Protein Kinase B Stimulates the Translocation of GLUT4 but Not GLUT1 or Transferrin Receptors in 3T3-L1 Adipocytes by a Pathway Involving SNAP-23, Synaptobrevin-2, and/or Cellubrevin

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Involving SNAP-23, Synaptobrevin-2, and/or Cellubrevin* An interaction of SNAP-23 and syntphin 4 on the plasma membrane with vesicle-associated synaptobrevin-2 and/or cellubrevin, known as SNAP (soluble N-ethyl-maleimide-sensitive factor attachment protein) receptors or SNAREs, has been proposed to provide the targeting and/or fusion apparatus for insulin-stimulated translocation of the GLUT4 isoform of glucose transporter to the plasma membrane. By microinjecting 3T3-L1 adipocytes with the Clostridium botulinum toxin B or E, which proteolyzed synaptobrevin-2/cellubrevin and SNAP-23, respectively, we investigated the role of these SNAREs in GLUT4, GLUT1, and transferrin receptor trafficking. As expected, insulin stimulated the translocation of GLUT4, GLUT1, and transferrin receptors to the plasma membrane. By contrast, a constitutively active protein kinase B (PKB-DD) only stimulated a translocation of GLUT4 and not GLUT1 or the transferrin receptor. The GLUT4 response to PKB-DD was abolished by toxins B or E, whereas the insulin-evoked translocation of GLUT4 was inhibited by approximately 65%. These toxins had no significant effect on insulin-stimulated transferrin receptor appearance at the cell surface. Thus, insulin appears to induce GLUT4 translocation via two distinct routes, only one of which involves SNAP-23 and synaptobrevin-2/cellubrevin, and can be mobilized by PKB-DD. The PKB-, SNAP-23-, and synaptobrevin-2/cellubrevin-independent GLUT4 translocation pathway may involve movement through recycling endosomes, together with GLUT1 and transferrin receptors.

In muscle, adipose tissue, and 3T3-L1 adipocytes, insulin primarily increases glucose uptake by promoting the trafficking of vesicles containing GLUT4 (glucose transporter isoform 4) to the plasma membrane (reviewed in Refs. 1 and 2). In the resting state, the majority of GLUT4 resides in vesicles distributed throughout the cell interior, with a fraction of GLUT4 undergoing cycling between the plasma membrane and several intracellular sorting compartments. Insulin triggers a substantial translocation of GLUT4-containing vesicles to the plasma membrane, a phenomenon that can largely explain the increase in V_max of the glucose uptake observed. A detailed molecular description of how insulin promotes this translocation is presently lacking, although some of the components involved in the signaling process and the fusion events have been identified.

Binding of insulin to its receptor activates the integral tyrosine kinase, which then elicits a cascade of cellular signaling responses, including phosphorylation of the cytosolic proteins of the insulin-receptor-substrate family (reviewed in Ref. 3). As a consequence of tyrosine phosphorylation, insulin-receptor substrates-1 and -2 bind several effectors; the resultant activation of phosphatidylinositol (4,5)-bisphosphate kinase (PI3-kinase) is of particular importance because it is known to play a key role in transducing the insulin signal leading to GLUT4 vesicle translocation (4–6). The lipid product of this enzyme, namely phosphatidylinositol 3,4,5-trisphosphate, promotes the phosphorylation and activation of the serine/threonine-kinase called protein kinase B (PKB; also known as RAC or Akt) via two protein kinases named PDK1 and PDK2 (7). Overexpression of constitutively active forms of PKB causes increased glucose uptake and GLUT4 translocation in adipocytes in the absence of insulin (8, 9), suggesting that this protein kinase may also be a crucial mediator of the effect of insulin on glucose transport, at least in part.

The molecular mechanism of GLUT4 vesicle fusion with the plasma membrane appears to share considerable similarity with Ca^2+-evoked exocytosis in neurons and neuroendocrine cells (reviewed in Ref. 10). In neurons, Söllner and co-workers (11) have proposed that the core of the synaptic-clear vesicle docking/fusion complex comprises two plasma membrane proteins, syntphin 1 and SNAP-25 (synaposomes protein with a molecular mass of 28 kDa), which interact with synaptobrevin (Sbr) on the vesicle. This provides the targeting specificity and/or the fusion apparatus necessary for neurotransmitter release at the active zones of nerve endings. Additionally, these three proteins serve as receptors for the cytosolic factors N-ethyl-maleimide-sensitive factor and SNAP (soluble N-ethyl-maleimide-sensitive factor attachment protein), which collectively regulate the ternary associations and, therefore, are referred to as SNAREs (SNAP receptors). SNAP-25 and syntphin 1, located predominantly on the targeted plasma mem-

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1 The abbreviations used are: PI3-kinase, phosphatidylinositol (4,5)-bisphosphate kinase; BoNT, botulinum neurotoxin; Cbr, cellubrevin; GFF, green-fluorescent protein; IRAP, insulin-responsive amino peptidase; TIR, transferrin receptor; hTIR, human TIR; HA, hemagglutinin; PKB, protein kinase B; Sbr, synaptobrevin; TeTx, tetanus toxin; TRITC, tetramethylrhodamine β-isothiocyanate; GTPyS, guanosine 5′-3-O-(thio)triphosphate; PIPES, 1,4-piperazinediethanesulfonic acid.
brane, are referred to as t-SNAREs, whereas vesicle located Sbr is the v-SNARE (11). An involvement of Sbr-2 and/or its ubiquitous non-neuronal homologue, Cbr (12), and syntaxin 4 (13) in insulin-stimulated GLUT4 vesicle fusion in 3T3-L1 adipocytes has been demonstrated. Their appropriate subcellular locations and protein associations support such a role (14–21); also, the process is inhibited by the cytosolic delivery of selective antibodies against syntaxin 4 (14) and overexpression of interfering SNARE mutants (16, 22). Recently, Martin and co-workers (23) demonstrated that introduction of Sbr-2, but not Sbr-1 or Cbr proteins, into these cells could block insulin-stimulated translocation. Moreover, botulinum neurotoxin (BoNT) serotypes B and D or tetanus toxins (TeTx), which selectively proteolyze Sbr isoforms and Cbr (see below), have been instrumental in establishing the involvement of these v-SNAREs in insulin-induced glucose uptake and GLUT4 trafficking (21, 24–26).

TeTx and serotypes A to G of BoNT are similar but immunologically distinct proteins produced by Clostridium tetani and Clostridium botulinum, respectively. They are composed of a heavy chain and light chain linked by a disulphide bond and noncovalent interactions. The heavy chain is responsible for high affinity binding to neuronal cholinergic ecto-acceptors, subsequent internalization, and translocation of the active moiety into the cytosol, where the light chain blocks exocytosis (reviewed in Refs. 27–30). The light chains of BoNTs and TeTx are Zn2+–dependent endoproteases (reviewed in Refs. 28–30). Sbr is proteolyzed by TeTx and BoNT/B, D/P and G (reviewed in Refs. 28–30); Cbr is also cleaved by TeTx and BoNT/B (12, 31). BoNT/A and E proteolyze SNAP-25 at specific C-terminally located sites, whereas BoNT/C cleaves both syntaxin 1 (reviewed in Refs. 28–30) and SNAP-25 (32, 33).

Using highly sensitive Western blotting methods, we (21) and others (18, 20, 25, 34, 35) have been unable to detect any SNAP-25 in 3T3-L1 adipocytes. This led to the suggestion that the recently cloned (from human (36) and mouse (37, 38)), non-neuronal homologue termed SNAP-23 (also referred to as Syndet), which is found at high levels and at the appropriate plasma membrane location in 3T3-L1 adipocytes (21, 37, 38), may substitute for SNAP-25 to provide the high affinity ternary docking/fusion complex described (39, 40). The association of SNAP-23 with Sbr-2/Cbr and syntaxin isoforms demonstrated in vitro (36, 37, 41) is consistent with a potential role of SNAP-23 in the fusion between GLUT4 vesicles and the plasma membrane. Indeed, recently, Rea and colleagues (42) have demonstrated that insulin-stimulated GLUT4 translocation to the plasma membrane was partially blocked upon the introduction into 3T3-L1 adipocytes of either anti-SNAP-23 antibodies or a synthetic peptide corresponding to the last 24 C-terminal amino acids of SNAP-23.

In this study we have investigated the mechanism by which insulin and a constitutively active PKB (PKB-DD) induce the translocation of GLUT4 to the plasma membrane of 3T3-L1 adipocytes. BoNT/B and BoNT/E, which specifically cleave Sbr-2/Cbr and SNAP-23, respectively, completely blocked the effect of PKB-DD but only partially blocked the effect of insulin. We also found that insulin, but not PKB-DD, caused GLUT1 and TFR translocation, in a manner that was insensitive to the actions of BoNT/B and E. Collectively, our results support the hypothesis (16, 23, 42, 43) that insulin may operate via two distinct pathways to promote GLUT4 vesicle fusion with the plasma membrane. In addition, our data suggest that only one of these trafficking pathways can be mobilized by a constitutively active PKB.

### EXPERIMENTAL PROCEDURES

#### Materials—A murine 3T3-L1 fibroblast clone (obtained from ATCC; number CCL 92.1) was supplied by the European Collection of Animal Cell Cultures (Salisbury, UK). Tissue culture reagents were from Life Technologies, Inc. or Sigma. High purity digitonin was purchased from Novabiochem (town, UK). Affinity-isolated anti-species-specific Ig conjugated with horseradish peroxidase, insulin and all other reagents were obtained from Sigma. Rabbit anti-SNAP-23 antibodies were a gift of Dr. S. Baldwin (University of Leeds). A monoclonal antibody specific for human transferrin receptor (hTfR) was provided by Dr. C. Holmes (University of Bristol). The plasmid pGFP–GLUT4 has been described elsewhere (44). The pCMV5-HTIR encoding the hTfR cDNA under the control of the cytomegalovirus promoter was kindly provided by Dr. H. Mellor (University of Bristol). The plasmid pGFP–GLUT4 has been described elsewhere (44). The pCMV5 vector was also used to express wild-type and constitutively active (I-D containing substitutions of Thr330 and Ser373 for aspartic acid residues) forms of PKB (45), and were kindly provided by Drs. Brian Hemmings (Friedrich Miescher Institute, Basel) and Dario Alessi (University of Dundee).

Antibodies were raised in rabbits against a soluble recombinant His6-tagged form of mouse syntaxin 1A, lacking the last 27 C-terminal residues, and were affinity-purified on the immobilized antigen (32). Affinity-purified Ig raised against residues 33–94 of human Sbr-2 (a region of amino acid sequence shared with Sbr and Cbr; Ref. 12) was prepared, as detailed elsewhere (32). Because only poor sequence identities exist between Sbr-2VAMP-2 and the other recently identified mammalian VAMP isoforms 4–8 (46), their immunoreactivity with this antibody is not expected. Moreover, VAMP isoforms 4–8 are unlikely to be proteolyzed by BoNT/B because of their sequence diversities (47). PKB (45), and were kindly provided by Drs. H. Mellor (University of Bristol). The plasmid pGFP–GLUT4 has been described elsewhere (44). The pCMV5 vector was also used to express wild-type and constitutively active (I-D containing substitutions of Thr330 and Ser373 for aspartic acid residues) forms of PKB (45), and were kindly provided by Drs. Brian Hemmings (Friedrich Miescher Institute, Basel) and Dario Alessi (University of Dundee).

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### Immunoblotting and Quantitation of Antigens—

#### Porcine Cells with Digitonin to Observe the Proteolytic Activities of BoNT, BoNT/B, BoNT/C1, or BoNT/E—Porcine chromaffin cells were prepared from adrenal glands and maintained in culture, as detailed elsewhere (50). Differen
tiated 3T3-L1 adipocytes were prepared as outlined before (21). Cells (5 × 10⁶ cells/well) were washed three times with buffer A (20 mM Tris, pH 7.4, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 1.2 mM NaH₂PO₄, 5 mM NaHCO₃, 100 mM KCl, 1.0 mM HEPES, pH 7.4). They were then permeabilized at 37 °C using 40 μM digitonin in KGE buffer (139 mM K⁺ glutamate, 20 mM PIPES–HCl, pH 7.0, 1 mM EGTA, 2 mM MgCl₂, 2 mM ATP, 0.25 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 10 μg/ml protease A) in the absence or presence of preincubated BoNT/B, BoNT/E, or BoNT/C1, or BoNT/E (at the concentrations given in the figure legends). More than 95% of the cells (of the three different lines employed) exhibited nuclear staining by trypan blue, confirming the efficacy of permeabilization. BoNTs were replaced with 20 mM dithiothreitol in 25 mM HEPES, pH 7.4, containing 0.15 mM NaCl for a minimum of 30 min at 37 °C before dilution in digitonin–KGE buffer and application to cells; neurotoxin-free control cells were treated to the same initial concentration of dithiothreitol, which never exceeded 1 mM. After incubation, the medium was aspirated and replaced with 50 mM NaHCO₃, pH 8.5, containing protease inhibitors and the cells were harvested and homogenized (detailed in Ref. 31). The homogenate was adjusted to 0.32 M sucrose and centrifuged at 800 × g for 10 min, and the resultant supernatant was subjected to 390,000 × g for 1 h to sediment all remaining membranes. The resultant pellets were solubilized in 150 mM Tris, pH 8.5, containing 2% (v/w) SDS plus 1 mM EDTA and solubilized by heating to 90 °C for 10 min.

#### Immunoblotting and Quantitation of Antibodies— Samples were subjected to immunoblotting as detailed in Ref. 21. Antibodies bound to the membranes were detected with anti-species-specific Ig conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence, using the ECL™ detection system (Amersham Pharmacia Bio-
Insulin and PKB-regulated GLUT4 Translocation

RESULTS

Murine, but Not Human, SNAP-23 Is Proteolyzed by BoNT/E—To investigate the role of SNAP-23 in GLUT4 vesicle docking and fusion with the adipocyte plasma membrane, the susceptibilities of the murine and human isoforms to proteolytic attack by BoNT/E were examined. A digitonin-based permeabilization method was used to introduce the toxin into cells, because it is known to allow efficient cleavage of other SNAREs (outlined in Ref. 21). Following incubation in the absence or presence of BoNTs, permeabilized cells were lysed and subjected to immunoblotting using primary antibodies reactive with the relevant SNAREs (detailed in “Experimental Procedures”). Indeed, application of BoNT/A, BoNT/C1, or BoNT/E to permeabilized bovine chromaffin cells resulted in cleavage of the majority of SNAP-25, type C1, additionally, proteolyzed syntaxin 1, whereas BoNT/B proteolized the Shb isoforms and Chr present (Fig. 1). Also, BoNT/B gave nearly complete cleavage of Chr and Shb in permeabilized mouse 3T3-L1 adipocytes and human CACO-2 cells (Fig. 1). These data confirm the high proteolytic activities and appropriate substrate selectivities of the BoNT serotypes used in this study.

The cleavage of SNAP-23 at its C terminus by BoNT/E was investigated using an antisera that recognizes the C-terminal 11 amino acids downstream of the putative cleavage site in murine SNAP-23 (potentially between Lys185-Ile186 (21)); the expectation was that this cleavage would result in the disappearance of its immunoreactivity on Western blots. Whereas treatment of permeabilized mouse and human cells for 30 min with 100 nM of either BoNT/A or BoNT/C1 failed to produce significant cleavage of either murine or human SNAP-23, an identical exposure to BoNT/E diminished the level of reactivity of murine SNAP-23 (Fig. 1). Quantitation by densitometric scanning revealed that BoNT/E removed 76.8 ± 2.9% (mean ± S.D from four separate experiments) of the immunoreactive SNAP-23 present, compared with toxin-free control. In contrast to this extensive cleavage of murine SNAP-23, BoNT/E consistently failed to yield detectable proteolysis of SNAP-23 in the human CACO-2 cell lines under the same conditions (Fig. 1); however, a very slow rate of cleavage could not be excluded. Furthermore, BoNT/E did not alter the abundance of Shb-2/Chr or syntaxin isoforms 1 or 4 in any of these cell lines, reaffirming its known selective proteolytic action (Fig. 1). The observed insensitivity of syntaxin 4 to proteolytic attack by type C1 (Fig. 1) confirms earlier studies (51).

A Requirement for Zn$^{2+}$ and Prereduction of the Interchain SNARE proteins plotted against band intensity were found to be linear. For 1% of the cell volume is delivered during microinjection, thus providing an intracellular concentration of toxin of 100–200 nM. After microinjection, the cells were incubated at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% (v/v) myokinase plus fetal calf serum for 16–24 h. Before further manipulation, adipocytes were incubated for 2 h at 37 °C in serum-free Dulbecco’s modified Eagle’s medium and then for 1 h in the presence of 200 nM insulin, as required. For immunoblotting using primary antibodies reactive with both semiautomatic system. Plasmids were microinjected at 20–100 μg/ml in the absence or presence of prereduced BoNT/B (2 μM) or BoNT/E (1 μM) in 10 mM HEPES, pH 7.5, containing 2 mM MgCl$_2$, 10 mM dithiothreitol, and 50 μg/mL ZnSO$_4$. It is estimated that approximately 10% of the cell volume is delivered during microinjection, thus providing an intracellular concentration of toxin of 100–200 nM. After microinjection, the cells were incubated at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% (v/v) myokinase plus fetal calf serum for 16–24 h. Before further manipulation, adipocytes were incubated for 2 h at 37 °C in serum-free Dulbecco’s modified Eagle’s medium and then for 1 h in the presence of 200 nM insulin, as required. Immunofluorescence Analysis—In some experiments, the cellular distribution of expressed PKB (which possesses a HA tag) was determined. Cells were fixed and permeabilized, using 4% paraformaldehyde and 1% Triton X-100. Unless otherwise stated, all subsequent steps were performed in phosphate-buffered saline supplemented with 3% bovine serum albumin. In some experiments, the cells were stained with monoclonal anti-HA antibodies (10 μg/ml of HA11; Berkeley Antibody Company, CA) for 30 min, followed by incubation in a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) or TRITC-conjugated rabbit anti-mouse IgG (Dako Laboratories) for 30 min. In other experiments, PKB was visualized using a 1:200 dilution of a rabbit polyclonal anti-HA antisera (Santa Cruz) followed by incubation in 1:200 dilution of TRITC-conjugated goat anti-rabbit IgG (Sigma) for 30 min.

To detect the insulin-responsive aminopeptidase, IRAP, the fixed and permeabilized cells were stained with a rabbit polyclonal anti-IRAP serum (5 μg/ml; a kind gift of Drs. Susanna Keller and Gus Lienhard) in phosphate-buffered saline with 3% bovine serum albumin for 30 min. This was followed by a 1:200 dilution of TRITC-conjugated goat anti-rabbit IgG for 15 min. GLUT1 was immunostained using the same procedure but with 25 μg/ml rabbit anti-GLUT1 antibody for 1 h followed by a 1:200 dilution of TRITC-conjugated goat anti-rabbit IgG for 30 min. Transferrin receptors were detected by staining fixed cells with undiluted monoclonal anti-transferrin receptor hybridoma supernatant in phosphate-buffered saline. Depending on the nature of the experiment, this was followed by a 1:200 dilution of TRITC-conjugated rabbit anti-mouse IgG or fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) for 30 min in phosphate-buffered saline with 3% bovine serum albumin.

Confocal Microscopy and Image Analysis—Confocal microscopy was performed with a Leica DM IRBE inverted confocal microscope controlled with TCS-NT4 software (Leica). Images were processed with Adobe Photoshop 3.0 and Freelance Graphics 95 (Lotus). The extent of GFP-GLUT4 translocation to the plasma membrane was quantified by marking a region of interest around the exterior and interior faces of the plasma membrane in the confocal image. The levels of fluorescence intensity within these areas were then computed using TCS-NT software, and the intensity of plasma membrane localized GFP-GLUT4 fluorescence (F$_{pm}$) expressed as a percentage of total cellular GFP-GLUT4 fluorescence intensity (F$_{t}$). This method corrects for variations in cell shape, size, and the expression level of GFP-GLUT4.

A Requirement for Zn$^{2+}$ and Prereduction of the Interchain

![Fig. 1. Proteolysis of murine SNAP-23 but not the human isoform by BoNT E. Bovine adrenomedullary chromaffin cells, 3T3-L1 adipocytes, or human CACO-2 cells were incubated with digitonin-KGE permeabilization buffer at 37 °C for 30 min in the absence or presence of 100 nM of the specified prereduced BoNT serotype. A total membrane fraction was prepared, and equal amounts of protein (20 μg for chromaffin cells and 50 μg for 3T3-L1 or CACO-2 cells) were immuno-blotted using the antibodies indicated. Binding of primary antibodies was detected with horseradish peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescence (see “Experimental Procedures”).](Image 369x493 to 494x729)
Disulphide of BoNT/E for Proteolysis of Murine SNAP-23 Establishes Its Selective Action—To reliably confirm that murine SNAP-23 is cleaved by BoNT/E, we investigated two physical requirements for the proteolytic activities of the other BoNT serotypes shared by type E, namely that Zn$^{2+}$ is essential and that reduction of the interchain disulphide bridge is necessary to activate the protease of the light chain (reviewed in Refs. 28–30). As shown in Fig. 2, prereduction of BoNT/E was found to be a prerequisite for proteolysis of SNAP-23 in permeabilized 3T3-L1 adipocytes. Moreover, incubation of reduced BoNT/E with the divalent cation chelators EDTA, dipicolinic acid, and 1,10-phenanthroline (which is highly selective for Zn$^{2+}$, compared with Ca$^{2+}$ or Mg$^{2+}$) before introduction into 3T3-L1 adipocytes removed the ability of BoNT/E to cleave SNAP-23 (Fig. 2); in contrast, the activity of the toxin was not altered by the protease inhibitors included in the permeabilization buffer to inhibit a broad range of other cellular proteolytic enzymes (see "Experimental Procedures"). Therefore, the unique protease activity of BoNT/E must be responsible for cleavage of murine SNAP-23 in 3T3-L1 adipocytes.

Murine SNAP-23 Is Not as Efficiently Proteolyzed as SNAP-25 by BoNT/E—Digitonin-permeabilized chromaffin cells that express SNAP-25 and 3T3-L1 adipocytes that contain SNAP-23 were exposed to various concentrations of BoNT/E. The extents of proteolysis of the respective targets were determined essentially as described in the legend to Fig. 1, by densitometric scanning of the resultant Western blots. The concentrations of BoNT/E extrapolated from the plot in Fig. 3 (circles) required to cleave 50% of SNAP-25 was 0.3 nM; in contrast, this extent of cleavage of murine SNAP-23 in permeabilized adipocytes required 30 nM BoNT/E (Fig. 3, squares). Thus, BoNT/E is 100-fold less effective in cleaving murine SNAP-23 than its homologue, SNAP-25. Nevertheless, BoNT/E could diminish the SNAP-23 content by 90.3 ± 2.8% (mean ± S.D., n = 3). While 2.4 nM BoNT/C1 was sufficient to proteolyze 50% of SNAP-25, longer term treatment of permeabilized adipocytes with high concentrations of this toxin (300 nM) caused insignificant proteolysis of murine SNAP-23 (Fig. 3). A small reduction (approximately 20%) in murine SNAP-23 by BoNT/A (280 nM) was observed (Fig. 3); however, because 0.04 nM of BoNT/A gave an equivalent degree of proteolysis of SNAP-25, cleavage of SNAP-23 requires approximately 7000 times more toxin. Thus, the ability of BoNT/E to selectively proteolyze SNAP-23, as well as the incubation conditions necessary to attain its efficient cleavage, have been established.

To conclude, our observations confirm the reported proteolysis of bacterially expressed murine SNAP-23 by native BoNT/E (52), although another group found murine recombinant SNAP-23 to be resistant to the toxin (25). The inability of
this toxin to proteolyze human SNAP-23 agrees with the finding of others (53). SNAP-23, while being 59% identical in amino acid sequence to SNAP-25, exhibits extensive diversity at its C terminus (36–38), the region which encompasses the cleavage sites of BoNT/A, BoNT/C1, and BoNT/E. Thus only small changes in amino acid sequence between SNAP-25 and human SNAP-23 could account for the inability of BoNT/A, BoNT/C1, or BoNT/E to proteolyze the latter. Similarly, human and murine forms of SNAP-23 also differ by 13% (36–38), perhaps explaining their differing proteolytic susceptibilities to BoNTs.

**GFP-GLUT4 Trafficking to the Plasma Membrane Occurs in Response to a Constitutively Active Form of PKB**—Having demonstrated the cleavage of SNAP-23 by BoNT/E, its effect on GLUT4 translocation was investigated after microinjection into 3T3-L1 adipocytes. To monitor GLUT4 translocation, we used a single cell assay that involved expression of a chimera linking GLUT4 to the C terminus of GFP from the jellyfish, *Aequorea victoria* (44). This entailed microinjecting a plasmid encoding GFP-GLUT4 into the nucleus, followed by laser scanning confocal microscopy 24 h later. We confirmed that the GFP-GLUT4 chimera was expressed in native GLUT4-containing vesicles, by demonstrating its efficient co-localization with endogenous IRAP, a bona fide GLUT4 vesicle-resident protein (54, 55). As shown in Fig. 4a, the majority of the GFP-GLUT4-containing vesicles co-localized with IRAP. In addition, GFP-GLUT4 was found in GLUT1-containing vesicles (presumably endosomes) but also in a population of vesicles that largely lacked GLUT1 (Fig. 4b). This is consistent with the known apparent distribution of GLUT4 between endosomes and specialized GLUT4-containing vesicles as determined previously (56, 57). In the basal state, the bulk of the GFP-tagged GLUT4 was distributed throughout the cytoplasm and just beneath the plasma membrane but was also concentrated close to the nucleus (Fig. 5a). Exposure to insulin resulted in a dramatic redistribution of GFP-GLUT4, such that a continuous line of fluorescence was observed on the plasma membrane (Fig. 5b; see also Ref. 44).

Adipocytes were also co-microinjected with pGFP-GLUT4 and plasmids encoding either wild-type PKB or a mutant PKB rendered constitutively active through substitution of Thr<sup>308</sup> and Ser<sup>473</sup> for aspartate residues (PKB-DD; both PKB constructs possessed a HA epitope tag for subsequent detection). The cells were fixed, stained with anti-HA antibodies, and imaged 24 h post-microinjection. Interestingly, both wild-type PKB and PKB-DD were located largely on the plasma membrane (Fig. 5, c and d); this is different from the predominantly cytosolic distribution of this kinase in nonstimulated fibroblasts and adipocytes as determined by either immunofluorescence staining or cell extraction followed by subcellular fractionation (9, 58–60). The reasons for the discrepancy are not known but could reflect the ability of the pleckstrin homology domain of PKB to bind phosphatidylinositol (4,5)-bisphosphate (61), which may be more abundant in the plasma membrane of resting 3T3-L1 adipocytes. Nevertheless, a plasma membrane location of the wild-type protein per se is not sufficient to promote the translocation of GLUT4-containing vesicles (Fig. 5e). PKB-DD, but not wild-type PKB, caused GFP-GLUT4 translocation to the plasma membrane to a similar extent to that induced by insulin (i.e. compare Fig. 5, f versus b). Thus, PKB activation mimicked by substitution of Thr<sup>308</sup> and Ser<sup>473</sup> for aspartate residues can promote GLUT4 translocation.

**BoNT/B and E Block GLUT4 Translocation Induced by Constitutively Active PKB More Efficiently than That Evoked by Insulin**—Because 100 nM BoNT/E was required to cause approximately 90% proteolysis of SNAP-23 in permeabilized 3T3-L1 adipocytes in a 45-min incubation (Fig. 3), concentrations of between 100 and 200 nM were delivered by microinjection. Because BoNTs can exert their activities over long periods...
in mammalian cells (reviewed in Refs. 21 and 30), most of the SNAP-23 would be cleaved by type E during the following 24 h period, while the GFP/GLUT4 was expressed from the coinjected GFP-GLUT4 plasmid. In validation of this approach, a concentration known in these cells to cause near complete proteolysis of Sbr-2 and Cbr. At the end of the experiments, the cells were fixed and stained with anti-HA antibody to confirm, where necessary, the expression and subcellular location of PKB variants.

The translocation of GFP-tagged GLUT4 was quantified in two ways. First, we visually examined each cell and determined whether they exhibited a continuous ring of fluorescence around the plasma membrane, indicative of translocation. On this basis, in the basal state, none of the cells showed a “translocated phenotype” (Fig. 6a). It was found that insulin and PKB-DD induced GFP-GLUT4 translocation in a similar fraction of the cells (Fig. 6a). (The reason underlying the heterogeneity in the insulin response is not known but has been noted in our previous studies on both insulin-stimulated GLUT4 translocation (44) and gene transcription (62), as well as by all others using the plasma membrane lawn technique to assess GLUT4 translocation.) As a second measure of the extent of GFP-GLUT4 translocation, the amount of GFP-GLUT4 found at the plasma membrane was calculated as a fraction of the total cellular GFP-GLUT4 measured. Thus, insulin and PKB-DD also caused a similar increase (approximately 4-fold) in the level of plasma membrane GFP-GLUT4.

The ability of PKB-DD to promote GLUT4 translocation to the plasma membrane in toxin-free cells (Figs. 6b and 7a) was completely blocked in the presence of microinjected BoNT/B (Figs. 6b and 7b) or BoNT/E (Figs. 6b and 7c), whereas the expressed PKB-DD was again mostly confined to the plasma membrane (Fig. 7, d–f). In the case of insulin, although neither toxin appeared to reduce the proportion of cells that responded to insulin (Fig. 6a), the fraction of GFP-GLUT4 that translocated in each individual cell was reduced by approximately 65% by either toxin (Fig. 6b). This partial inhibition of the insulin-stimulated GLUT4 translocation event by BoNT/B is consistent, therefore, with our previous studies (21). Similarly, Rea et al. (42) reported that a C-terminal peptide from SNAP-23 or specific antibodies reactive to the latter only blocked insulin-stimulated GLUT4 translocation by approximately 40% in permeabilized cells (see “Discussion”).

Insulin, but Not PKB-DD, Stimulates the Translocation of the Transferrin Receptor and GLUT1—The data presented above suggest that insulin stimulates GLUT4 translocation via two distinct pathways, only one of which is blocked by BoNT/B or BoNT/E. The latter may represent the trafficking pathway that responds to the introduction of the constitutively active PKB-DD mutant. We thus investigated the nature of the toxin-sensitive GLUT4 trafficking pathway.

In the basal state, GLUT4 is found in a vesicle compartment termed a GLUT4 storage vesicle (43), which contains Sbr-2 but is apparently devoid of GLUT1 and the TfR, as well as in the recycling endosomal pool that also contains the TfR and
GLUT1 (Refs. 23, 56, and 57; see also “Discussion”). Therefore, we investigated the effect of insulin or PKB-DD on GLUT1 and TfR translocation. Immunofluorescence staining showed that the majority of cells exhibited relatively low levels of endogenous GLUT1 on the plasma membrane in the basal state, i.e. few cells exhibited a continuous ring of GLUT1 immunofluorescence in the plasma membrane (Figs. 8a and 9a). Insulin treatment caused an increase in GLUT1 translocation such that approximately 70% of the cells exhibited a continuous ring of GLUT1 immunoreactivity at the plasma membrane (Figs. 8b and 9b). Conversely, expression of the constitutively active PKB-DD had no apparent effect on the subcellular distribution of GLUT1 or the ability of insulin to cause a translocation to the plasma membrane (Figs. 8, c and d, and 9a). The extent of GLUT1 translocation was quantified and expressed as the fraction of cellular GLUT1 present in the plasma membrane (% of total). In cells expressing wild-type PKB, 8.9 ± 0.9% (n = 46) of the GLUT1 was expressed in the plasma membrane (mean ± S.E. for the number of cells shown in parentheses and pooled from three independent experiments). In the presence of PKB-DD but the absence of insulin, 6.2 ± 0.6% (n = 41) of the GLUT1 was found in the plasma membrane; however, this increased to 22.1 ± 1.7% (n = 37) in the simultaneous presence of insulin and PKB-DD (p < 0.001 versus control without insulin).

To investigate the distribution of the TfR and because our antisera do not detect endogenous murine TfR, 3T3-L1 adipocytes were microinjected with a plasmid encoding the hTfR. This was subsequently detected by immunofluorescence staining with a highly avid monoclonal antibody specific for the human form of the receptor. In the basal state, the expressed hTfR was predominantly located intracellularly (Fig. 8e) and exhibited a considerable co-localization with endogenous GLUT4 (data not shown), although this was incomplete, as would be expected (57). Unlike GLUT1, however, in some cells (approximately 20–30%; Fig. 9b) a continuous ring of hTfR immunofluorescence was found in the plasma membrane probably as a result of overexpression. Exposure to insulin caused a pronounced translocation of hTfR to the plasma membrane such that approximately 70% of the cells now exhibited a continuous ring of hTfR around the plasma membrane (Figs. 8f and 9c). But, as with GLUT1, the constitutively active PKB-DD mutant did not promote any detectable translocation of hTfR to the plasma membrane or alter the ability of insulin to cause translocation of the latter (Figs. 8g and 9c).

The effects of BoNT/B and BoNT/E on insulin-stimulated transferrin receptor translocation were quantified; the ectopic expression of the hTfR allowed us to easily detect the toxin injected cells. However, neither BoNT had any significant ef-
fect on insulin-stimulated translocation of hTfR (Fig. 9b). In support of the validity of our results, a recent study by Martin and co-workers (23) found that transferrin-horseradish peroxidase-mediated ablation of recycling TfR-containing endosomes inhibited the subsequent insulin-stimulated translocations of TfR, as well as GLUT1. Despite this, insulin was still able to elicit large increases in the cell surface levels of GLUT4, presumably through activation of an alternate vesicle trafficking pathway (i.e. distinct from the fusion of recycling endosomes with the plasma membrane).

**DISCUSSION**

The molecular basis underlying the ability of insulin to promote the translocation of GLUT4 and thus stimulate glucose uptake remains incompletely defined. Our results comparing the effects of insulin and a constitutively active PKB mutant, together with the use of two botulinum toxins, support the proposal (16, 23, 42, 43, 57) that at least two pathways are involved in insulin-stimulated GLUT4 trafficking to the plasma membrane. Notably, our results suggest that only one of these pathways is sensitive to the action of a constitutively active PKB.

Insulin stimulated the translocation of GLUT1, GLUT4, and the TfR to the plasma membrane of 3T3-L1 adipocytes. This contrasted with the effect of overexpressing a constitutively active PKB-DD mutant (rendered active via substitution of the PDK1 and PDK2 phosphorylation sites with aspartate residues), which promoted only a translocation of GLUT4 but not GLUT1 or TfR. The effect of PKB-DD on GLUT4 translocation is consistent with the known effects on glucose transport of the constitutively active gag-Akt oncogene and a PKB mutant possessing a myristoylation signal sequence at its N terminus (8, 9). Although this suggests that PKB activation can mimic insulin-stimulated GLUT4 translocation, its precise role in the effect of insulin remains controversial. For example, Cong and co-workers (63) found that a dominant-negative PKB blocked insulin-stimulated glucose transport by approximately 20%, whereas Kitamura et al. (64) found no apparent effect. Regardless of this controversy, constitutively active PKB-DD is clearly a useful tool to investigate the mechanism by which insulin may promote GLUT4 translocation.

We investigated the role of SNARE proteins in the insulin- and PKB-mediated translocations of GLUT4, GLUT1, or TfR. Numerous studies have highlighted the essential requirement of syntaxin 4 and Sbr-2/Cbr for insulin-stimulated GLUT4 vesicle translocation in adipocytes (see the Introduction). To form a high affinity interaction between vesicle-derived Sbr and plasma membrane syntaxin-1, SNAP-25 is required (39, 40). However, numerous laboratories (20, 25, 34, 35, 38), including our own (see the Introduction) have been unable to detect the expression of SNAP-25 in 3T3-L1 adipocytes, despite the use of extremely sensitive Western blotting and high affinity selective antibodies. This prompted us to investigate the role of SNAP-23, which exhibits the appropriate molecular characteristics (see the Introduction), is abundant in 3T3-L1 adipocytes and was reported by James and colleagues (42) to be involved in insulin-stimulated glucose uptake.

BoNT/E cleaves murine, but not human, SNAP-23 in permeabilized cells, albeit at 100-fold higher concentrations than required for equivalent cleavage of SNAP-25. However, when employed at a sufficient concentration, BoNT/E can proteolyze >90% of SNAP-23, thus enabling an assessment of the role of this SNARE in GLUT4 vesicle trafficking. To do this, we microinjected BoNT/B or BoNT/E and performed single cell imaging of GLUT4 translocation using a GFP-tagged GLUT4 construct. Although insulin-stimulated GLUT4 translocation to the plasma membrane was partially blocked (approximately 65%) by BoNT/E, intriguingly this toxin completely blocked the effect of PKB-DD on GLUT4 translocation (Figs. 6 and 7). The same result was obtained following introduction of BoNT/B into cells to cleave the Sbr-2/Cbr present. Collectively, these observations suggest that PKB-dependent GFP-GLUT4 translocation is completely dependent on Sbr-2/Cbr and SNAP-23.

Although a variety of methods have been used to interfere with SNARE interactions in adipocytes, only partial inhibitions (maximally between 65–80%) of insulin-stimulated glucose uptake and GLUT4 translocation have been reported. These include the use of botulinum or tetanus toxins (21, 24, 26), interfering SNARE fragments, and peptides (14, 16, 22, 42) or inhibitory anti-SNARE antibodies (14, 42). Failure to achieve a complete blockade of the insulin response thus led to the proposal that insulin uses both BoNT-sensitive and -insensitive pathways to promote GLUT4 translocation. Our data suggest that PKB-DD exclusively stimulates GLUT4 translocation via the BoNT-sensitive pathway and from a vesicle pool that lacks GLUT1 and TfR. Such a vesicle population probably represents the post-endocytic GLUT4 storage compartment termed GLUT4 storage vesicles by Rea and James (43) and that resembles small synaptic vesicles with respect to its SNARE content and recycling characteristics.

Although GLUT4 and GLUT1 have been shown to segregate into distinct vesicle pools in adipocytes (56, 56, 66), there is also considerable evidence to suggest that a substantial fraction of GLUT4 (approximately 40–50% (56, 57)) also resides in recycling endosomes that contain GLUT1 and the TfR. Insulin-stimulated translocation of GLUT1 from this latter pool to the plasma membrane occurs independently of Sbr-2/Cbr and SNAP-23 (16, 23, 42), observations that are consistent with our own data in which neither BoNT/B nor BoNT/E blocked insulin-stimulated TfR translocation (Fig. 9b). It is curious to note that despite the known presence of Cbr in approximately 60% of transferrin receptor-containing vesicles in 3T3-L1 adipocytes (57), we observe no significant inhibition of insulin-stimulated translocation when Cbr function is prevented. Although an earlier study (21) suggested that Cbr may mediate insulin-stimulated GLUT4 translocation, more recently Martin and co-workers (23) found that only Sbr-2 was likely to be involved. Therefore, the precise function of Cbr in 3T3-L1 adipocytes is yet to be defined.

Although the exocytosis of GLUT1- and TfR-containing vesicles recycling via the endocytic pathway is not sensitive to the actions of PKB-DD (Fig. 8), inhibitors of PI3-kinase have been reported to block insulin-stimulated translocation of the transferrin receptor (67) and GLUT1 (4). Taken together with our own data, this suggests that PI3-kinase is central to the translocation of GLUT4 from both pools. However, the signal may then diverge such that PKB then mobilizes GLUT4 to the plasma membrane from the GLUT4 storage vesicles and an as yet unknown pathway stimulates mobilization of GLUT4, GLUT1, and the TfR from recycling endosomes. If the two pools of GLUT4 are in dynamic equilibrium, in the presence of dominant-negative PKB, GLUT4 may be able to traffic to the plasma membrane by returning into recycling endosomes. The extent to which insulin utilizes these two trafficking mechanisms in any one cell type and the degree to which these pools might equilibrate may help to explain why dominant-negative PKBs only partially suppress insulin-stimulated GLUT4 translocation (63), if they have any effect at all (64). This possibility requires further investigation.

**Concluding Comments**—Our data lend support to the proposal that GLUT4 translocation occurs via at least two distinct trafficking pathways. One is Sbr-2/Cbr and SNAP-23-dependent and may represent GLUT4 storage vesicles that can be
mobilized to the plasma membrane in response to constitutively active PKB-DD. This pathway may correspond to the pool of GLUT4 in adipocytes reported to resemble small synaptic vesicles in neurons (based on morphology and recycling characteristics). The other trafficking pathway is Sbr-2/Cbr- and SNAP-23-independent (or can at least use alternative BoNT-insensitive SNARE proteins) and is not stimulated by PKB-DD. The latter pathway may represent the translocation of GLUT4 present in GLUT1- or Tfr-containing endosomes.

Other trafficking pathways and/or signaling mechanisms may also exist. For example, GLUT4 translocation induced by osmotic shock or GTP-γ-S in adipocytes or contraction in muscle has been reported to occur independently of PI3-kinase and protein kinase B (22, 65, 68, 69). Clearly, identifying the events between PI3-kinase activation and SNARE-dependent docking/fusion of GLUT4 vesicles, as well as the other “putative” pathways utilized by insulin that may operate independently of Sbr-2/Cbr- and SNAP-23, are important avenues for future exploration.

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