RESEARCH ARTICLE

Proteome analysis of adipocyte lipid rafts reveals that gC1qR plays essential roles in adipogenesis and insulin signal transduction

Ki-Bum Kim[1], Bong-Woo Kim[1], Hyo-Jung Choo[1], Young-Chan Kwon[1], Byung-Yoon Ahn[1], Jong-Soon Choi[2], Jae-Seon Lee[3] and Young-Gyu Ko[1]

[1] College of Life Sciences and Biotechnology, Korea University, Seoul, Korea
[2] Proteomic Team, Korea Basic Science Institute, Daejeon, Korea
[3] Division of Radiation Molecular Oncology, Korea Institute of Radiological and Medical Sciences, Seoul, Korea

Since insulin receptors and their downstream signaling molecules are organized in lipid rafts, proteomic analysis of adipocyte lipid rafts may provide new insights into the function of lipid rafts in adipogenesis and insulin signaling. To search for proteins involved in adipocyte differentiation and insulin signaling, we analyzed detergent-resistant lipid raft proteins from 3T3-L1 preadipocytes and adipocytes by 2-DE. Eleven raft proteins were identified from adipocytes. One of the adipocyte-specific proteins was globular C1q receptor (gC1qR), an acidic 32 kDa protein known as the receptor for the globular domain of complement C1q. The targeting of gC1qR into lipid rafts was significantly increased during adipogenesis, as determined by immunoblotting and immunofluorescence. Since the silencing of gC1qR by small RNA interference abolished adipogenesis and blocked insulin-induced activation of insulin receptor, insulin receptor substrate-1 (IRS-1), Akt, and Erk1/2, we can conclude that gC1qR is an essential molecule involved in adipogenesis and insulin signaling.

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1 Introduction

Various receptors and their downstream signaling molecules are organized into specific membrane compartments within the plasma membrane [1–3]. Since the membrane compartments are not solubilized by nonionic detergents and are mainly composed of cholesterol and glycosphingolipids, they are called detergent-resistant lipid rafts [4]. In addition to cellular signaling, lipid rafts are involved in various cellular events such as endocytosis, pathogenic invasion, immune responses, and cellular migration [5–7]. Caveolae, omega-typed plasma membrane invaginations, are formed from the lipid rafts with the help of caveolin-1 (Cav-1), a protein that is highly expressed in endothelial cells, muscle cells, fibroblasts, and adipocytes [8]. Thus, lipid rafts and caveolae are not distinguishable by their biochemical properties such as detergent insolubility and low density even though caveolae are specifically involved in caveolin-dependent endocytosis, transcytosis, and potocytosis [6, 9]. Since the detergent-resistant lipid rafts and caveolae have relatively small protein

Correspondence: Dr. Young-Gyu Ko, School of Life Sciences and Biotechnology, Korea University, 1, 5-ka, Anam-dong, Sungbuk-gu, Seoul 136-701, Korea
E-mail: ygko@korea.ac.kr
Fax: +82-2-927-9028

Abbreviations: Cav-1, caveolin-1; **C/EBPβ,** CCAAT/enhancer-binding protein β; **gC1qR,** globular C1q receptor; **IR,** insulin receptor; **IRS-1,** insulin receptor substrate-1; **PC-1,** glycoprotein 1; **PKC,** protein kinase C; **PPARγ,** peroxisome proliferator-activated receptor γ; **PTP-1P,** protein tyrosine phosphatase-1B; **siRNA,** small interfering RNA

complexity with about 700 proteins [10], and are easily isolated from other cellular organelles, they are good target organelles for proteomic analysis. Indeed, protein lists of lipid rafts have been reported in various mammalian cells and tissues including liver cells, HeLa cells, fibroblasts, and primary neurons [10–14].

Lipid rafts and caveolae are major plasma membrane platforms initiating insulin signaling since they harbor a quantity of insulin receptor (IR) and IR substrate-1 (IRS-1) [15]. Since Cav-1 is associated with and stabilizes the IR, its disruption in mice leads to the degradation of IR in adipose tissue along with insulin resistance [16, 17]. In adipose tissue, glucose transporter GLUT4-containing intracellular vesicles are fused to the detergent-resistant lipid rafts immediately after insulin stimulation [18, 19]. The GLUT4 translocation into the plasma membrane requires the formation of a flotillin-CAP-Cbl complex following the recruitment of Crk and C3G into lipid rafts [20–22], indicating that lipid rafts are essential for insulin-stimulated GLUT4 translocation and glucose uptake in adipose tissue. By focusing on adipocyte lipid rafts, it may be possible to propose the precise molecular mechanism for insulin signaling with the identification of novel insulin signaling molecules.

Since the activation of different receptors and their downstream molecules are regulated by their localization in lipid rafts, the identification of such proteins in lipid rafts would provide a good insight into their functions and interactions. Here, we identified 11 adipocyte-specific raft proteins by differential 2-DE of detergent-resistant lipid rafts from 3T3-L1 preadipocytes and adipocytes. One of the proteins was globular C1q receptor (gC1qR)/HABP-1/p32/p33, a 32 kDa protein with affinity to various ligands such as the globular head of C1q, high-molecular-weight kininogen, hyaluronic acid, and fibronectin [23]. In addition to the lipid-raft association of gC1qR, our data indicate that its expression is induced during adipogenesis and that it is required for the differentiation and insulin signaling of adipocytes.

2 Materials and methods

2.1 Antibodies and reagents

Anti-flotillin-1, Cav-1, IRS-1, and phosphotyrosine protein antibodies were purchased from Transduction Laboratories (Lexington, KY, USA); anti-IRβ and gC1qR antibodies from Upstate Biotechnology (Charlottesville, VA, USA); anti-Akt, phospho-Akt (ser 473 specific), CCAAT/enhancer-binding protein β (C/EBP-β) and peroxisome proliferator-activated receptor γ (PPAR-γ) antibodies from Cell Signaling Biotechnology (Beverly, MA, USA). Anti-cytochrome c, LAMP-1, Erk, phospho-Erk, GAPDH, β-actin, and HA antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-perilipin antibody from Abcam (Cambridge, MA, USA). Protease inhibitor cocktail and phosphatase inhibitor cocktail were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). Insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were from Sigma (St. Louis, MO, USA), and 2-deoxy-D-[^{3}H] glucose from Amersham Biosciences (Piscataway, NJ, USA). Small interfering RNA (siRNA) against gC1qR (Si-gC1qR) and negative control, scrambled RNA (Si-Con) were purchased from Ambion (Austin, TX, USA). Protein-A-agarose was from Calbiochem (Cambridge, MA, USA).

2.2 Cell culture, adipocyte differentiation, electroporation, and Oil-Red O staining

3T3-L1 fibroblasts were purchased from ATCC and grown in DMEM supplemented with 1% penicillin–streptomycin (Jeil Biosciences Inc.) and 10% FCS (Jeil Biosciences, Seoul, Korea). Differentiation of postconfluent NIH 3T3-L1 fibroblasts was carried out according to the previous report [24]. For siRNA-mediated knockdown experiments, 3T3-L1 preadipocytes and adipocytes were electroporated with Si-gC1qR (120 pmol/3×10^5 cells) using MicroPorator MP-100 system (Digital Bio Technology, Suwon, Korea) according to the manufacturer's instructions. Maturation-dependent triglyceride deposit was visualized by Oil-Red O staining and quantified by measurement of its optical absorbance at 520 nm.

2.3 The isolation of detergent-resistant lipid rafts

Lipid raft isolation was performed as previously described [25]. Briefly, 3T3-L1 preadipocytes and adipocytes grown in five 150 mm dishes were lysed with 1 mL of lysis buffer (1% Triton X-100, 25 mM HEPES, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail), and applied to discontinuous sucrose gradient ultracentrifugation using SW41 Ti rotor for 18 h at 39 000 rpm, 4°C. After centrifugation, the sucrose gradients were fractionated into 13 fractions including pellet for further immunoblotting analysis. Alternatively, the floating buoyant band corresponding to lipid rafts was obtained, washed with washing buffer (25 mM HEPES, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail), pelleted, and lysed with 2-DE sample lysis buffer (9 M urea, 2 M thiourea, 100 mM DTT, 2% CHAPS, 2% IPG buffer, and protease inhibitor cocktail) for 2-DE analysis.

2.4 IEF and 2-DE

2-DE analysis was conducted as described previously [12]. The IEF of each protein sample (150 μg) was carried out in a linear wide-range IPG (pH 3.10; 24 cm long IPG strips) with a total focusing time of 81 780 Vh, at 20°C, using the IPGphor system according to the manufacturer's instruction (Amersham Biosciences). The second dimension was then carried out under constant current, in three steps (step 1: 5 W/gel; step 2: 10 W/gel; step 3: 15 W/gel) at 20°C, using an Ettandalt 6 system (Amersham Biosciences). The resultant analytical gels were visualized by silver staining.

2.5 In-gel protein digestion and ESI-MS/MS

Protein spots selected as being of interest from the 2-DE analysis were excised from the gel, destained, and subjected to tryptic digestion as described previously [12]. After desalting the peptide solution through a C_{18} nanocolumn (IN2GEN, Seoul, Korea), the MS/MS of peptides was conducted by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The source temperature used was 80°C. A 1 kV potential was then applied to precoated borosilicate nanoelectrospray needles (EconoTip™, New Objective, USA) in an ion source, combined with a 0–5 psi nitrogen back pressure, in order to ensure a stable flow rate (10–30 nL/min). The cone voltage used was 40 V. In addition, a quadrupole analyzer was applied in order to select precursor ions for fragmentation within the hexapole collision cell. Ar was used as a collision gas at a pressure of $6–7 \times 10^5$ mbar, with collision energy of 20–30 V. The product ions were analyzed with an orthogonal TOF analyzer, and were fitted with a reflector, a microchannel plate detector, and a time-to-digital converter. These data were processed with a Mass Lynx Windows NT PC system.

2.6 Database search

In order to identify the proteins, the peptide mass list was searched against amino acid sequences from the NCBInr databases (NCBInr 20041204), using the MASCOT search program (www.matrixscience.com). Search parameters for MASCOT were as follows: trypsin cleavage; allow up to one missed cleavage; no restriction on protein MW and the p*I*; peptide mass tolerance of 100 ppm; quantitative modification, carbamidomethyl (C); and nonquantitative modification, oxidation (M). A protein was identified when it had a significant MASCOT probability score ($p<0.05$).

2.7 Immunoblotting, immunoprecipitation, and immunofluorescence

Preadipocytes and adipocytes were lysed with SDS lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, and protease cocktail) for 20 min at 4°C. After microcentrifugation (14 000 rpm) for 10 min at 4°C, the whole cell lysates (supernatant) were separated on SDS-PAGE and transferred to a NC membrane. The membranes were blocked for 1 h at 37°C with 5% w/v dry milk in TBS buffer. Subsequent incubation with primary and secondary antibodies was conducted for 1 h at room temperature, respectively. The signals were visualized with an automatic image analyzing system. (LAS 3000, Fujifilm Life Science). For immunoprecipitation, whole cell lysates were incubated with 2 μg of anti-IRβ and IRS-1 antibodies for 16 h at 4°C, and then with 30 μL of protein-A-agarose (50% w/v) for 1 h at 4°C. The immunoprecipitates were analyzed by immunoblotting with an anti-tyrosine phosphoprotein antibody. For immunofluorescence, cells were fixed with 10% formaldehyde for 20 min, blocked with blocking buffer (5% BSA in PBS), and incubated with primary antibodies. The primary antibodies were detected with fluorescein-conjugated secondary antibodies. Cells were observed with a confocal microscope (Zeiss LSM 510 META).

2.8 Glucose uptake assay

Glucose uptake measurement was followed by a previous report [26] with slight modifications. Briefly, after serum starvation for 3 h, 3T3-L1 adipocytes in a 12-well plate were washed three times with PBS and incubated in 0.5 mL/well Krebs-Ringer-phosphate-HEPES buffer (10 mM HEPES, pH 7.4, 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 2.5 mM NaH_2PO_4) in the absence and presence of 100 nM insulin for 20 min at 37°C. The cells were then incubated with 2-[^{3}H]deoxyglucose (0.1 μCi, final concentration of 0.1 mM) for 15 min at room temperature. The cell-associated radioactivity was determined by scintillation counting.

3 Results

3.1 Isolation of detergent-resistant lipid rafts from preadipocytes and adipocytes

In order to identify novel lipid raft proteins involved in adipocyte differentiation, we analyzed the changes in raft protein profiles during 3T3-L1 adipogenesis. Fibroblast-like 3T3-L1 preadipocytes were differentiated into round-shaped mature adipocytes by incubating them with MDI (3-isobutyl-1-methylxanthine, dexamethasone, and insulin) media for eight days. After confirming adipogenesis with the increased expression level of adipogenic marker proteins such as PPAR-γ, C/EBP-β, perillipin, and Cav-1 (Fig. 1A), we isolated detergent-resistant lipid rafts from preadipocytes and adipocytes using discontinuous sucrose gradient ultracentrifugation. Each fraction from sucrose gradient was analyzed by immunoblotting. In both preadipocytes and adipocytes, the raft/caveolae marker proteins, flotillin-1, and Cav-1 were predominantly found in raft fractions whereas nonraft marker proteins such as LAMP-1 (a lysosomal protein) and cytochrome c (a mitochondrial protein) were in bottom fractions (Fig. 1B). These results demonstrate that detergent-resistant lipid rafts were isolated from preadipocytes and adipocytes without contaminations from other subcellular organelles.

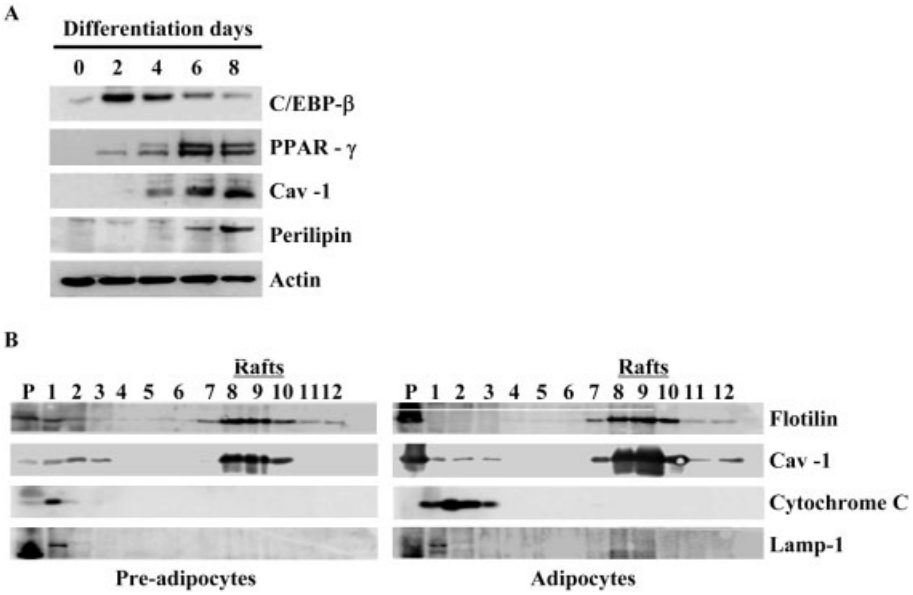

Figure 1. The isolation of detergent-resistant lipid rafts from preadipocytes and adipocytes. (A) 3T3-L1 cells were differentiated by hormonal cocktail treatment for the indicated times as described in Section 2. The whole cell lysates were analyzed by immunoblotting using anti-C/EBP-β, PPAR-γ, Cav-1, and perillipin antibodies. β actin was used for a loading control. (B) Detergent-resistant lipid rafts from 3T3-L1 preadipocytes and adipocytes (differentiated for eight days) were isolated on the basis of detergent insolubility and low density. After sucrose gradient ultracentrifugation, the sucrose gradient was fractionated from the bottom to the top. Each fraction was analyzed by immunoblotting using anti-Cav-1, flotillin-1, cytochrome c, and LAMP-1 antibodies. "P" indicates pellet. Each panel is a representative result of three separate experiments.

3.2 Adipocyte-specific raft proteins are identified by differential 2-DE analysis

The detergent-resistant lipid raft proteins from 3T3-L1 preadipocytes and adipocytes were analyzed by 2-DE. As shown in Fig. 2, many adipocyte-specific raft protein spots were revealed by the differential 2-DE analysis. Eleven adipocyte-specific spots were excised, trypsinized, and subjected to ESI-MS/MS analysis. The resultant MS/MS spectra were then used for subsequent database searches. The identified peptides were employed in the calculation of sequence coverages of the original proteins. In a database search using the MS/MS data, we were able to identify 11 adipocyte-specific raft proteins.

Table 1 lists the adipocyte-specific raft proteins identified by ESI-MS/MS. These proteins were found to correspond with the mass and p*I* values of selected protein spots in the gel images. Among these adipocyte-specific raft proteins, mitochondrial proteins such as NADH-ubiquinone oxidoreductase 42 kDa subunit, voltage-dependent anion channel 2 (VDAC2), cytochrome b-c1 complex subunit 1, mitofilin, and mortalin have been repeatedly observed in the plasma membrane as well as the detergent-resistant lipid rafts isolated from various mammalian cell lines [11–13], indicating that these mitochondrial proteins found in rafts are not simple contaminants.

One of adipocyte-specific raft proteins was gC1qR (spot no. 10), a highly acidic (p*I* of 4.7) protein which is known to interact with the globular domain of complement C1q [27]. Figure 3 shows the amino acid sequence and the representative MS/MS spectrum for gC1qR. This 32 kDa protein is ubiquitously expressed in many cell types [28] and shows affinity for a broad range of extracellular molecules such as high molecular weight kininogen, vitronectin, fibrinogen, and hyaluronic acids [29–32].

3.3 Raft targeting of gC1qR is increased during adipogenesis

Although gC1qR is known to be a multi-functional, multiligand binding protein involved in various cellular events such as viral infections and blood coagulation cascade [28, 33], its role in adipocytes has never been investigated. In order to assess the role of gC1qR in adipocytes, we determined its expression level during 3T3-L1 adipogenesis. As shown in Fig. 4A, the expression level of gC1qR was gradually increased during the adipocyte differentiation, and maintained at a high level thereafter in the fully matured adipocytes. To confirm its association with lipid rafts, we isolated the detergent-resistant lipid rafts from preadipocytes and adipocytes and analyzed gC1qR by immunoblotting. As expected, a considerable amount of gC1qR was found in the raft fractions of adipocytes but not in those of preadipocytes (Fig. 4B). Moreover, the localization of gC1qR in adipocyte lipid rafts was found to be dependent on cholesterol because gC1qR disappeared from lipid raft fractions after methyl-β-

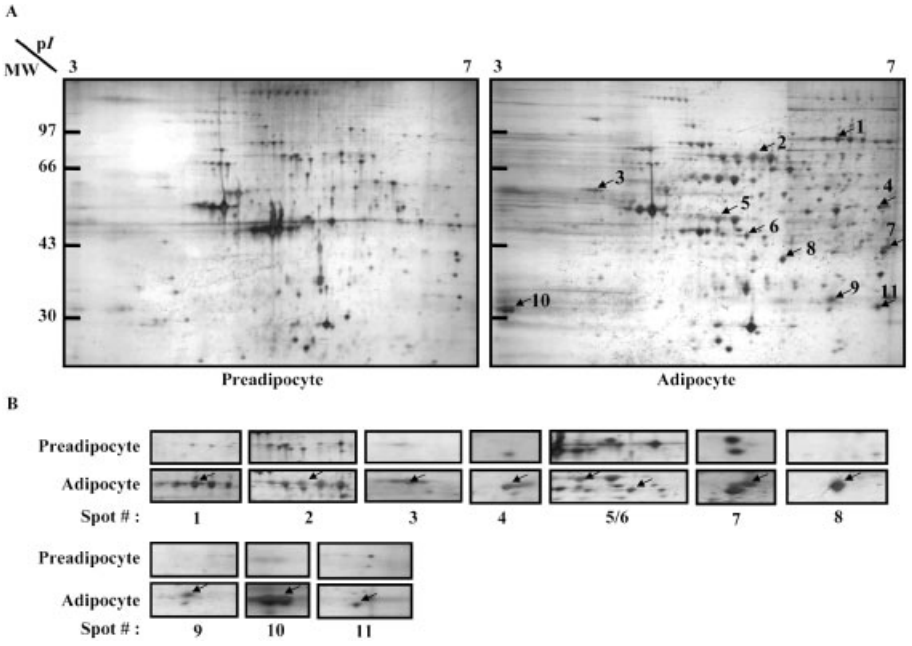

Figure 2. Differential 2-DE profiles of lipid rafts from 3T3-L1 preadipocytes and adipocytes. (A) The detergent-resistant lipid rafts were isolated from 3T3-L1 preadipocytes and adipocytes (differentiated for eight days), washed with washing buffer, and then pelleted by ultracentrifugation at 25 000 rpm, 4°C for 30 min. The pelleted lipid rafts were then solubilized with 300 μL of 2-DE sample lysis buffer containing 9 M urea and 2% CHAPS. The raft proteins (150 μg each) were subjected to large scale 2-DE and then visualized by silver staining. The adipocyte-specific spots were indicated by black arrows with numbers on the right panel. (B) The magnified view of spots indicated in A. Both 2-DE gel figures are representative of three independent experiments.

Table 1. List of adipocyte-specific lipid raft proteins identified by differential 2-DE analysis of 3T3-L1 preadipocytes and adipocytes

Description	MW (kDa)/p*I*	Gene symbol	Swiss-Prot/ TrEMBL accession no.	Sequence coverage (%)	Location
1) Mitofilin	84.2/6.18	Immt	Q8CAQ8	8	MT, PM, LR
2) GRP75; Mortalin	73.7/5.81	Hspa9	P38647	11	MT, PM, LR
3) Prolyl 4-hydroxylase, β polypeptide	57.4/4.77	P4hb	Q3TF72	6	ER
4) Dihydrolipoamide branched chain transacylase E2	53.2/8.78	Dbt	Q3TMF5	12	MT
5) Cytochrome b-c1 complex subunit 1	53.4/5.75	Uqcrc1	Q9CZ13	9	MT, LR
6) SPFH domain family member 2	38.1/5.37	Spfh2	Q8BFZ9	21	ER
7) NADH-ubiquinone oxidoreductase 42 kDa subunit	40.6/7.63	Ndufa10	Q99LC3	23	MT, LR
8) Isocitrate dehydrogenase 3 (NAD+) α	40.1/6.27	Idh3a	Q9D6R2	6	MT, LR
9) Voltage-dependent anion channel 2	35.6/5.9	Vdac2	Q60930	14	MT, LR
10) gC1qR/p32/HABP-1	31.3/4.77	C1qbp	O35658	19	MT, PM, LR
11) 3-Hydroxylisobutyrate dehydrogenase	35.8/8.37	Hibadh	Q99L13	13	MT, PM, LR

LR, lipid rafts; MT, mitochondria; PM, plasma membrane.

cyclodextrin (MβCD) treatment. Remarkably, immunofluorescence also shows that gC1qR localization changed from perinuclear to the plasma membrane during adipogenesis (Fig. 4C). Interestingly, gC1qR was co-localized with Cav-1, a caveolar marker protein in adipocytes (Fig. 4D), indicating that gC1qR is a real raft/caveolar protein in adipocytes.

3.4 Down-regulation of gC1qR blocks adipogenesis and impairs insulin signaling

In order to investigate the function of gC1qR during 3T3-L1 adipogenesis, adipogenesis was monitored by staining with Oil-Red O after down-regulating gC1qR using small RNA interference. As shown in Fig. 5A and B, the staining intensity of Oil-Red O was decreased by more than 75% in cells treated with Si-gC1qR, compared to control cells. Accordingly, the expression level of adipogenic marker proteins such as PPAR-γ and Cav-1 were largely decreased in the cells treated with Si-gC1qR (Fig. 5C). Considering transient nature of the siRNA-mediated gC1qR suppression, these results suggest that the expression of gC1qR is required in the early stage of 3T3-L1 adipogenesis.

Next, we tested the possible involvement of gC1qR in insulin signaling cascade after down-regulating gC1qR in

A

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  1 SARGHTVPWP GRPSLCTCPP RDAPSAALRA PRPRRRRPPA SRTAIPAQPL
 51 RHLLQPAPRP CLRPFGLLSV RAGSARRSGL LQPPVPCACG CGALHTEGDK
101 AFVEFLTDEI KEEKKIQKHK SLPKMSGDWE LEVNGTEAKL LRKVAGEKIT
151 VTFNINNSIP PTFDGEEEPS QGQKAEEQEP ELTSTPNFVV EVTKTDGKKT
201 LVLDCHYPED EIGHEDEAES DIFSIKEVSF QATGDSEWRD TNYTLNTDSL
251 DWALYDHLMD FLADRGVDNT FADELVELST ALEHQEYITF LEDLKSFVKN
301 Q
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B

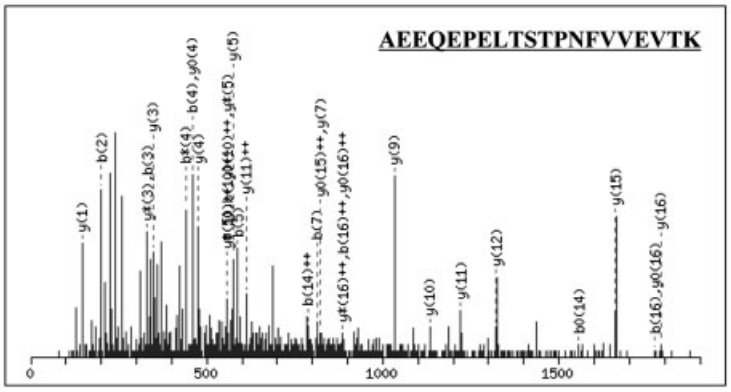

Figure 3. Peptide sequence and MS spectrum of gC1qR. (A) Peptide sequences were obtained from the tryptic digest of gC1qR (spot no. 10). The identified peptide sequences were indicated by bold letters. (B) A representative mass spectrum of the peptide underlined and in bold in A.

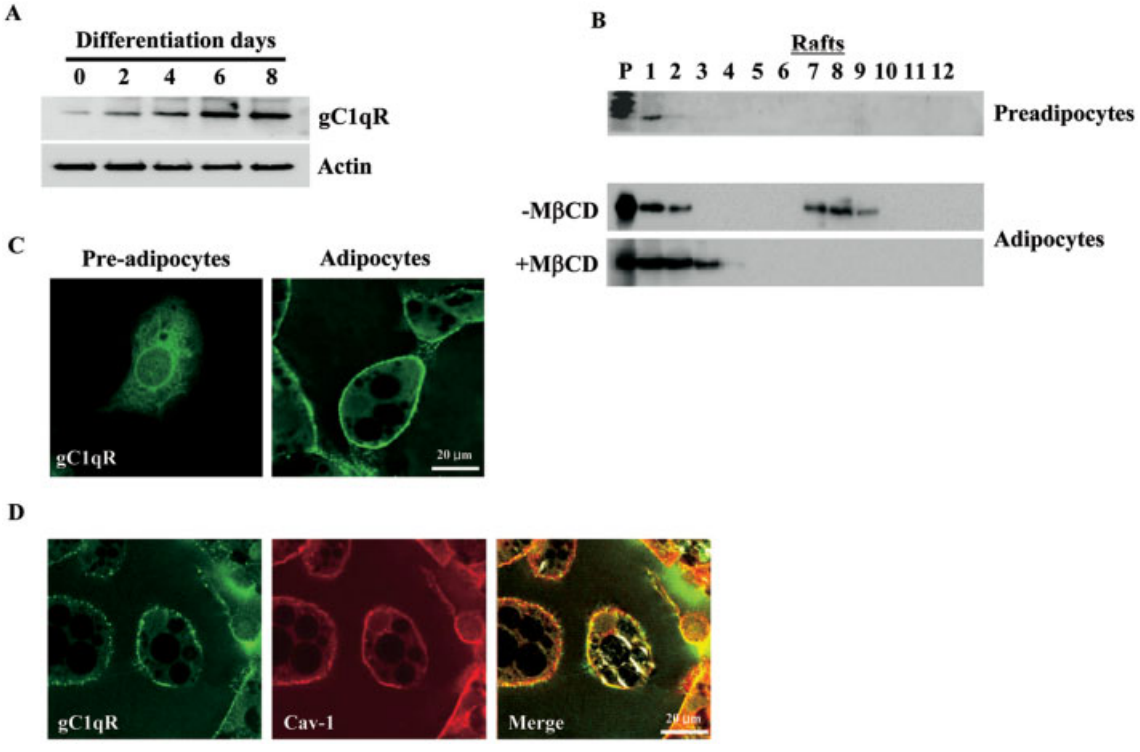

Figure 4. The expression of gC1qR is increased in lipid rafts during adipogenesis. (A) 3T3-L1 preadipocytes were differentiated into adipocytes for the indicated days and followed by the immunoblot analysis of whole cell lysates using anti-gC1qR and β-actin antibodies. (B) Detergent-resistant lipid rafts were isolated from 3T3-L1 preadipocytes and adipocytes (differentiated for eight days) with or without the treatment of 15 mM MβCD for 60 min. Each fraction from the sucrose gradient was analyzed by immunobotting with an anti-gC1qR antibody. "P" indicates pellet. (C) The localization of gC1qR was determined from preadipocytes and adipocytes by immunofluorescence with anti-gC1qR. (D) Co-localization of gC1qR with Cav-1 was determined in adipocytes by immunofluorescence. Each panel is the representative of three separate experiments.

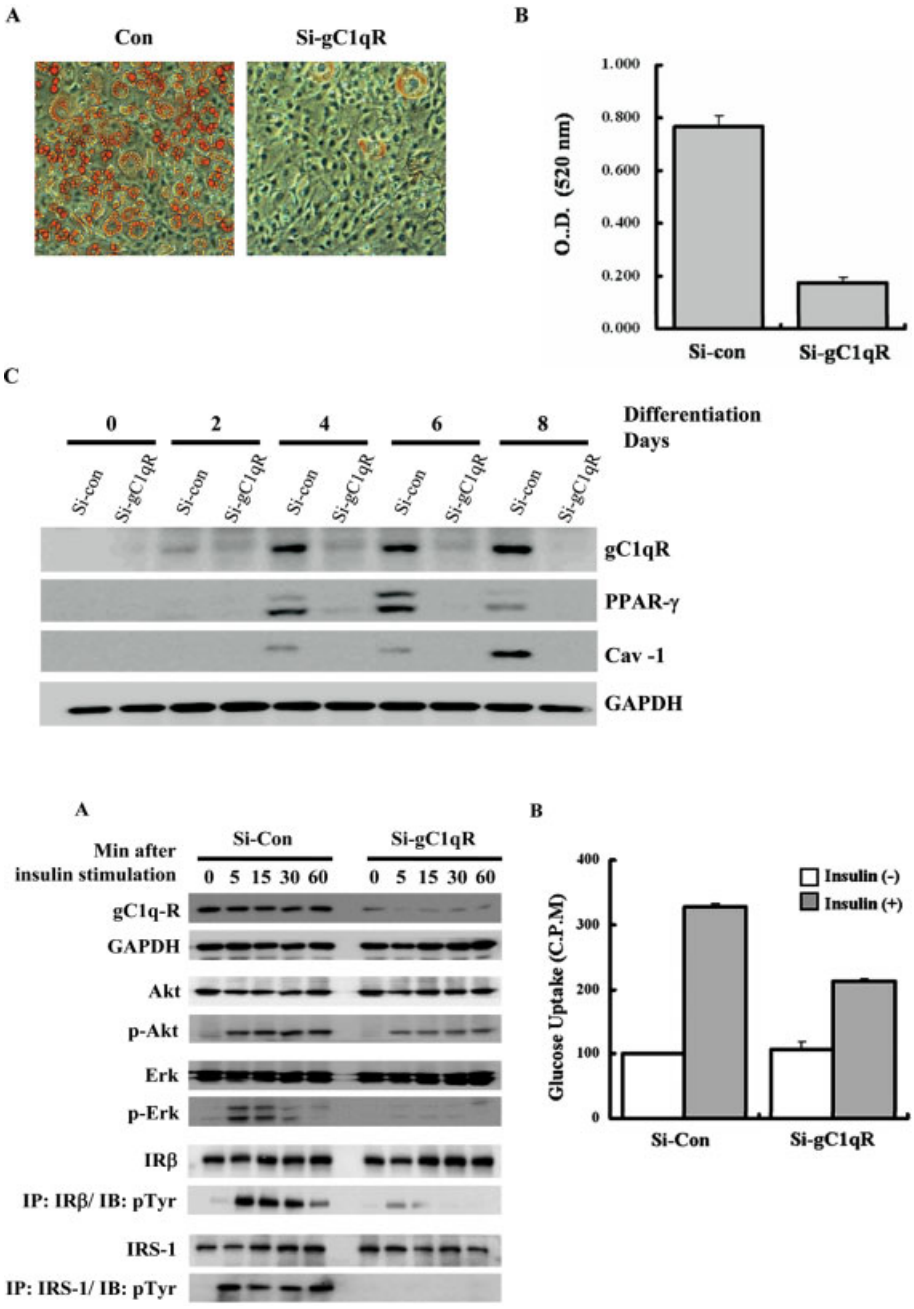

Figure 5. The down-regulation of gC1qR inhibits adipocyte differentiation. (A) 3T3-L1 preadipocytes were treated with negative control siRNA (Si-Con), and siRNA against gC1qR (Si-gC1qR) as described in Section 2. After 48 h, cells were differentiated into adipocytes for 8 days. The cells were stained with Oil-Red O for 30 min for monitoring maturation-dependent triglyceride deposit. (B) Quantification of lipid accumulation was achieved from the extracts of Oil-Red O-stained cells by measuring optical density at 520 nm. (C) 3T3-L1 preadipocytes were treated with Si-gC1qR and then differentiated for the indicated days. Adipogenesis was monitored by immunobotting with anti-gC1qR, PPAR-γ, and Cav-1 antibodies. Each panel is the representative result of three separate experiments.

Figure 6. Down-regulation of gC1qR blocks insulin signaling. (A) 3T3-L1 adipocytes (differentiated for eight days) were electroporated with 120 pmol (*per* 3×10^5 cells) of Si-Con or Si-gC1qR for 48 h, serum-starved for 16 h, and then stimulated with insulin (100 nM) for the indicated times. The level of gC1qR, Akt, phospho-Akt (p-Akt), Erk1/2, and phospho-Erk1/2 (p-Erk1/2) were analyzed by immunoblotting. Tyrosine phosphorylation of IRβ and IRS-1 was determined by immunoprecipitations with anti-IR and IRS-1 antibodies, respectively, and then by immunoblotting with an anti-tyrosine phosphoprotein antibody. (B) Adipocytes down-regulating gC1qR were serum-starved for 3 h, treated with insulin (100 nM) for 20 min, and then incubated with 0.1 μCi of 2-[^{3}H]deoxyglucose for 10 min. Cell-associated radioactivity was measured as described in Section 2. Each panel is the representative result of three separate experiments.

adipocytes. Insulin-induced IRS-1, Akt, and Erk1/2 phosphorylation was prevented in the adipocytes down-regulating gC1qR (Fig. 6A). Strikingly, gC1qR down-regulation had a direct effect on the activation of IR itself since the tyrosine phosphorylation of IR after insulin stimulation was abrogated in the adipocytes treated with Si-gC1qR (Fig. 6A). We also demonstrated that insulin-induced glucose uptake was significantly decreased in the adipocytes down-regulating

gC1qR (Fig. 6B). Taking into account all these findings, we conclude that gC1qR is an essential insulin signaling molecule in adipocytes.

4 Discussion

Differential proteomic studies have been conducted using 3T3-L1 preadipocytes and adipocytes, focusing on whole cell lysates and secreted proteins [34, 35]. Moreover, in-depth proteomic analysis of the cytosol, mitochondria, and plasma membrane reveal that targeted proteomics is a very useful approach for the identification of adipocyte proteins involved in proteasome degradation, cytoplasmic ribosomal proteins, and insulin signaling molecules [36]. Although detergent-resistant lipid rafts have the reduced protein complexity and harbor many signaling proteins, there has been no differential proteomic approach using the rafts isolated from 3T3-L1 preadipocytes and adipocytes.

Our differential proteomics of rafts revealed that many mitochondrial proteins, including NADH-ubiquinone oxidoreductase 42 kDa subunit, voltage-dependent anion channel 2 (VDAC2), cytochrome b-c1 complex subunit 1, 3-hydroxyisobyutyrate dehydrogenase, mitofilin, and mortalin were present in the rafts during adipogenesis (Table 1). These results may raise a concern that the detergent-resistant lipid rafts could contain contaminants originating from mitochondria. However, these nontraditional lipid raft proteins might not be simple intracellular contaminants since these proteins have been demonstrated to be localized within the plasma membrane lipid rafts. For example, in-depth proteomic analysis shows that the plasma membrane of 3T3-L1 adipocytes contains many mitochondrial proteins including oxidative phosphorylation (OxPhos) proteins [36]. Indeed, mitochondrial ATP synthase α and β are unambiguously demonstrated to be in the outer leaflet of plasma membrane lipid rafts in adipocytes and hepatocytes, as determined by cellular fractionation, immunofluorescence, and surface biotin labeling [11, 12, 24]. Furthermore, other mitochondrial oxidation–reduction chains such as Oxphos I, III, and IV are also exposed to the extracellular environment, as determined by immunofluorescence in nonpermeabilized embryonic kidney 293 cells [12]. Thus, the existence of mitochondrial proteins in the lipid rafts could usher us in a new door to investigate their novel functions in the plasma membrane.

Among adipocyte-specific raft proteins, gC1qR is localized in cell surface as well as in mitochondria where it functions as a multi-ligand binding protein [33]. The surface gC1qR regulates the activation of immune cells by binding to C1q [27], and is also necessary for the entry of various bacteria and viruses into host cells *via* association with its recognition molecules such as IlnB of *Listeria monocytogenes*, HIV-1 Rev and Tat, core protein V of adenovirus, and HCV core protein [37–40]. In addition, the binding of gC1qR with various extracellular molecules such as high molecular weight kininogen, vitronectin, fibrinogen, thrombin, and hyaluronic acid [29–32] indicates that the surface gC1qR is necessary for the cell attachment to extracellular matrix.

We unambiguously demonstrated that gC1qR down-regulation significantly prevented adipogenesis as indicated by the significant reduction of lipid accumulation and adipogenic marker proteins (Fig. 5). Moreover, gC1qR down-regulation had a drastic effect on insulin signaling in mature adipocytes (Fig. 6). Strikingly, gC1qR down-regulation directly inhibited the activation of IR itself through the inhibition of insulin-induced tyrosine phosphorylation of IR. Consequently, gC1qR down-regulation in adipocytes prevented insulin-induced glucose uptake as well as IRS-1, Akt, and Erk1/2 activation. This is the first report on the involvement of gC1qR in adipogenesis and insulin signaling as well as its association with lipid rafts.

From our findings of gC1qR in association with the adipocyte lipid rafts which enrich for IR and IRS-1, and the inhibitory effect of gC1qR down-regulation on the insulin-induced tyrosine phosphorylation of IR, it is tempting to speculate that gC1qR might directly regulate IR activation by its molecular association with the IR. However, we failed to demonstrate the molecular association of gC1qR with IR by co-immunoprecipitation (data not shown). IR activation is negatively regulated by plasma cell membrane glycoprotein 1 (PC-1), protein tyrosine phosphatase-1B (PTP-1B), and protein kinase C (PKC) [41]. For example, plasma membrane PC-1 blocks autophosphorylation of IR *via* a direct interaction [42–44]. PTP-1B contributes to IR inactivation by dephosphorylating tyrosine residues of IR [45, 46]. PKCβ1, β2, and θ are reported to be involved in the phosphorylation of the IR on serine and threonine residues in order to inactivate IR autophosphorylation on tyrosine residues [47–49]. Thus, it will be interesting to investigate whether gC1qR fortifies IR activation by binding and interfering with these IR-inhibiting proteins (PC-1, PTP-1B, and PKC).

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