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Association of Carboxyl Esterase with Facilitative Glucose Transporter Isoform 4 (GLUT4) Intracellular Compartments in Rat Adipocytes and Its Possible Role in Insulin-induced GLUT4 Recruitment*

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Wan Lee, Jiwon Ryu, Jongsik Hah‡, Takahiro Tsujita§, and Chan Y. Jung¶

From the Biophysics Laboratory, Veterans Affairs Medical Center, Department of Physiology and Biophysics, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14215 and the §Department of Medical Biochemistry, School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791-02, Japan

Facilitative glucose transporter isoform 4 (GLUT4) in rat adipocytes is largely sequestered in intracellular sites, and insulin recruits GLUT4 from these sites to the cell surface. The process is known to involve multiple intracellular compartments and associated proteins, many of which are yet to be identified. Recently, we purified three distinct insulin-sensitive intracellular GLUT4 compartments ($G4T_L$, G4H, and G4L) in rat adipocytes and unraveled several new resident proteins in these compartments. Here, we describe one of them, a 62-kDa protein, purified and identified as rat adipose tissue carboxyl esterase (p62/CE) by matrix-assisted laser desorption/ionization time of flight mass spectroscopy, reverse transcription-polymerase chain reaction, gene cloning, and immunological and enzymatic activity measurements. p62/CE in rat adipocytes was 80% cytosolic and 20% microsome-associated. It was found in all of the three insulin-sensitive intracellular GLUT4 compartments, and particularly enriched in $G4T_L$, a compartment thought to represent GLUT4 endocytic vesicles. Significantly, an antibody against p62/CE introduced into rat adipocytes completely abolished the insulin-induced GLUT4 recruitment to the plasma membrane in host cells without affecting the basal GLUT4 distribution. Together, these findings suggest that p62/CE plays a key role in insulin-induced GLUT4 recruitment in rat adipocytes, probably by hydrolyzing acylglycerols or acyl-CoA esters to the respective free acids that are required for GLUT4 transport vesicle budding and/or fusion.

The uptake of glucose by muscle and adipose cells is primarily catalyzed by GLUT4,[1] a facilitative glucose transporter isoform, and further regulated by hormones, specifically by insulin (1, 2). GLUT4 in these cells constantly and rapidly recycles between the cell surface and intracellular compartments, is found mostly in intracellular compartments, and is recruited to the plasma membrane in response to insulin (3–5). In morphological studies, GLUT4 is found in association with tubulovesicular structures, small vesicules, clathrin-coated lattices, and clathrin-coated vesicles (6, 7), indicating that multiple subcellular compartments are involved in GLUT4 sequestration, recycling, and recruitment. Exact roles of these intracellular compartments or organelles in GLUT4 regulation remain largely undefined. Biochemical studies, on the other hand, identified a number of proteins that are associated with immunopurified GLUT4-containing vesicles (8–18), implying that some of these proteins may play a role in GLUT4 regulation. Compartment-specific association of these proteins and their role in GLUT4 recycling and recruitment, however, are also unknown.

By using hypotonic lysis, differential centrifugation, and glycerol gradient velocity sedimentation, we (19) have recently separated GLUT4 in rat adipocytes into four distinct fractions, namely, T_H, T_L, H, and L. Data of endosomal and organelle-specific marker distribution revealed that the fraction T_H contains both intracellular and plasma membrane GLUT4 compartments (designated as $G4T_H$ and G4PM, respectively), whereas GLUT4 in the fractions T_L, H, and L represents exclusively intracellular compartments (designated as $G4T_L$, G4H, and G4L, respectively). Furthermore, GLUT4 compartments in these fractions were distinct in response to insulin; insulin treatment reduced G4H and G4L compartments and increased $G4T_L$ compartment. Based on these findings, we suggested that G4H and G4L represent the putative GLUT4 storage endosomes and GLUT4 exocytic vesicles, respectively, while $G4T_L$ represents GLUT4 endocytic vesicles. $G4T_L$, G4H, and G4L have been purified by immunoadsorption and shown to be distinct in protein composition.

In the present study, we describe an association of a 62-kDa protein with these immunopurified GLUT4 compartments. MALDI-TOF MS, RT-PCR, gene cloning, immunoreactivity, and enzyme activity assay identified this protein as a neutral carboxyl esterase expressed in rat adipocytes (20). Most importantly, the p62/CE antibody selectively abolished the insulin-induced GLUT4 redistribution to the cell surface in semipermeabilized rat adipocytes, without affecting the basal GLUT4 distribution. Based on these findings, we propose a working hypothesis that p62/CE modulates lipid metabolites in GLUT4 compartments and regulates membrane budding and fusion

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‡ Visiting Scientist from the Department of Physiology, School of Medicine, Ewha Womans University, Seoul 120-750, Korea.

¶ To whom correspondence should be addressed. Tel.: 716-862-6540; Fax: 716-862-6526; E-mail: cyjung@acsu.buffalo.edu.

[1] The abbreviations used are: GLUT4, facilitative glucose transporter isoform 4; CE, carboxyl esterase; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectroscopy; nCEH, neutral cholesterol ester hydrolase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; p62/CE, 62-kDa rat adipocyte carboxyl esterase; PAGE, polyacrylamide gel electrophoresis; SLO, streptolysin O; PM, plasma membrane; LDM, low density microsome; FAEE, fatty acid ethyl ester; ER, endoplasmic reticulum.

required for the insulin-induced GLUT4 recruitment in rat adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Collagenase (type I) was obtained from Worthington. 1F8 was purchased from Biogenesis Ltd. (Sandown, NH). Horseradish peroxidase-labeled protein A and anti-mouse IgG were from Zymed Laboratories Inc. (San Francisco, CA). Antibody against rat adipose tissue carboxyl esterase (p62/CE) was raised in rabbits using purified rat adipose tissue carboxylesterase (21). Oligonucleotides primers were from IDT (Lexington, KY).

Preparation and Hypotonic Lysis of Adipocytes—Adipocytes were isolated from epididymal fat pads of male Harlan Sprague-Dawley rats and stabilized as described (22). Adipocytes were then lysed as described (19) using hypotonic lysing medium (0.25 mM ATP, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM NAD, 0.05 mM NADP, and 1 mM $KHCO_3$, pH 7.4) (23).

Subcellular Fractionation by Differential Centrifugation and Glycerol Gradient Velocity Sedimentation—The procedures have been described (19). Briefly, adipocyte lysates (from 3–5 rats) were centrifuged at 900 × *g* for 15 min and the resulting pellet (900 × *g* pellet) was resuspended in 600 μl of NaCl/HEPES buffer (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM $MgCl_2$, pH 7.4). The 900 × *g* supernatant was centrifuged at 185,000 × *g* for 2 h, and the pellet (185,000 × *g* pellet) was resuspended in 600 μl of NaCl/HEPES buffer. Suspension of 900 × *g* or 185,000 × *g* pellet, without or after sonication (in a bath type sonicator, for 2 h at 4 °C), was then layered onto 9 ml of 5–30% glycerol gradient prepared in NaCl/HEPES buffer over 400 μl of 50% (w/v) sucrose pad in 10-cm height. Suspension on gradient was centrifuged in a 40.2SW rotor (Beckman) at 60,000 × *g* for 1 h, and 13 fractions were collected from the bottom to the top (P and 1–12). This typically separated GLUT4 into two peaks: a sharp, heavy peak (fractions P, 1, and 2) and a broad, light peak (mostly recovered in fractions 8–10). They were separately pooled, and designated as fractions T_H and T_L for 900 × *g* pellet and fractions H and L for 185,000 × *g* pellet, respectively.

Immunoadsorption—1F8 or normal mouse IgG was coupled to Tris acrylic beads (Reacti-Gel GF2000; Pierce) at a concentration of 0.7 mg of antibody/ml of resin according to the manufacturer's instructions. The antibody on beads was quenched by 2 M Tris (pH 8.0) for 1 h, incubated with 2% bovine serum albumin in phosphate-buffered saline (134 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4) for 2 h to block nonspecific binding and washed five times with 1 ml of phosphate-buffered saline at room temperature. Fractions T_L and L and sonicated fraction H (100 μg of protein each) were incubated with 30 μl of beads overnight at 4 °C, beads were settled down spontaneously, and unbound supernatants were collected for analysis. Beads were washed five times with 1 ml of phosphate-buffered saline at 4 °C, and then the adsorbed materials were eluted with SDS (2%)-containing Laemmli buffer without β-mercaptoethanol for 1 h at room temperature.

Gel Electrophoresis and Immunoblotting—Solubilized membrane in Laemmli solution was subjected to SDS-PAGE on 8, 10, 12, or 15% resolving gels as described (24). Separated proteins were electrophoretically transferred to nitrocellulose membrane (Bio-Rad), blocked with 5% nonfat milk in Tris-buffered saline, and incubated with primary antibodies in TTBS (Tris-buffered saline containing 0.05% Tween 20) containing 1% nonfat milk. After overnight incubation, membranes were washed with TTBS and incubated with horseradish peroxidase-labeled protein A (for the detection of polyclonal antibodies) or horseradish peroxidase-labeled anti-mouse IgG (for the detection of monoclonal antibodies). Proteins were visualized using enhanced chemiluminescent substrate kit (NEN Life Science Products). Immunoblot intensities were quantitated by densitometry using an analytical scanning system (Molecular Dynamics, Inc., Sunnyvale, CA).

MALDI-TOF MS Analysis—Proteins resolved by gel electrophoresis were visualized by silver staining (25) and rinsed with 1% acetic acid. Selected bands, including the one corresponding to 62 kDa, were excised into Eppendorf tube with a clean scalpel, and 10 μl of 1% acetic acid was added. Protein samples were subjected to protein identification based on MALDI-TOF MS analysis after tryptic digestion at Borealis Biosciences Inc. (Toronto, Canada) as described (26).

RT-PCR—Poly(A)$^+$ RNA of rat adipose tissue was prepared using the FastTract 2.0 kit (Invitrogen, San Diego, CA) according to the manufacturer's recommendation. 2.5 units of avian myeloblastosis virus reverse transcriptase, *Tfl* DNA polymerase (Promega, Madison, WI), primers and 1.5 μg of poly(A)$^+$ RNA of rat adipose tissue were used for reverse transcription and amplification with DNA Thermal Cycler (Perkin-Elmer Cetus, Warrington, United Kingdom). Primer sets were derived from sequences located in three different conserved sites of rat carboxyl esterase (27): CE-S1–5′, GGAGGTGGACTGGTAGTGGGT (469–489); CE-S1–3′, AATGATCCAGCCAAACTCTTG (1102–1122); CE-S2–5′, TGCCTCTACCTGAACGTTTAT (394–414); CE-S2–3′, ACCTCCTGCAGATTCTCC (703–720); CE-S3–5′, GGAGAATCTGCAGGAGGT (703–720); CE-S3–3′, GAAGAGTTCATCACCATG (1444–1461).

Northern Blot Analysis—Membranes preblotted with poly(A)$^+$ RNA from rat adipose tissue were used for detection of carboxyl esterase. The RT-PCR product from rat adipocytes poly(A)$^+$ RNA was labeled with ^{32}P using the Prime-a-Gene labeling kit (Promega) and used as the probe. Hybridization, washing, and stripping of the blots were done according to instructions provided by CLONTECH.

Cloning of p62 Gene and Transformation—The general procedure has been described (22). 3 ml of LB broth (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) containing ampicillin (100 μg/ml) was inoculated with single colony of *Escherichia coli* DH5α containing pGEX-3X plasmid DNA. After incubation at 37 °C for 15 h, the cells were collected from the culture by centrifugation (11,000 rpm, 15 min, 4 °C) and used for double-stranded DNA preparation (Promega Wizard DNA purification kit). p62 full sequence flanked with the *Sma*I restriction site on both ends was generated by RT-PCR. *Sma*I-restricted vector DNA and p62/*Sma*I fragment were ligated at 14 °C and 16 h. The ligation mix was transformed into the competent *E. coli* DH5α or BL21. From the transformant *E. coli* colony, we purified double-stranded DNA and carried out DNA sequencing.

p62/CE Fusion Protein Preparation and Purification—Seed cultures in 100 ml of LB medium containing ampicillin (100 μg/ml) were obtained by inoculating one loop of *E. coli* BL21 containing pGEX-3X/p62 and incubating at 200 rpm and 37 °C for 15 h. 1 liter of LB medium containing ampicillin (100 μg/ml) was inoculated with the seed cultures and cultivated at 37 °C and 200 rpm for 2.5 h. At that time, isopropyl-1-thio-β-D-galactopyranoside (final concentration to 0.2 mM) was added and incubated for another 4 h. The cells were collected by centrifugation, washed with phosphate-buffered saline buffer (pH 7.3) on a GS-3 rotor for 10 min at 7700 × *g* at 4 °C, and were stored at −70 °C until use. The p62 protein was purified by sonication, 1% Triton X-100 treatment, glutathione-agarose bead (Sigma) treatment, and factor Xa digestion, essentially as described (22). The degree of purification was determined by using 10% SDS-PAGE and Western blot analysis.

Carboxyl Esterase Assay—Carboxyl esterase activity against the water-soluble substrate *para*-nitrophenyl butyric acid was determined by spectrophotometric method (28). In this procedure, the samples were diluted with 0.5 M Tris-HCl (pH 7.4 or 8.0) or sodium acetate (pH 5.0) in the presence of 6 mM taurocholate. The assay was initiated by adding a freshly prepared *para*-nitrophenyl butyric acid solution (100 μg/ml in sodium acetate, pH 5.0). Carboxyl esterase activity was then monitored and compared with control autohydrolysis of the substrate using an Amersham Pharmacia Biotech spectrophotometer set at 405 nm.

Streptolysin O (SLO) Permeabilization and Antibody Treatment—Adipocytes were permeabilized with SLO as described (29) with minor modifications. Adipocytes were washed with cold intracellular buffer (140 mM potassium glutamate, 20 mM HEPES, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM NaCl, pH 7.2) and incubated with intracellular buffer containing 200 units/ml of SLO at room temperature for 15 min. Adipocytes were washed twice with intracellular buffer containing 1 mg/ml of bovine serum albumin and ATP-regenerating system (40 IU/ml creatine phosphokinase, 5 mM creatine phosphate, and 1 mM ATP) and incubated with 0.5 mg/ml anti-carboxyl esterase antibody or normal rabbit IgG for 40 min at 37 °C. When specified, adipocytes were treated with insulin (100 nM) for the last 20 min of the permeabilization protocol above.

Other Methods—DNA sequencing was done at the SUNY at Buffalo Sequencing Laboratory. Protein was determined with a BCA kit (Pierce) according to the manufacturer's instructions. Adipocyte membrane fractions enriched with plasma membranes (PMs) and low density microsomes (LDMs) were prepared after homogenization as described (3).

RESULTS

Particulate fractions T_H, T_L, H, and L (19) were prepared from rat adipocytes by hypotonic lysis, differential centrifugation, and glycerol gradient velocity sedimentation (see "Experimental Procedures"). The GLUT4 compartments in fractions T_L, H, and L, ($G4T_L$, G4H, and G4L, respectively) (19) were purified by immunoadsorption using 1F8 (see "Experimental Procedures"). GLUT4 in the fraction T_H ($G4T_H$) was not immu-

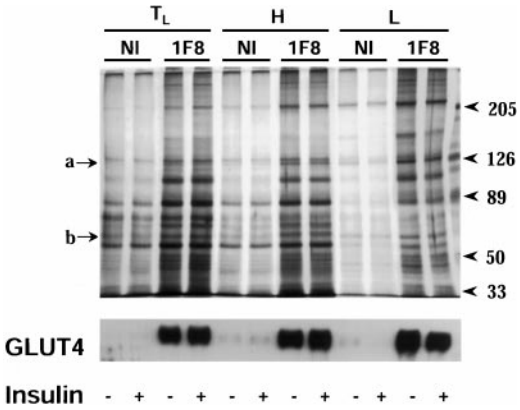

FIG. 1. **Protein composition and GLUT4 content of GLUT4 compartments immunopurified from fractions T_L, H, and L.** Fractions T_L, H, and L were isolated from basal (−) or insulin-stimulated (+) adipocytes, and each (100 μg of protein) was subjected to immunoadsorption with 1F8 (*1F8*) or normal mouse IgG (*NI*) (see "Experimental Procedures"). Adsorbed materials were eluted with SDS-containing Laemmli buffer without β-mercaptoethanol for 1 h at room temperature. After adding β-mercaptoethanol (1% final), the proteins were resolved in SDS-PAGE in 8% gel and subjected to silver staining for protein analysis (*upper panel*) and GLUT4-immunoblotting (*lower panel*). *Bands a* and *b* (*upper panel*) were 120- and 62-kDa proteins, respectively, that were purified and analyzed by MALDI-TOF MS.

noadsorbable by 1F8 and thus was not studied further here. Typical separation of protein species and GLUT4 in fractions T_L, H, and L (100 μg of protein each) during immunoadsorption is illustrated in Fig. 1. With each of these fractions, very little protein was found in materials bound to nonimmune IgG, while a number of distinct protein bands were visible in materials bound to 1F8. Furthermore, the proteins in immunoadsorbed vesicles to 1F8 differed in species and relative abundance between the fractions. GLUT4 was detected only in 1F8 adsorbates in immunoblot (Fig. 1), clearly indicating that the interaction of some of these proteins with GLUT4 is compartment-specific. A protein with an apparent molecular mass of 120 kDa (*band a* in Fig. 1) showed differential enrichment between individual GLUT4 compartments, being highest in G4L and lowest in $G4T_L$. A protein with apparent molecular mass of 62 kDa (*band b* in Fig. 1) was clearly detectable in all of these purified GLUT4 compartments, although it was particularly enriched in $G4T_L$.

The immunoadsorption procedure described in Fig. 1 was scaled up to purify proteins in sufficient quantities for sequence identification, using fraction H because of its large protein yield and GLUT4 compartment size. Proteins in 1F8-immunoadsorbed materials were separated by SDS-PAGE and visualized by silver staining as in Fig. 1. Selected protein bands (marked as *a* and *b* in Fig. 1) in gel were excised and digested with trypsin, and resulting peptide fragments were analyzed by MALDI-TOF MS (see "Experimental Procedures"). Such an analysis performed for 62-kDa protein (*b* in Fig. 1) is shown in Table I. From the linear tryptic spectrum, 19 potent peptide fragments were matched with rat hepatic neutral cholesterol ester hydrolase (nCEH; EC 3.1.1.13) (GenBank™ accession no. L46791) (30). Similarly, MALDI-TOF MS of tryptic fragments of 120-kDa protein (*a* in Fig. 1) indicated its potential identity with KIAA0339 (GenBank™ accession no. AB002337), a protein containing ATP/GTP binding site motif A, whose function is not known (31). Further characterization of this protein will be considered in a separate report.

To confirm the expression of nCEH in rat adipocytes, we performed RT-PCR in rat adipocytes using three different primer sets (see "Experimental Procedures") designed from three highly conserved regions of nCEH sequence, CE-S1, -2, and -3 (27), using β-actin as a control. RT-PCR using the primer sets for nCEH produced corresponding size of DNA from rat adipose tissue mRNA (Fig. 2*A*). To further confirm the identity of p62 with nCEH, we sequenced the CE-S1-, CE-S2-, and CE-S3-amplified DNA segments from poly(A)$^+$ RNA of rat adipose tissue obtained above. The sequence was found to be 99.7% identical to that of rat hepatic nCEH mRNA (with five nucleotide differences out of 1695 nucleotides).

TABLE I
Analysis of p62 by MALDI-TOF MS

Mass	Matching sequences with nCEH
2845.8	Glu^{106}–Lys^{129}
1628.5	Phe^{65}–Lys^{78}
1709.1	Leu^{172}–Arg^{186}
3232.6	Ala^{243}–Lys^{274}
1375.8	Thr^{275}–Lys^{286}
2891.5	Thr^{275}–Lys^{299}
1534.7	Gln^{287}–Lys^{299}
2662.7	Ser^{378}–Lys^{400}
3998.2	Ser^{378}–Lys^{412}
1071.7	Tyr^{401}–Lys^{410}
2800.0	Asp^{437}–Lys^{460}
3918.9	Thr^{461}–Lys^{496}
927.5	Tyr^{497}–Arg^{503}
2863.0	Tyr^{497}–Lys^{512}
1244.8	Ile^{527}–Lys^{538}
2604.5	Ile^{527}–Arg^{549}
1621.4	Leu^{537}–Arg^{549}
1136.7	Leu^{537}–Lys^{551}
830.4	Glu^{541}–Arg^{549}

Next, CE-S1, -2, and -3 above were labeled by random priming with [^{32}P]dATP and used as probes for Northern blot analysis. A typical result of the Northern blot with CE-S1 is illustrated in Fig. 2*B*, again using β-actin as a control. p62 mRNA was detected in rat adipose tissue at appropriate size (about 1.9 kb), demonstrating that a nCEH-like protein p62 (hereafter referred to as p62/CE) is indeed expressed in rat adipose tissue. We cloned p62/CE (GenBank™ accession number AF171640). Deduced amino acid sequence of cloned p62/CE was identical to that of rat liver carboxyl esterase (ES-10) (32) with an exception of one residue (Asn instead of Lys at position 265). It was also identical to that of rat hepatic nCEH (30) with an exception of three (Ala, Ser, and Lys instead of Glu, Thr, and Gln, at 420, 491, and 492, respectively) residues.

We expressed the clone and purified the recombinant protein. The recombinant p62/CE possessed carboxyl esterase activity; it hydrolyzed *para*-nitrophenyl butyric acid *in vitro* (Fig. 3). The esterase activity displayed a simple Michaelis type of kinetics with estimated K_m and V_{max} values of 0.76 mM and 1.45 units/mg, respectively. The enzyme activity was approximately 2.5-fold greater at pH 8 than at pH 5 (not illustrated), indicating that p62/CE is a neutral carboxyl esterase (20).

A carboxyl esterase, known as FAEE synthase/CE, has been purified from rat adipose tissue by one of us (20) and partially characterized. A polyclonal antibody was raised using the purified protein of this carboxyl esterase (21). The antibody has been shown to be highly specific to the enzyme; it reacted with only one protein in rat adipose tissue extract with no other cross-reactive protein. Furthermore, The antibody inhibited CE activity in rat adipose tissue but not other carboxyl ester-hydrolyzing enzymes in the tissue including lipoprotein lipase, hormone-sensitive lipase, cholesterol esterase, and cholinesterase.

This antibody cross-reacted with purified recombinant p62/CE in immunoblot (Fig. 2*C*). Although the DNA sequence has not been reported, the N-terminal 27-amino acid sequence of FAEE synthase/CE has been known (21), which is identical to that of p62/CE, suggesting that the two are the same protein.

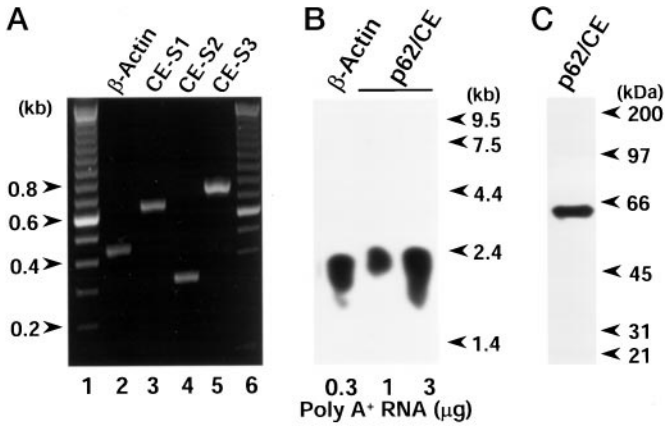

FIG. 2. **Identification of p62/CE in rat adipose tissue by RT-PCR, Northern blot, and Western blot analyses.** *A*, RT-PCR analysis in poly(A)$^+$ RNA from rat adipocytes was done using the primers derived from sequences located in different conserved sites of CE as described under "Experimental Procedures." 25 μl of PCRs were analyzed in a 1% agarose gel. CE-S1, CE-S2, and CE-S3 were amplified by CE-S1–5′ and S1–3′ primers (*lane 3*), CE-S2–5′ and CE-S2–3′ primers (*lane 4*), and CE-S3–5′ and CE-S3–3′ primers (*lane 5*), respectively. *Lanes 1* and *6* were DNA size markers, while *lane 2* contained a β-actin fragment as a control. *B*, the CE-S1 labeled with [^{32}P]dATP from *A* was used as a probe for Northern blot analysis to detect cholesterol esterase mRNA poly(A)$^+$ RNA of rat adipose tissue as described under "Experimental Procedures." Blots were analyzed by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA) after 3-day exposure. The images have been digitally processed to facilitate qualitative comparisons. *C*, recombinant p62/CE was produced from BL21 cells containing pGEX-3X/p62 construct. Recombinant p62/CE protein (100 ng) was subjected to 10% SDS-PAGE separation and then Western blot using the antibody specific to p62/CE as described under "Experimental Procedures." Molecular marker positions are indicated by *arrowheads*.

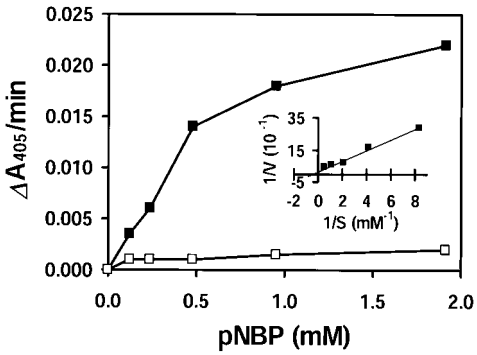

FIG. 3. **Carboxyl esterase activity of p62/CE and its inactivation by p62/CE antibody.** The esterase activity was measured as described under "Experimental Procedures." Each reaction mixture contained 3.5 μg of recombinant p62/CE without (*closed squares*) and with (*open squares*) 20 μg of rat adipose tissue carboxyl esterase antibody added 1 h prior to initiation of the reaction. Each point represents an average of triplicate measurements from three independent experiments. A double reciprocal plot of the data obtained with no antibody is shown in the *inset*.

The antibody inhibited the esterase activity of purified p62/CE recombinant protein *in vitro* (Fig. 3). The antibody also reacted with the rat liver carboxyl esterase (ES-10) and rat hepatic nCEH (21). We refer to this antibody as anti-p62/CE antibody and have used it here for characterization of p62/CE in rat adipocytes.

Direct testing of the identity of p62 purified from the GLUT4 compartment with cloned p62/CE was felt to be imperative at this point. To this end, we immunoadsorbed fraction H by 1F8, separated the adsorbed proteins by SDS-PAGE, and dissected the 62-kDa protein band in gel. This is the protein sample that we used for MALDI-TOF MS analysis discussed above. When the protein in gel was microeluted and immunoprecipitated

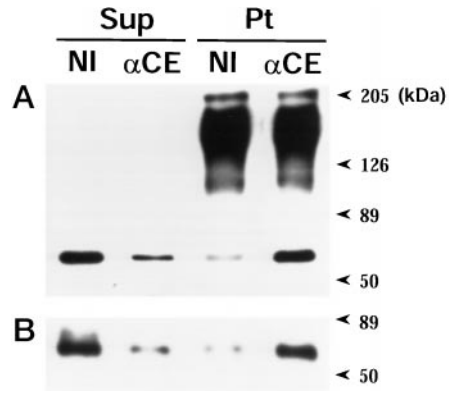

FIG. 4. **Immunoprecipitation of purified p62 by p62/CE antibody.** Protein p62 was purified from GLUT4-containing vesicles in fraction H after immunoadsorption with 1F8 as described in the legend to Fig. 1. The p62 was microeluted from SDS gel and subjected to immunoprecipitation with 3 μg of rat carboxyl esterase antibody (αCE) and protein A-agarose beads. 3 μg of normal rabbit IgG (*NI*) were used for nonimmune control. ⅕ of the supernatant (*Sup*) and ½ of the precipitates (*Pt*) were separated on SDS-PAGE, and p62/CE was detected by immunoblotting using rat carboxyl esterase antibody (*A*) and by silver stain (*B*). Positions of molecular size markers are indicated in kDa. Similar results were reproduced in two other experiments.

with FAEE synthase/CE antibody (Fig. 4), more than 80% of p62 was precipitated as assessed by either immunoblotting or protein staining, while none was precipitated by normal IgG. This clearly establishes the identity of purified p62 with cloned p62/CE, and further justifies the use of the antibody for characterization of p62 or p62/CE in rat adipocytes below.

Subcellular fractionation followed by immunoblotting revealed that p62/CE was mostly in the cytosol, and 18–20% was associated with particulate fraction (not illustrated). Relative distribution of p62/CE within particulate fraction was assessed by immunoblotting fractions T_H, T_L, H, and L in comparison with the respective GLUT4 compartment sizes (Fig. 5). Relative abundance of p62/CE was approximately 40, 10, 45, and 5% for T_H, T_L, H, and L, respectively, for basal adipocytes. Exposure of adipocytes to insulin did not change p62/CE association significantly in these fractions. This is in contrast to the changes in GLUT4 compartment size seen after cellular exposure to insulin, an increase in T_H and T_L, and a decrease in H and L. This would indicate that the association of p62/CE with the GLUT4 compartment represents a relatively minor portion of the p62/CE in each of these particulate fractions.

To assess direct association of p62/CE with GLUT4 compartments, we purified GLUT4 compartments in fractions T_L, H, and L by immunoadsorption using basal and insulin-treated adipocytes as in Fig. 1, and adsorbed materials were eluted and subjected to immunoblotting analysis using p62/CE antibody (Fig. 6). A single band with an apparent molecular mass of 62 kDa was immunolabeled in each of the 1F8 adsorbates but not in any of the nonimmune IgG adsorbates. When normalized against GLUT4 contents, the relative labeling intensities differed significantly between the three purified GLUT4 compartments; it was highest in G4T_L and lowest in G4L. The relative immunostaining intensities (Fig. 6) largely paralleled with the p62 band protein staining intensities (Fig. 1). Insulin did not show any significant and reproducible effect on the p62/CE immunoreactive band intensities. The protein staining intensity of p62 in G4H was reduced significantly in some (two out of four independent experiments), but not all, insulin-treated adipocytes, the significance of which is not immediately clear.

To investigate the functional significance of the association of p62/CE with GLUT4 compartments in insulin-induced GLUT4 recruitment, we introduced anti-p62/CE antibody into

rat adipocytes and studied its effect on the steady-state distribution of endogenous GLUT4 between the PM and the LDM. Basal and insulin-stimulated adipocytes were permeabilized using SLO in the absence and in the presence of normal rabbit IgG or anti-p62/CE antibody (0.5 mg/ml each) (see "Experimental Procedures"), and PM and LDM fractions were isolated and subjected to immunoblot with GLUT4 antibody. In IgG-incorporated adipocytes, insulin increased the PM GLUT4 content 5–6-fold, with a concomitant reduction (by 25–30%) in the LDM GLUT4 level (Fig. 7). These are essentially identical to the GLUT4 subcellular distributions observed in intact (nonpermeabilized) adipocytes (3). Similar insulin effects on PM and LDM GLUT4 levels were observed in SLO-treated adipocytes in the absence of nonimmune IgG or antibody (data not illustrated). In p62/CE antibody-incorporated adipocytes, however, there was no observable difference in GLUT4 levels in PM or LDM between the basal and insulin-stimulated states (Fig. 7), indicating that the p62/CE antibody completely abolished insulin-stimulated GLUT4 redistribution. This occurred without affecting basal GLUT4 level (Fig. 7).

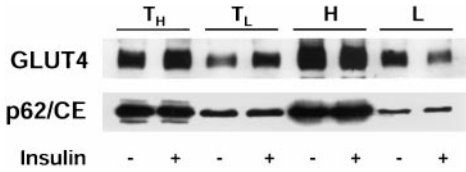

FIG. 5. **Subcellular distribution of p62/CE in rat adipocytes.** Fractions T_H, T_L, H, and L were prepared as described under "Experimental Procedures" and subjected to immunoblot using antibodies raised against purified protein of rat adipose tissue carboxyl esterase (p62/CE) or GLUT4 (GLUT4). Each lane contained a fixed portion (1/40 rat equivalent) of fractions obtained from basal (−) or insulin-stimulated (+) cells. The results were reproduced in four independent experiments.

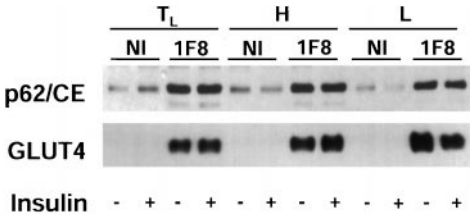

FIG. 6. **Colocalization of p62/CE in immunopurified GLUT4 compartments in basal (−) and insulin-stimulated (+) rat adipocytes.** Fractions T_L, H, and L were subjected to immunoadsorption (see "Experimental Procedures") using 1F8 (1F8) or normal mouse IgG (*NI*) as a control, and resulting immunoadsorbates were immunoblotted by antibodies against rat adipose tissue carboxyl esterase (p62/CE) or GLUT4 (GLUT4). Each lane contained a fixed portion (1/10) of adsorbates originating from 100 μg of protein of the specified fraction. Similar results were obtained in two other experiments.

DISCUSSION

GLUT4 recycling and its insulin-induced recruitment to the cell surface in adipocytes are known to involve multiple protein functions in multiple intracellular compartments (5, 6). Clear identification of the individual intracellular GLUT4 compartments and the associated protein functions involved in these processes has been elusive. Recent success in separation and partial purification of three distinct, intracellular GLUT4 compartments from hypotonically lysed rat adipocytes (19) allowed us to detect and study novel association of certain proteins with GLUT4 compartments that hitherto escaped from identification. Among these was a 62-kDa protein (Fig. 1), which we have identified here as adipose tissue neutral carboxyl esterase, p62/CE. This identity was first suggested by MALDI-TOF MS tryptic fragmentation spectrum profile analysis (Table I) and subsequently confirmed by RT-PCR (Fig. 2*A*), Northern blotting (Fig. 2*B*), gene cloning (GenBank™ accession no. AF171640), enzyme activity assay (Fig. 3), and immunological cross-reactivity (Figs. 2*C* and 4). Adipose tissue carboxyl esterase (FAEE synthase/CE) has been purified and partially characterized (20, 21). It is a member of the mammalian carboxyl esterase (CE) superfamily (EC 3.1.1.1) (27) and hydrolyzes a variety of esters including endogenous substrates such as short- and long-chain acylglycerols and long-chain acyl-CoA esters to the respective free acids with broad substrate specificity.

The association of p62/CE with GLUT4 compartments is unequivocal both in protein staining (Fig. 1) and in immunoblotting (Fig. 6). p62/CE, like many other CE isoforms, contains the C-terminal four amino acids capable of conferring retention in the ER (32). There are exceptions to this ER retention signal rule, however (30); ES-10 and nCEH, for example, contain the N-terminal cleavable signal sequence and the C-terminal ER retention signal sequence, HVEL, yet ES-10 is exclusively found in ER lumen, whereas nCEH is mostly (about 80%) in the cytosol. It is quite possible that p62/CE may also reside in the ER, and this by itself would not necessarily contradict the

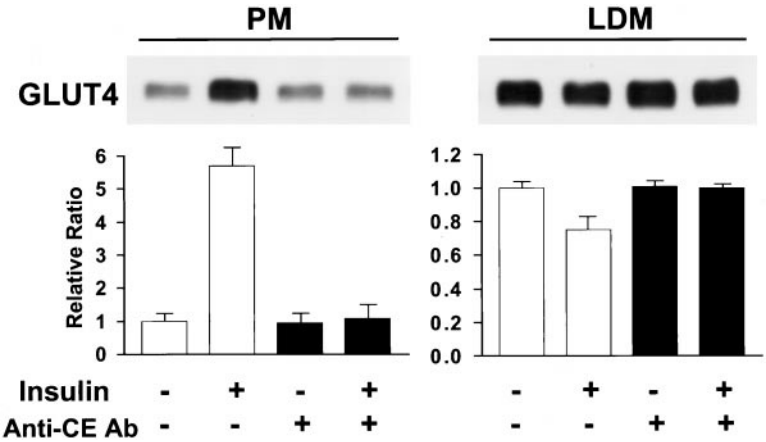

FIG. 7. **Effects of p62/CE antibody on the basal and insulin-stimulated GLUT4 subcellular distribution in permeabilized rat adipocytes.** Adipocytes were permeabilized with SLO (200 unit/ml) and treated without (−) or with (+) anti-CE antibody (0.5 mg/ml) as described under "Experimental Procedures." When insulin was used, adipocytes were treated with insulin (100 nM) for last 20 min during incubation. PM and LDM fractions were isolated from adipocytes after homogenization and subjected to GLUT4 immunoblot (*upper panel*). Each lane contained a fixed portion of subcellular fractions (1/10 rat equivalence). Immunoblot intensities were quantitated by densitometry (see "Experimental Procedures") and expressed in arbitrary units where the intensity of basal PM was 1. Results from three independent experiments were pooled, and the averages are shown with S.D. in *vertical bars* (*lower panel*).

presence of p62/CE in cytosol and its association with GLUT4 vesicles. Please also note that GLUT4-containing compartments purified by 1F8 in fractions T_L, H, and L were free of ER, yet showed p62/CE immunoreactivity. In preliminary experiments, we have noted that 1 M KCl or alkaline wash reduces p62/CE association with GLUT4 vesicles, suggesting cytoplasmic, rather than luminal, interaction of p62/CE with the vesicles.

Quantitative assessment of the association of p62/CE with each of the GLUT4-compartments, however, was less than unequivocal, a problem not uncommon in assessment of extrinsic membrane protein binding to membranes. Notably, immunoblot data showed no clear insulin effect on p62/CE association in each GLUT4 compartment (Fig. 6), while an apparent reduction in p62/CE protein staining intensity in G4H was observed in some (19) but not all insulin-stimulated adipocytes (Fig. 1). In protein staining data, p62/CE appeared more abundant in $G4T_L$ than in G4H or G4L (Fig. 1), although such a difference was not clearly seen in immunoblots (Fig. 6). However, when normalized against GLUT4 content, p62/CE was significantly more concentrated in $G4T_L$ in both assays (Figs. 1 and 6). $G4T_L$ is a compartment composed exclusively of small vesicles, free of integrin but enriched in transferrin receptor and Rab5, and thought to represent GLUT4 endocytic vesicles (19).

p62/CE antibody in permeabilized rat adipocytes completely abolishes insulin-induced recruitment of GLUT4 without affecting basal GLUT4 distribution (Fig. 7). This, together with the fact that the antibody inhibits the esterase activity of p62/CE (Fig. 3), strongly suggests the importance of p62/CE or a p62/CE-catalyzed hydrolysis in the insulin-induced GLUT4 recruitment in adipocytes. The role of p62/CE in insulin-induced GLUT4 recruitment, however, is yet to be determined. An interesting possibility is that this neutral esterase hydrolyzes certain lipid component(s) in nonacidic, GLUT4 compartments such as sorting and/or recycling endosomes and regulates GLUT4 transport vesicle sorting, budding, or fusion. The high p62/CE concentration observed in $G4T_L$ and G4H, the presumed endocytic vesicles and recycling/storage endosomes, respectively, is consistent with this possibility. Endosomal lipids are known to undergo enzymatic processing (33). The hydrolysis of endosomal cholesterol ester by nCEH, for example, was implicated in free cholesterol transport vesicle sorting, budding, or fusion in cholesterol homeostasis (34). Recently, insulin-sensitive phospholipid signal pathways have been implicated in insulin-induced GLUT4 recruitment in rat adipocytes (35). Thus, the activation of phospholipase D-dependent phosphatidylcholine hydrolysis and *de novo* phosphatidic acid synthesis by insulin result in formation of phosphatidic acid- and phosphatidylinositol (4,5)P_2-enriched microdomains in endosomes, which may promote vesicle formation, coat protein release, and/or fusion to target membranes (36). At least two more enzymes that are involved in lipid metabolism, namely acyl-CoA synthetase-1 (14) and L-3-hydroxyacyl-CoA dehydrogenase (37), have been suggested to play a role in GLUT4 regulation. How at the molecular level the endosomal lipid metabolism catalyzed by these enzymes and p62/CE regulates GLUT4 recycling and recruitment is an important question that deserves systematic investigation.

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Association of Carboxyl Esterase with Facilitative Glucose Transporter Isoform 4 (GLUT4) Intracellular Compartments in Rat Adipocytes and Its Possible Role in Insulin-induced GLUT4 Recruitment

Wan Lee, Jiwon Ryu, Jongsik Hah, Takahiro Tsujita and Chan Y. Jung

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