Characterization of the Role of the Rab GTPase-activating Protein AS160 in Insulin-regulated GLUT4 Trafficking

Mark Larance, Georg Ramm, Jacqueline Stöckli, Ellen M. van Dam, Stephanie Winata, Valerie Wasinger, Fiona Simpson, Michael Graham, Jagath R. Junutula, Michael Guilhaus, and David E. James

From the Diabetes and Obesity Program, Garvan Institute of Medical Research, Sydney 2010, Australia; Bioanalytical Mass Spectrometry Facility, University of New South Wales, Sydney 2052, Australia; Institute for Molecular Biology, University of Queensland, Brisbane 4072, Australia; Benitec, Inc., Mountain View, California 94043, and Genentech Inc., South San Francisco, California 94080

Insulin stimulates the translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane. In the present study we have conducted a comprehensive proteomic analysis of affinity-purified GLUT4 vesicles from 3T3-L1 adipocytes to discover potential regulators of GLUT4 trafficking. In addition to previously identified components of GLUT4 storage vesicles including the insulin-regulated aminopeptidase insulin-regulated aminopeptidase and the vesicle soluble N-ethylmaleimide factor attachment protein (v-SNARE) VAMP2, we have identified three new Rab proteins, Rab10, Rab11, and Rab14, on GLUT4 vesicles. We have also found that the putative Rab GTPase-activating protein AS160 (Akt substrate of 160 kDa) is associated with GLUT4 vesicles in the basal state and dissociates in response to insulin. This association is likely to be mediated by the cytosolic tail of insulin-regulated aminopeptidase, which interacted both in vitro and in vivo with AS160. Consistent with an inhibitory role of AS160 in the basal state, reduced expression of AS160 in adipocytes using short hairpin RNA increased plasma membrane levels of GLUT4 in an insulin-independent manner. These findings support an important role for AS160 in the insulin regulated trafficking of GLUT4.

Glucose transport into mammalian muscle and fat cells is an important step in insulin action and is critical for the maintenance of glucose homeostasis within the body (1). In mammalian muscle and fat cells, insulin stimulation activates a phosphorylation cascade, which in turn causes intracellular vesicles that contain the glucose transporter GLUT4, to translocate to the plasma membrane (PM) and fuse (2, 3). In the basal state GLUT4 is distributed between the endosomal system, the trans-Golgi network (TGN), and a GLUT4 storage vesicle (GSV) compartment that is highly insulin-responsive (4–6).

The protein kinase Akt is activated in response to insulin and plays a critical role in GLUT4 translocation (1, 7). However, the link between the insulin signaling pathway and GLUT4 translocation is not fully understood. The insulin-dependent movement of GLUT4 vesicles to the PM is an Akt-independent process, and this is followed by an Akt-dependent step likely involving the docking and fusion of vesicles with the PM (7–9). The mechanism by which Akt controls the docking and fusion of GLUT4 vesicles with the PM is not known. However, it was previously shown that a Rab GTPase-activating protein (RabGAP) known as AS160 is phosphorylated by Akt in response to insulin (10). How AS160 functions in GLUT4 trafficking and its cognate Rab proteins are not known. The role of a variety of Rab proteins in GLUT4 trafficking including Rab3d, Rab4, Rab5, and Rab11 has been examined (11–16). However, although these Rab proteins may participate in some aspects of GLUT4 trafficking, no compelling evidence for specific involvement in the insulin-regulated trafficking of GLUT4 has been found.

In this study we describe four key findings that add to our understanding of GLUT4 trafficking. Using mass spectrometry we have identified three Rab proteins on GLUT4 vesicles that could potentially be substrates of AS160 and, thus, play an important role in insulin-regulated glucose transport. In addition, we have shown that AS160 is present on GLUT4 vesicles in the basal state and dissociates with insulin. We have gone on to show that the association of AS160 with GLUT4 vesicles is mediated at least in part via a direct interaction with the cytosolic tail of IRAP, and this interaction appears to be insulin-dependent. Furthermore, a decrease in AS160 expression by shRNA leads to increased levels of GLUT4 at the PM in the basal state. Together, these results provide novel insights into the regulatory mechanism of insulin-stimulated GLUT4 translocation.

EXPERIMENTAL PROCEDURES

Materials—3T3-L1 murine fibroblasts, C2C12 fibroblasts, and Chinese hamster ovary (CHO) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Dulbecco’s modified Eagle’s medium and newborn calf serum were obtained from Invitrogen, Myoclone-Plus fetal calf serum from Trace Scientific (Melbourne, VAMP, vesicle-associated membrane protein; LDM, low density microsomes; PBS, phosphate-buffered saline; Puro, puromycin; Hygro, hygromycin; HA, hemagglutinin; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; CI-MPR, cation-independent mannose 6-phosphate receptor; CD-MPR, cation-dependent mannose 6-phosphate receptor; APTPA, copper-transporting ATPase 1; SCAMP, secretory carrier-associated membrane protein; GST, glutathione S-transferase; MES, 4-morpholineethanesulfonic acid; GSV, GLUT4 storage vesicle.

Received for publication, April 11, 2005, and in revised form, August 31, 2005 Published, JBC Papers in Press, September 8, 2005, DOI 10.1074/jbc.M503897200

The abbreviations used are: GLUT4, glucose transporter 4; PM, plasma membrane; TGN, trans-Golgi network; GSV, GLUT4 storage vesicle; RabGAP, Rab GTPase-activating protein; IRAP, insulin-regulated aminopeptidase; AS160, Akt substrate of 160 kDa; Akt, protein kinase Akt; v-SNARE, vesicle soluble N-ethylmaleimide factor attachment protein; v-SNARE, v-SNARE; Rab10, Rab11, Rab14, Rab3d, Rab4, Rab5, Rab11.
AS160 and GLUT4 Trafficking

Australia), and antibiotics were from Invitrogen. Paraformaldehyde was from ProSciTech (Thuringowa Central, Australia). Insulin was obtained from Calbiochem, and bovine serum albumin from United States Biochemical Corp. (Cleveland, OH). Bicinchoninic acid reagent, GF-2000 beads, Supersignal West Pico chemiluminescent substrate, and protein G-agarose beads were from Pierce. Lipofectamine 2000 was from Invitrogen. Centrifuge tubes (11 × 60 mm) were from Beckman Instruments. Polyvinylidene difluoride membrane was from Millipore (Billerica, MA). C18 Tagetips were from Proxeon Biosystems (Odense, Denmark). Trypsin was from Promega (Madison, WI). Strong cation exchange and C18 cartridges were from Michrom (Auburn, CA). Magic C18 material was from (Alltech, Deerfield, IL). Complete protease inhibitor mixture tables were from Roche Applied Science. All other materials were obtained from Sigma. Antibodies were kindly provided by Dr. Gwyn Gould (CI-MPR and CD-MPR, University of Glasgow, Glasgow, UK), Dr. Claus Munck Peterson (sortulin, Weizmann Institute of Science, Rehovot, Israel), Dr. P. Chavrier (Rab5 and Rab7, INSERM CNRS, Marseille, France), Dr. Robert Parton (Rab11, University of Queensland, Brisbane, Australia), Dr. Bernad (PKL12, Campus de la Universidad Autonoma de Madrid, Madrid, Spain), Dr. Castle (SCAMP2 and SCAMP3, University of Virginia, USA), Dr. Wanjin Hong (syntaxin 16, IMCB, Singapore), and Dr. Julian Mercer (ATP7A, Deakin University, Burwood, Australia). Antibodies were purchased from Santa Cruz Biotecnology (IRS-1; Santa Cruz, CA), Cayman Chemical (CD36), BD Biosciences Pharmingen (Vti1b), Synaptic Systems (VAMP2, VAMP3, and mvP45; Goettingen, Germany), Zymed Laboratories Inc.; San Francisco, CA (TIR, Sigma (FLAG M2), Transduction Laboratories (syntaxin 6; Lexington, KY), and Berkeley Antibody Co., Inc. (HA peptide (16B12); Richmond, CA). Antibodies against GLUT4 (17), syntaxin 4 (18), IRAP (5), Rab14 (19), and VAMP3 (18) have been described previously. ALEXA 488 and Cy3-conjugated secondary antibodies were obtained from Molecular Probes (Leiden, The Netherlands) or Jackson ImmunoResearch (West Grove, PA), respectively. Horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences.

Production of a Rabbit AS160 Antibody—Rabbit polyclonal antibodies against human AS160 (TBC1D4) were produced as previously described (18) using a region of human AS160 from amino acids 621–766 fused with GST. Serum from these rabbits was affinity-purified using the AS160 antigen coupled to a GF-2000 column according to manufacturer’s instructions.

Cell Culture—3T3-L1 fibroblasts, CHO, and C2C12 fibroblasts were cultured as described previously (5). Briefly, cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum or fetal calf serum, 2 mM l-glutamine, 100 units/liter penicillin, and 100 μg/liter streptomycin at 37 °C in 10% CO2 and passaged at 60% confluence. In the case of 3T3-L1 fibroblasts, confluent cells were differentiated into adipocytes and used between days 8 and 10 post-differentiation and between passages 10 and 20. To establish basal conditions before use, cells were incubated in serum-free Dulbecco’s modified Eagle’s medium for 2 h at 37 °C in 10% CO2.

Subcellular Fractionation of Adipocytes—3T3-L1 adipocyte fractionation was carried out as described previously (5). Briefly, cells were incubated for 2 h in Dulbecco’s modified Eagle’s medium containing 25 mM glucose at 37 °C and lysed using 12 passes through a 22-gauge needle followed by 6 passes through a 27-gauge needle in HES buffer (20 mM HEPES, 10 mM EDTA, 250 mM sucrose pH 7.4) containing Complete protease inhibitor mixture and phosphatase inhibitors (2 mM sodium orthovanadate, 1 mM pyrophosphate, 1 mM ammonium molybdate, 10 mM sodium fluoride) at 4 °C. The lysate was then centrifuged at 500 × g for 10 min to remove unbroken cells and at 10,080 × g for 12 min, 15,750 × g for 17 min, and 175,000 × g for 75 min at 4 °C to obtain the PM, mitochondria and nuclei high density microsomal pellet containing the endoplasmic reticulum and large endosomes, and the low density microsomal (LDM) pellet which contained intracellular transport vesicles and the majority of GLUT4 vesicles, respectively. The LDM was then resuspended in PBS-containing inhibitors for use in immunoprecipitations.

Cationic Colloidal Silica Plasma Membrane Isolation—Plasma membranes were purified as per Chaney and Jacobson (21) with modifications. Briefly, basal or insulin-stimulated 3T3-L1 adipocytes were washed twice with ice-cold PBS and twice in ice-cold coating buffer (20 mM MES, 150 mM NaCl, 280 mM sorbitol, pH 5.0–5.5). Cationic silica 1% (stored as a 30% stock and diluted to 1% in coating buffer) was added to the cells in coating buffer for 2 min on ice. Excess silica was removed by washing once with ice-cold coating buffer. Sodium polyacrylate (1 mg/ml, pH 6–6.5) was added to the cells in coating buffer and incubated at 4 °C for 2 min. Cells were washed once in ice-cold coating buffer and then washed with modified HES (20 mM HEPES, 250 mM sucrose, 1 mM dithiothreitol, 1 mM magnesium acetate, 100 mM potassium acetate, 0.5 mM zinc chloride, pH 7.4) at 4 °C and lysed as described above. Nycodenz (100%) in modified HES buffer was added to the lysate to a final concentration of 50%. The lysate was layered onto 0.5 ml of 70% Nycodenz in modified HES and centrifuged in a swing-out rotor at 41,545 × g for 20 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 0.5 ml of modified HES buffer and centrifuged at 500 × g for 5 min at 4 °C. The pellet was resuspended in SDS-PAGE sample buffer and heated to 65 °C for 10 min. The sample was then centrifuged at 10,000 × g at 24 °C, and the supernatant was retained for analysis by immunoblotting.

Immunooisolation of GLUT4 Vesicles and Mass Spectrometry Analysis—GLUT4 vesicles were immuno-isolated using the protocol of Hashimoto et al. (22) with the exception that either control mouse IgG or the anti-GLUT4 monoclonal 1F8 was covalently coupled to GF-2000 beads according to manufacturer’s instructions. LDM (2.5 mg) was resuspended in 100 μl of PBS and added to 50 μl of both control mouse IgG and 1F8 beads and incubated overnight with rotation at 4 °C in the presence of 0.1% bovine serum albumin and PBS in a final volume of 250 μl. The beads were then washed 5 times with PBS, and the proteins were eluted with 100% formic acid. The eluate was removed and vacuum-dried. Ammonium bicarbonate (500 mM) was added to adjust the pH to 8 and vacuum-dried again. Urea (10 μl) and 5 mM dithiothreitol were then added, and samples were incubated at 37 °C for 1 h. Cysteines were subsequently alkylated using a 10-fold molar excess of iodoacetamide and incubated at 37 °C for 1 h. Proteins were digested by the addition of modified trypsin (12.5 ng/μl) in 100 μl NH4HCO3 and incubated overnight at 37 °C. The digestion was stopped by the addition of 5% formic acid, and peptides were desalted using C18 Stage tips. Peptides were analyzed by tandem mass spectrometry using a CapLC high performance liquid chromatography system (Waters, Milford, MA) by binding to a strong cation exchange cartridge and sequential elution of the peptides using salt steps of 5, 10, 15, 20, 25, 30, 40, 50, 75, 150, 300, 1000 mM ammonium acetate. After each salt step eluted peptides were desalted on a capillary C18 cartridge and resolved in a 100-mm × 75-μm C18 Magic reverse phase analytical column with a flow rate of 200 nL/min. Peptides were ionized by nanoelectrospray at 2.8 kV from the end of the column, which was pulled to an inner diameter of 5 μm by a P-2000 laser puller (Sutter Instruments Co). Tandem mass spectral analysis was carried out on a Waters (Milford, MA) quadrupole time-of-flight Ultima mass
spectrometer. A data-dependent acquisition method was used for all experiments where precursor ions needed to have an intensity higher than 10 counts and be in the +2, +3, or +4 charge state. MS/MS spectra were searched against a metazoan data base generated from Swiss-Prot and Trembl containing 574734 sequences using Sequest (Thermo Electron Corporation, Waltham, MA). Peptides were counted as valid if in the +2 charge state they had an Xcorr value >2 or in the +3 charge state, an Xcorr >3.5. All peptides also needed a ACN value greater than 0.08. One missed cleavage per peptide was tolerated, and peptides could be partially tryptic. Proteins identified by less than two peptides were validated manually.

**Immunoblotting**—All samples were subjected to SDS-PAGE analysis on 10% resolving gels according to Laemmli (23). Equal amounts of proteins were loaded for each sample in a single experiment, with 1–10 μg per lane unless otherwise stated. Separated proteins were electrophoretically transferred to poly(vinylidene difluoride) membrane, blocked with BB (2% nonfat skim milk in 0.1% Tween 20 in PBS), and incubated with primary antibody in BB. After incubation, membranes were washed 3 times in BB and incubated with horseradish peroxidase-labeled secondary antibodies in BB. Proteins were visualized using Supersignal West Pico chemiluminescent substrate and imaged using a Versadoc 5000 imager (Bio-Rad).

**Sucrose Gradient Fractionation Experiments**—LDM (165 μl) obtained from 3T3-L1 adipocytes as described above was mixed with 835 μl of 70% sucrose solution in HES to a final concentration of 60% sucrose (24) and placed in an 11 × 60-mm centrifuge tube overlaid with 1 ml of 50, 30, and 10% of sucrose and 0.4 ml of 5% sucrose. The sample was then centrifuged for 18 h at 111,132 × g in a Beckman SW61 swing-out rotor. Fractions (0.4 ml) were collected from the bottom of the tube by gravitational flow.

**Confocal Laser Scanning Microscopy**—3T3-L1 adipocytes were cultured as described above on glass coverslips. The cells were serum-depleted for 2 h at 37 °C, after which they were incubated in the absence or presence of 200 nM insulin for 20 min. Cells were then fixed with 3% paraformaldehyde in PBS, permeabilized with anti-HA antibody as described previously (26). The surface HA-GLUT4 was measured in non-permeabilized adipocytes with anti-HA antibody as described previously (26). For double labeling, fluorophores were scanned separately and analyzed by confocal laser scanning microscopy using a Leica TCS SP system. For double labeling, fluorophores were scanned separately and overlaid using Adobe Photoshop software. Images were generated by the maximum projection of a stack sections from the middle of each cell.

**GLUT4 Recycling Experiments**—3T3-L1 adipocytes expressing a control shRNA or the AS160 shRNA and HA-GLUT4 were incubated in the presence or absence of 100 nM insulin for 20 min. Anti-HA antibody (60 μg/ml) was then added for 10 and 60 min. Subsequently the cells were fixed, permeabilized, and incubated with a secondary Cy3-conjugated anti-mouse antibody. Confocal laser scanning microscopy was carried out as described above.

**Transfection of C2C12 Cells and CHO Cells**—C2C12 and CHO cells were transiently transfected with DNA constructs for expression of FLAG-tagged AS160 and the AS160 and control shRNA constructs using Lipofectamine 2000 according to manufacturer’s instructions.

**Immunoprecipitation and GST Pull-down of FLAG-tagged AS160**—FLAG-AS160-expressing CHO cells were lysed in extraction buffer (1% Nonidet P-40, 137 mM sodium chloride, 10% glycerol, 25 mM Tris, pH 7.4), centrifuged at 18,000 × g for 20 min, anti-FLAG antibody and protein G beads were added to the supernatant and incubated for 2 h at 4 °C with mixing. The beads were then washed extensively and boiled in SDS-PAGE sample buffer. For GST pull-downs, 100 μg of lysis was incubated with either GST alone or GST-IRAP1-109 GST-IRAP1-58 GST-IRAP1-27 GST-GLUT4466–509 GST-VAMP21–94 coupled to CNBr-activated Sepharose 4B beads according to the manufacturer’s instruction (Amersham Biosciences). Beads were washed extensively and boiled in sample buffer.

**Membrane Protein Isolation**—Protein G beads were added to the supernatant and incubated for 2 h at 4 °C with mixing. The beads were then washed extensively and boiled in SDS-PAGE sample buffer. For GST pull-downs, 100 μg of lysis was incubated with either GST alone or GST-IRAP1-109 GST-IRAP1-58 GST-IRAP1-27 GST-GLUT4466–509 GST-VAMP21–94 coupled to CNBr-activated Sepharose 4B beads according to the manufacturer’s instruction (Amersham Biosciences). Beads were washed extensively and boiled in sample buffer.
TABLE ONE
Summary of GLUT4 vesicle proteins identified by mass spectrometry from 3T3-L1 adipocytes
Proteins shown were the consensus of three separate experiments. -, no significant translocation; +, <2-fold translocation; ++, >2-fold translocation. Summary of localization of protein identified in GLUT4 vesicles was based on either the present study (†) or other published data.

| LocusID      | Accession No. | Description                                    | Perinuclear Overlap | Peripheral Overlap | Translocation to the PM with insulin | Comments                  |
|--------------|---------------|------------------------------------------------|---------------------|--------------------|--------------------------------------|---------------------------|
| Vesicle transport proteins |              |                                                 |                     |                    |                                      |                           |
| A2A1_MOUSE   | (P17428)      | Adaptor-related protein complex 2 alpha 1 subunit (Alpha-adaptin A) | -                   | -                  | PM †                                 |                           |
| A2A2_MOUSE   | (P17427)      | Adaptor-related protein complex 2 alpha 2 subunit (Alpha-adaptin C) | -                   | -                  | PM †                                 |                           |
| DYL1_HUMAN   | (Q15701)      | Dynamin light chain 1, cytoplasmic (93 kDa dynamin light chain) (DLC8) | +                   | -                  | -                                    | Endosomal †               |
| C704D04      | (P70404)      | Vamp8 (Endobrevin)                             | +                   | -                  | -                                    | Cytosol (64)              |
| C14677       | (Q14677)      | Hypothetical protein KIAA0171 (Epsin 4) (Entrophin) |                     |                    |                                      |                           |
| C9ER00       | (Q9ER00)      | Syntaxin 12                                    |                     |                    |                                      |                           |
| C9ER00       | (Q9ER00)      | Syntaxin 12                                    |                     |                    |                                      |                           |
| C9ER00       | (Q9ER00)      | Syntaxin 8                                     | ++                  | +                  | Perinuclear (47)                     |                           |
| C4656        | (Q4656)       | SNAP-29 protein                                | +                   | -                  | Cytosol †                            |                           |
| C560K1       | (Q560K1)      | Syntaxin 8                                     | ++                  | -                  | -                                    | TGN (66)                  |
| RB1A1B_MOUSE | (P62492)      | Ras-related protein Rab-11A (F24.394) Ras-related protein Rab-11A | +                   | -                  | -                                    | Endosomal (67)            |
| RB10_RAT     | (P35281)      | Ras-related protein Rab-10                     |                     |                    |                                      |                           |
| RB14_HUMAN   | (P35287)      | Ras-related protein Rab-14                     | +++                 | +                  | Perinuclear (19)                     |                           |
| SCA3_MOUSE   | (Q56069)      | Secretory carrier-associated membrane protein 3 | ++                  | +                  | -                                    | Perinuclear (47)          |
| SNA1_MOUSE   | (Q90986)      | Alpha-soluble NSF attachment protein (SNAP-alpha) |                     |                    |                                      |                           |
| SNA2_MOUSE   | (Q90987)      | Gamma-soluble NSF attachment protein (SNAP-gamma) |                     |                    |                                      |                           |
| STX1_HUMAN   | (Q14662)      | Syntaxin 16 (STX16)                            | +++                 | +                  | -                                    | TGN (69)                  |
| VAMP2_MOUSE  | (Q64357)      | Vesicle-associated membrane protein 2 (VAMP-2) (Synapticbrevin 2) | +++                 | +                  | GSVs †                               |                           |
| VAMP3_MOUSE  | (Q64271)      | Vesicle-associated membrane protein 3 (VAMP-3) (Cotubrevin) | +++                 | +                  | Endosomes (70)                       |                           |
| VP46_MOUSE   | (Q87390)      | Vacuolar protein sorting-associated protein 45 (mVps45) | +                   | -                  | -                                    | TGN (71)                  |
| VT1B_MOUSE   | (O83381)      | Vesicle transport through interaction with 1-SNAREs homolog 1B (VT1b) | +++                 | +                  | -                                    | Perinuclear †             |
| Proteins from the secretory pathway |
| APM1_MOUSE   | (Q65994)      | Adipokinetic precursor                         |                     |                    |                                      | Secreted (72)             |
| CBP3_MOUSE   | (O89001)      | Carboxypeptidase D precursor (EC 3.4.17.1) (GP1B0) |                     |                    |                                      | TGN (73)                  |
| CYTC_MOUSE   | (P21460)      | Cystatin C precursor (Cystatin 3)               |                     |                    |                                      | Secreted (74)             |
| LIP1_MOUSE   | (P11152)      | Lipoprotein lipase precursor (EC:3.1.1.34) (LPL) |                     |                    |                                      | Secreted (72)             |
| RSN_MOUSE    | (Q99878)      | Resistin precursor                             |                     |                    |                                      |                           |
| Recycling membrane proteins |
| AT2A_MOUSE   | (Q64330)      | Cotransporting ATPase 1 (ATP7A)                | +++                 | +                  | -                                    | TGN (36)                  |
| CD36_MOUSE   | (Q08857)      | Plasma glycoprotein IV (GPIV) (GPIVII) (CD36 antigen) | -                   | -                  |                                      | Plasma Membrane †         |
| LDLR_MOUSE   | (P35951)      | Low-density lipoprotein receptor precursor (LDL receptor) | -                   | -                  | -                                    | Endosomes (75)            |
| LRR1_MOUSE   | (P98156)      | Very low-density lipoprotein receptor precursor (VLDL-receptor) | -                   | -                  | -                                    | Endosomes (75)            |
| GTR1_MOUSE   | (P17809)      | Solute carrier family 2, facilitated glucose transporter, member 1 (GLUT1) | ++                  | +                  | +                                    | Endosomes (76)            |
| MPRD_MOUSE   | (P34868)      | Carbohydrate-dependent mannose-6-phosphate receptor precursor (CD-MPR) | ++                  | +                  | -                                    | Perinuclear (37)          |
| MPRFE_MOUSE  | (Q07113)      | Carbohydrate-independent mannose-6-phosphate receptor precursor (C-MPR) | +++                 | +                  | Perinuclear (77)                     |                           |
| TFR1_MOUSE   | (Q93581)      | Transferrin receptor protein 1 (TIR1) (TIR1) (TIR1b) | +++                 | +                  | Endosomes (78)                       |                           |
| VAO4_MOUSE   | (P51861)      | Vacuolar ATP synthase subunit d (EC 3.6.3.14) | +++                 | +                  | Endosomes (78)                       |                           |
| VAA1_MOUSE   | (P60516)      | Vacuolar ATP synthase catalytic subunit A (EC 3.6.3.14) | +++                 | +                  | Endosomes (79)                       |                           |
| VP1_MOUSE    | (Q92144)      | Vacuolar proton translocating ATPase 116 kDa subunit A | +++                 | +                  | Endosomes (79)                       |                           |
| Proteins with unknown vesicle transport functions |
| CAY1_MOUSE   | (P48871)      | Caveolin-1                                      | +                   | -                  | -                                    | PM (80)                   |
| CAY2_MOUSE   | (Q89423)      | Caveolin-2                                      | +                   | -                  | -                                    | PM (80)                   |
| MYDM_MOUSE   | (Q66882)      | Myeloid-associated differentiation marker       | +                   | -                  | Endosomal †                          |                           |
| Q6R1Q7       | (Q6R1Q7)      | Potassium channel protein 2                    | -                   | -                  | PM (81)                               |                           |
| STXG_MOUSE   | (O88697)      | Serin/threonine protein kinase 16 (EC 2.7.1.37) (PKG1) | +++                 | +                  | -                                    | Cytosol (82)              |
| TBC4_HUMAN   | (O62843)      | TBC1 domain family member 4 (AS160)             | +++                 | +                  | -                                    | GSVs/Cytosol †            |
We next wanted to determine which of these proteins might play a functional regulatory role in GLUT4 trafficking or indeed determine which proteins may be targeted with GLUT4 into insulin-responsive vesicles. To accomplish this we divided these proteins into integral versus peripheral membrane proteins.

Characterization of Integral Membrane Proteins Found in GLUT4 Vesicles—It has previously been shown that GLUT4 is found in GSVs, endosomes, and the TGN (28–30). Therefore, it was not surprising to find a large number of endosomal and TGN-derived proteins in immuno-isolated GLUT4 vesicles. To determine which of these were localized to GSVs, we used two separate criteria. First, we examined insulin-responsive movement to the PM, as this is a distinguishing feature of GSV constituents (31). Second, we performed co-localization experiments to compare the intracellular distribution of each integral membrane protein with GLUT4.

To quantify the insulin-dependent movement of proteins to the PM, we developed a method to isolate highly purified plasma membranes (21). Electron microscopy studies of these membranes revealed that they contained large PM sheets with very little contamination from other organelles (data not shown). The only other organelle we could detect by electron microscopy was mitochondria. We were unable to detect Golgi or endoplasmic reticulum membranes proteins in the purified PM fraction by immunoblotting (data not shown), which suggests that this is a highly purified fraction. In contrast PM markers such as syntaxin 4 or caveolin 1 were highly enriched in this fraction (data not shown). We observed very little GLUT4 in the PM in the absence of insulin, whereas after insulin stimulation there was a time-dependent movement of GLUT4 to the PM, and this effect was inhibited by the phosphatidylinositol 3-kinase inhibitor wortmannin (Fig. 1A). The level of the t-SNARE syntaxin 4 in the PM remained constant under these conditions (Fig. 1A). Among the integral membrane proteins examined GLUT4, IRAP and VAMP2 exhibited the largest insulin-dependent increase at the PM, showing 10-, 6-, and 2.5-fold increases, respectively (Fig. 1B) (n = 4). The protein that exhibited the next most significant change with insulin was the TIR, showing a 1.5-fold increase in cell surface levels (Fig. 1B). In contrast, other endosomal proteins such as VAMP3 did not undergo any significant change in PM localization with insulin. Consistent with previous studies (22, 32–35) we observed a slight effect of insulin on PM levels of CI-MPR, CD-MPR, and sortilin, but this was much lower than that observed for GLUT4, IRAP, or VAMP2 (Fig. 1b). Intriguingly, the copper-transporting ATPase (ATP7A), which was identified in GLUT4 vesicles and can translocate to the plasma membrane with copper-stimulation (36), did not translocate with insulin-stimulation.

We next compared the intracellular localization of these proteins with GLUT4. A defining feature of the intracellular localization of GLUT4 is its concentration in peripheral vesicles that are thought to represent the insulin-responsive vesicles (37). Our laboratory has previously described quantitative immunoelectron microscopic labeling of basal versus insulin-treated 3T3-L1 and primary rat adipocytes (4, 37). We observed a 53% reduction in GLUT4 labeling of cytosolic vesicles in response to insulin, whereas only an 18% reduction in GLUT4 labeling was seen for the TGN or perinuclear region. These data indicate that the peripheral cytosolic vesicles correspond to the insulin-responsive GSVs. In agreement with this conclusion, we observed significant colocalization between GLUT4, IRAP, and VAMP2 in cytosolic vesicles (Fig. 2A). However, this was not the case for any of the other integral membrane proteins examined. For example some proteins such as sortilin (Fig. 2A) and syntaxin 16 (data not shown) were highly concentrated in the perinuclear area, presumably corresponding to TGN labeling, but were not concentrated in the peripheral cytosolic vesicles. Some proteins like the copper-transporting ATPase ATP7A (Fig. 2A) or VAMP3 (data not shown) exhibited both perinuclear and peripheral cytosolic vesicular staining (Fig. 2A). However, the peripheral cytosolic vesicular staining did not correspond to that containing GLUT4, indicating that these proteins reside in separate vesicles. Therefore, these studies indicate that the major integral membrane proteins in GSVs are GLUT4, IRAP, and VAMP2.

Characterization of Peripheral Membrane Proteins Found in GLUT4 Vesicles—Several novel peripheral membrane proteins were discovered associated with GLUT4 vesicles in this study by mass spectrometry. Perhaps the most interesting among these were 3 Rab proteins Rab10, Rab11, Rab14, and the RabGAP AS160. Rab11 has previously been studied in the context of GLUT4 trafficking, but there is little evidence to indicate a role for this protein in the insulin-regulated trafficking of GSVs to the PM (11). In contrast, Rab10 and Rab14 have not previously been described in GLUT4 vesicles. To verify our mass spectrometry data, we obtained antibodies against Rab11 and Rab14; however, no Rab10-specific antibodies are currently available. We immunoblotted GLUT4 vesicles isolated from adipocytes that had been incubated in the absence or presence of insulin and found that both Rab11 and Rab14 remained associated with the GLUT4 vesicles with and without insulin stimulation (Fig. 3). Rab7, which had been found to non-specifically associate with GLUT4 vesicles in the mass spectrometry analysis, is also shown (Fig. 3). Rab5, which has previously been shown not to associate with GLUT4 vesicles (38), was neither detected in the control nor in GLUT4 immuno-isolations (Fig. 3). We next wanted to examine the intracellular localization of the Rab7 and confocal immunofluores-
AS160 and GLUT4 Trafficking

**FIGURE 1.** Translocation of GLUT4 vesicle proteins identified by mass spectrometry to the plasma membrane with insulin-stimulation in 3T3-L1 adipocytes. Plasma membranes were isolated for each condition using the cationic silica isolation method. A, translocation of GLUT4 and syntaxin 4 to the plasma membrane after 2 or 30 min of stimulation with 100 nM insulin or 30 min with 100 nM wortmannin before a 30-min insulin stimulation with 100 nM insulin. Protein levels were detected by immunoblotting. B, translocation of proteins to the plasma membrane after 30 min of stimulation with 100 nM insulin. C, quantification of protein translocation to the plasma membrane after 30 min of stimulation with 100 nM insulin. Error bars are S.E. Immunoblots are from a representative experiment (n = 4).

The identification of AS160 on the same membranes as the three Rab proteins was particularly interesting and raises the possibility that they may be in vivo substrates for the GAP activity of AS160. To examine the function of AS160 in GLUT4 trafficking, we generated a rabbit polyclonal antibody against a central region of AS160. Consistent with previous studies by Sano et al. (40), our antibody against AS160 specifically recognizes immunoprecipitated FLAG-tagged AS160 (Fig. 4A). This antibody also immunolabeled a protein of 160 kDa in adipocytes that were enriched in the LDM fraction (Fig. 4B), which contains the majority of GLUT4 vesicles (22). We have previously shown that the LDM fraction is comprised of both membrane vesicles and large protein complexes (24). To clarify which structures AS160 is associated with in the LDM, we subjected this fraction to sucrose density flotation analysis. Intriguingly, although the majority of AS160 was found at the bottom of the gradient, a significant amount was found in the vesicle-enriched fraction, migrating at a similar position to GLUT4. To confirm the mass spectrometry data showing the association of AS160 with GLUT4 vesicles, we immunoblotted purified GLUT4 vesicles with the AS160 antibody. Consistent with the mass spectrometry data, AS160 was detected in the GLUT4 vesicles but was not detected in control IgG samples. In addition, we found that insulin stimulation induced a marked decrease in the amount of AS160 associated with GLUT4 vesicles (Fig. 4D). The dissociation of AS160 from GLUT4 vesicles was blocked by treatment with the phosphatidylinositol 3-kinase inhibitor wortmannin before insulin stimulation (Fig. 4E).

**Identification of IRAP as the Binding Partner for AS160 on GLUT4 Vesicles**—The above data are consistent with a model whereby the interaction of AS160 with GLUT4 vesicles plays a key role in their intracellular sequestration in the basal state. Therefore, we next wanted to identify the molecular basis for this interaction. In view of our data showing that GLUT4, IRAP, and VAMP2 are the major integral membrane proteins in GLUT4 vesicles, we next explored the possibility that AS160 may interact with one or more of these proteins. To test this we performed in vitro GST pull-down experiments using lysates from CHO IR/IRS-1 cells that had been transiently transfected with FLAG-tagged AS160. As shown in Fig. 5A, whereas we were unable to observe any specific binding of AS160 to GST alone, GST fused to the cytosolic tail of VAMP2, or GST fused to the carboxyl tail of GLUT4, there was a significant interaction between AS160 and the GST-IRAP1–109 fusion protein (data not shown) and a GST-IRAP1–58 fusion protein (Fig. 5A). In contrast, we were unable to observe an interaction between AS160 and a truncated IRAP cytosolic tail comprising the first 27 amino acids (Fig. 5A). Moreover, the interaction between AS160 and the IRAP tail was significantly higher using basal compared with insulin-treated cell lysate. To further confirm this interaction, we next endeavored to show an interaction between AS160 and IRAP in vivo. LDM was prepared from basal or insulin-stimulated 3T3-L1 adipocytes and solubilized in 60 mM β-octylglucoside, 1% Triton X-100, and the soluble fraction was incubated with either control antibodies or antibodies against AS160 or
IRAP coupled to CNBr beads. As shown in Fig. 5B, IRAP co-immunoprecipitated with AS160, and this interaction was only observed in basal but not in insulin-stimulated cells. Similarly, AS160 co-immunoprecipitated with IRAP from adipocyte lysate, but in contrast to that observed in the AS160 immunoprecipitate, this interaction was only slightly reduced by insulin (Fig. 5C). The explanation for this difference is not clear; however, these data suggest that the effect of insulin on the interaction between AS160 and IRAP may be more complex than a simple dissociation event.

Co-localization of AS160 with GLUT4 and Characterization of AS160 shRNA Knockdown—To further characterize AS160 in 3T3-L1 adipocytes, we determined the co-localization of AS160 with GLUT4 by confocal immunofluorescence microscopy. AS160 showed a predominantly peripheral vesicular staining pattern (Fig. 2B). Co-localization of AS160 with GLUT4 indicated some overlap of the two proteins in peripheral vesicles and the perinuclear area (Fig. 2B). The insulin-dependent release of AS160 from vesicles suggests a model whereby the targeting of AS160 to GLUT4 vesicles in the absence of insulin may play an important role in the intracellular sequestration of this compartment. To test this we used RNA interference to suppress the expression of AS160. We constructed a hairpin vector specifically targeting AS160 that was able to suppress AS160 levels by greater than 80% after transient transfection in C2C12 cells (Fig. 6A). To express the shRNA in insulin-responsive adipocytes we employed the pBabe retrovirus. As indicated in Fig. 6A adipocytes infected with this retrovirus demonstrated a 40% reduction in AS160 protein levels. To measure GLUT4 translocation in cells in which AS160 levels were suppressed, we co-infected cells with retroviruses expressing HA-GLUT4 and AS160 shRNA. Because of the variable reduction in AS160 levels, we used a single cell analysis to study the relationship between total AS160 expression and cell surface levels of HA-GLUT4. This analysis revealed that overall, cells infected with the AS160 shRNA had an 8-fold increase in surface HA-GLUT4 labeling compared with control cells ($p < 0.005$). Analysis of the individual data points clearly indicates that there is a threshold level of AS160 expression, below which surface levels of HA-GLUT4 are significantly elevated. Importantly, the increase in PM levels

![Figure 2. Localization of integral and peripheral membrane proteins with GLUT4. 3T3-L1 adipocytes were serum-starved for 2 h and fixed and processed for immunofluorescence. GLUT4 was detected using a Cy3-conjugated secondary antibody, and the other proteins shown were detected using ALEXA 488-conjugated secondary antibodies. A, co-localization of GLUT4 vesicle integral membrane proteins with GLUT4 vesicles in the basal state. B, co-localization of GLUT4 vesicle peripheral membrane proteins with GLUT4 vesicles in the basal state. Images are from a representative experiment ($n = 3$).](image-url)
of HA-GLUT4 upon reduced AS160 expression were similar to that observed in control cells treated with insulin (Fig. 6B). It has previously been shown that overexpression of the AS160 4P mutant in adipocytes principally affects GLUT4 translocation by perturbing the exocytosis of GLUT4 (41). We wanted to determine whether the AS160 shRNA was functioning in a similar manner. To determine this, recycling experiments were performed in which either control cells or cells expressing the AS160 shRNA were incubated with anti-HA antibody at 37 °C for 10 min or 60 min. As shown in Fig. 6C in non-stimulated control cells there was little antibody uptake over the course of the 60-min incubation, consistent with previous studies showing that the basal recycling rate of HA-GLUT4 is very slow (6, 42). In contrast, in cells expressing the AS160 shRNA there was a significant increase in antibody uptake even in the absence of insulin, and labeling was detected at an intracellular localization consistent with increased exocytosis of GLUT4 upon AS160 reduction.

**DISCUSSION**

In this study we have made several novel observations about insulin-regulated GLUT4 trafficking in adipocytes. First, using a comprehensive proteomic and biochemical analysis of affinity-purified GLUT4 vesicles we have found that the major membrane protein constituents of GSVs are GLUT4, IRAP, and VAMP2. The remaining constituents are probably endosomal or TGN-derived proteins. Second, we have identified three Rab proteins associated with GLUT4 vesicles, Rab10, Rab11, and Rab14, two of which have not previously been reported to associate with GLUT4 vesicles. Third, we have shown that the putative RabGAP AS160 is associated with GLUT4 vesicles in 3T3-L1 adipocytes and dissociates from GLUT4 vesicles with insulin stimulation. Fourth, we have demonstrated that the binding of AS160 to GLUT4 vesicles in the basal state is mediated at least in part via an interaction with the integral membrane protein IRAP and that this interaction is insulin-regulated. Finally, we show that knockdown of AS160 in adipocytes with shRNA causes insulin-independent movement of GLUT4 to the PM.

Previous studies attempting to identify proteins associated with GLUT4 vesicles used analytical techniques much less sensitive than that used in the present study and have, therefore, only identified the most abundant proteins. The proteins identified previously include GLUT4, IRAP (43), VAMP2 (44), TIR (45), CD-MPR (37), CI-MPR (46), sortilin (35), copper amine oxidase (34), SCAMPs (47), and VAMP3 (for review, see Bryant et al. (11)). We have identified all of these proteins and many other novel proteins not previously identified in GLUT4 vesicles. The large number and the diverse localizations of the proteins identified demonstrate that we have achieved a comprehensive analysis of the majority of proteins associated with GLUT4 vesicles. Based on our morphological and biochemical analysis of a large number of these proteins, we have concluded that GLUT4, IRAP, and VAMP2 are the major proteins that are uniquely targeted to peripheral GLUT4 vesicles in adipocytes, and these studies likely explain the unique responsiveness of these...
proteins. Notably, insulin also has a smaller effect on the translocation of other proteins including the TSR (Fig. 1b) and GLUT1 (48). There are at least three possible explanations for this effect (1). First, insulin may inhibit general endocytosis. This is unlikely because it has been shown that insulin has no effect on TR endocytosis in adipocytes (49). Second, a small proportion of the TR may be targeted to GSVs and undergo exocytosis together with GLUT4, VAMP2, and IRAP. However, Akt is not involved in TR exocytosis, which suggests that the mechanism for its regulated release is different compared with these other proteins (50). Third, insulin may increase endosomal recycling. This seems likely because a number of endosomal recycling proteins including MPR, GLUT1, and TR have been shown to undergo a relatively small increase in translocation to the PM with insulin (1). We cannot rule out that some of the other proteins identified for which we do not have antibodies may be in GSVs. However, studies of synaptic vesicle proteins have also shown that synaptic vesicles are quite simple in composition, as some of the other proteins identified for which we do not have antibodies may be substrates for the RabGAP AS160 and play an integral role in insulin-stimulated GLUT4 translocation (11, 14, 15). Expression of a dominant negative mutant of Rab4α in adipocytes inhibits GLUT4 translocation by ~50%, and the only direct evidence of a role for Rab4α in GLUT4 translocation is its binding to syntaxin 4 (14). However, the significance of this observation is not clear, as Rab proteins have not been found to bind directly to t-SNAREs in other vesicle transport pathways. Moreover, we failed to observe Rab4α in our GLUT4 vesicle fraction, suggesting that the majority of Rab4α probably does not co-localize with GLUT4 in adipocytes. Rab11 is known to be involved in traffic through recycling endosomes, and it has previously been shown to be associated with GLUT4 vesicles (11). Rab11 translocates to the PM slightly with insulin stimulation (11). However, Rab11 does not translocate to the PM with GTPγS stimulation, indicating that the PM may not be the Rab11 target organelle (13). Rab11 function has been implicated in insulin-stimulated GLUT4 translocation through interactions with the Rab11bp (55). However, the mechanism of action of Rab11 is unknown. The discovery in this study that Rab10, Rab11, and Rab14 are associated with GLUT4 vesicles is exciting, because one or more of these three Rab proteins may be substrates for the RabGAP AS160 and play an integral role in insulin action. Rab14 has been shown to be involved in TGN to endosomal trafficking (19); however, significant amounts of Rab14 are found at the PM. Therefore, Rab14 may have other more specialized roles in exocytosis such as insulin-stimulated GLUT4 translocation. The role of Rab10 has yet to be elucidated. Intriguingly, it is one of the closest mammalian homologues of the yeast sec4 protein (56) and, therefore, may function in the post-Golgi secretory pathway. Sec4 has been shown to regulate assembly of the tethering complex known as the exocyst (57). This is of interest because the exocyst has been implicated in insulin-regulated GLUT4 trafficking in adipocytes (58). Amino acid sequence alignment of the three Rab proteins identified in this study shows that Rab14 and Rab11 are much more closely related to each other than to Rab10, which may indicate that Rab10 has a more unique role in GLUT4 exocytosis. Further work will be required to examine the role of each Rab in GSV translocation and their association with AS160. Intriguingly, Lienhard and co-workers (59) have recently shown in an in vitro GAP assay that the GAP domain of AS160 shows GAP activity toward Rab10 and Rab14.

Recently, we and others have suggested that the movement of GLUT4 vesicles toward the PM is Akt-independent, whereas a step close to the PM, likely involving docking and/or fusion of GLUT4 vesicles, is the major Akt-dependent step (7, 9, 40, 41, 60). In this study we have shown that AS160 dissociates from GLUT4 vesicles with insulin stimulation. Furthermore, we have shown that there is a specific association between AS160 and the cytosolic tail of IRAP. This observation is intriguing for a number of reasons. First, it has previously been shown that microinjection of a fusion protein comprising the cytosolic N terminus of IRAP into adipocytes was sufficient to trigger GLUT4 translocation to the PM (61). A similar effect was observed using a fusion protein encompassing the first 58 amino acids of the IRAP tail, and we have also observed that this peptide interacts with AS160 in vitro. Hence, it is tempting to speculate that overexpression of the IRAP cytosolic tail in adipocytes blocks
the interaction of AS160 with the GLUT4 vesicles, thus allowing their constitutive translocation to the PM. Second, it has been shown that GLUT4 trafficking is unaffected in adipocytes from IRAP knock out mice, suggesting that IRAP is not necessary for the intracellular sequestration of the vesicles (62). A likely explanation for this is that AS160 may be capable of interacting with other proteins associated with GLUT4 vesicles, possibly even its putative Rab substrate. Cargo binding may be a general function of RabGAPs in view of the large number of RabGAPs in the human genome, and one could envisage that if each member of this family encoded unique cargo binding specificity, this could represent a novel mechanism for regulating protein trafficking at different locations in eukaryotic cells. Each cargo-RabGAP interaction could be controlled by discrete signaling inputs. In the case of AS160, this appears to involve phosphorylation by Akt, and as described above, this would place the action of AS160 close to the PM. In this way the dissociation of AS160 from GSVs may trigger GTP loading of a relevant Rab protein. Although Rabs have been implicated at several steps in vesicle transport, one of their major functions appears to involve regulation of vesicle docking at the target membrane (63). The fact that the down-regulation of AS160 led to insulin-independent movement of GLUT4 to the PM indicates the involvement of AS160 before fusion. A recent study by Zeigerer et al. (41) has also shown that a step before GLUT4 vesicle fusion is AS160-dependent. These studies also suggest that AS160 plays an important role in the intracellular sequestration of GLUT4 in the absence of insulin. As reported previously (6, 26), GLUT4 has a very low rate of cell surface recycling in the absence of insulin as indicated in Fig. 6C. Suppression of AS160 levels enhance this basal recycling rate (Fig. 6C). This is unlikely due to an effect of AS160 on endocytosis because we observed rapid internalization of GLUT4 in cells expressing the AS160 shRNA. This is consistent with experiments performed by Zeigerer and co-workers (41) showing that overexpression of the AS160 4P mutant inhibited GLUT4 exocytosis with no effects on GLUT4 endocytosis. These studies are consistent with a role of AS160 in GLUT4 exocytosis. This demonstrates that AS160 is a specific regulator for the exocytosis of insulin-responsive GLUT4 vesicles in adipocytes. We propose a model of GLUT4 translocation such that in the basal state AS160 is associated with GSVs by interacting with IRAP and possibly other vesicle cargo, and its GAP activity maintains a key GTPase(s) in an inactive GDP-bound form. Upon stimulation with insulin the GSVs move toward the PM, and AS160 is phosphorylated by Akt, leading to its dissociation from GSVs. This allows GTP loading of one or more Rab proteins, enabling the docking and fusion of the GSVs with the PM.

Acknowledgments—We thank Jonathan Davey for help with the shRNA experiments, Donna Stolz for providing the cationic silica particles, Gus Lienhard for the AS160 constructs, and all those colleagues who provided antibodies used in this study.

REFERENCES

1. Bryant, N. J., Gover, R., and James, D. E. (2002) Nat. Rev. Mol. Cell Biol. 3, 267–277
2. Cushman, S. W., and Wardzala, L. J. (1980) J. Biol. Chem. 255, 4758–4762
3. Suzuki, K., and Kono, T. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 2542–2545
4. Martin, S., Millar, C. A., Lyttle, C. T., Meelmo, T., Marsh, B. J., Gould, G. W., and James, D. E. (2000) J. Cell Sci. 113, 3427–3438
5. Shewan, A. M., van Dam, E. M., Martin, S., Luen, T. R., Hong, W., Bryant, N. J., and James, D. E. (2003) Mol. Biol. Cell 14, 973–986
