCUUUACUU-3′, scramble control; 5′-GGAAAGACUGUUCCAAAAA-3′. Transfection of the siRNA duplexes was carried out using DharmaFECT Transfection Reagents (Dharmacon) for 48 h.

2.7. Confocal microscopy

Hirc-B cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, and permeabilized with 0.1% Triton X-100 in PBS for 20 min. Permeabilized cells were rinsed with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature (RT). Cells were rinsed with PBS and incubated with anti-cav-2 and anti-IR antibodies in 1% BSA in PBS for overnight at 4 °C. After washing three times with PBS, the cav-2 and anti-IR antibodies were detected with TRITC- and FITC-conjugated anti-mouse and anti-rabbit secondary antibodies, respectively in 1% BSA in PBS for 2 h at RT. The coverslips were washed and mounted on glass slides. Fluorescent images were obtained using an Olympus Fluoview 1000 confocal microscope attached to an Olympus BX61 vertical microscope equipped with PlanApo 60×/1.40 oil immersion objective (Olympus). Quantitation of colocalization of cav-2 with IR was performed with the Line Profile Tool of Image-ProPlus 6.1 (Media Cybernetics). To analyze colocalization of cav-2 with cavin-1 or cavin-2, cavin-1-GFP or cavin-2-GFP transfected Hirc-B cells were subjected to confocal microscopy analysis using anti-cav-2 primary and anti-TRITC-conjugated anti-mouse secondary antibodies as above.