In the present study, we examined the possible interaction between Rab4 and syntaxin 4, both having been implicated in insulin-induced GLUT4 translocation. Rab4 and syntaxin 4 were coimmunoprecipitated from the lysates of electrically permeabilized rat adipocytes. The interaction between the two proteins was reduced by insulin treatment and increased by the addition of guanosine 5’-O-(3-thiotriphosphosphate) (GTPγS). An in vitro binding assay revealed that the bacterially expressed Rab4 was bound to a glutathione S-transferase fusion protein containing the cytoplasmic domain of syntaxin 4 (GST-syntaxin 4-(1–273)) but not to syntaxin 1A or vesicle-associated membrane protein-2. The interaction between Rab4 and syntaxin 4 seemed to be regulated by the guanine nucleotide status of Rab4, because 1) GTPγS treatment of the cells significantly increased, but guanosine 5’-O-(2-thiodiphosphate) (GDPβS) treatment decreased the amount of Rab4 pulled down with GST-syntaxin 4-(1–273) from the cell lysates; 2) GTPγS loading on Rab4 caused a marked increase in the affinity of Rab4 to syntaxin 4 whereas GDPβS loading had little effect; and 3) a GTPase-deficient mutant of Rab4 (Rab4Q67L), but not a GTP-binding-defective mutant (Rab4S22N), was bound to GST-syntaxin 4-(1–273). Although insulin stimulated [γ32P]GTP binding to Rab4 in a time-dependent fashion, its effect on the Rab4 interaction with syntaxin 4 was apparently biphasic; an initial increase in Rab4 associated with syntaxin 4 was followed by a gradual dissociation of the GTPase from syntaxin 4. Finally, the binding of Rab4Q67L to GST-syntaxin 4-(1–273) was inhibited by munc-18c in a dose-dependent manner, indicating that GTP-loaded Rab4 binds to syntaxin 4 in the open conformation. These results suggest that 1) Rab4 interacts with syntaxin 4 in a direct and specific manner, and 2) the interaction is regulated by the guanine nucleotide status of Rab4 as well as by the conformational status of syntaxin 4.

Insulin stimulates glucose transport in skeletal/cardiac muscles and adipose cells primarily by inducing translocation of a facilitative glucose transporter isofrom, GLUT4, from the intracellular compartments to the plasma membrane (1–3). Although the molecular mechanisms of insulin-regulated GLUT4 translocation still remain obscure, there is evidence of a role for Rab4, a member of the Ras-related small GTP-binding protein family, in the insulin action. Rab4 was found by Cormont et al. (4) on immunoadsorbed GLUT4-containing vesicles in adipocytes, and later in skeletal muscles (5). In these cells, insulin stimulation causes a subcellular shift of Rab4 from the membrane fraction to the cytosolic fraction in concert with recruitment of GLUT4 to the plasma membrane (4, 5).

Recent studies have shown that insulin-induced translocation of GLUT4 is a dynamic event that consists of accelerated exocytosis and constitutive, or weakly decelerated endocytosis of the transporter (6–8) and that the former is rate-limiting for GLUT4 recruitment onto the plasma membrane (9). Involvement of Rab4 in exocytosis of GLUT4 was indicated by the observation that a synthetic peptide corresponding to the C-terminal domain of Rab4 inhibits insulin- or GTPγS-induced exocytotic recruitment of GLUT4 to the plasma membrane in rat adipocytes (10). Consistent with this, Vollenweider et al. (11) showed that microinjection of a GTP-binding defective mutant of Rab4 or anti-Rab4 antibodies inhibited insulin-evoked GLUT4 translocation by 50% in 3T3-L1 adipocytes. It was also reported that a Rab4 mutant lacking the geranylgeranylation sites inhibited insulin-induced recruitment of GLUT4 to the cell surface in cultured rat adipocytes (12). On the other hand, a study by Bortuluzzi et al. (13) revealed GTPase-activating protein (GAP) activity for Rab4 in the plasma membrane fraction of 3T3-L1 adipocytes, although they did not find any effect of insulin on the Rab4-GAP activity or its subcellular localization. Since the action of Rab family GTPases would be terminated on GTP hydrolysis, the presence of Rab4-GAP activity suggests that the plasma membrane would be one of the destinations for Rab4-mediated vesicle transport. Consistent with this, a recent work by Millar et al. (14) demonstrated an accumulation of Rab4 at the cell surface in GTPγS-treated adipocytes. Furthermore, we recently found that insulin accelerates GTPγS binding to Rab4 in naphosphatidylinositol (PI)3-kinase-dependent manner (15). All of these observations have indicated a critical role for Rab4 in the insulin-regulated subcellular trafficking of GLUT4. The mechanism of action of Rab4, however, remains to be defined.

Although Rab family GTPases are implicated in the directional transport of vesicles from one subcellular compartment to another (16–18), it has become apparent in recent years that, in addition to Rab, diverse families of functional proteins are involved in the vesicle transport. Among them, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins play a critical role in the late stage(s) of vesicle transport, i.e. docking and/or fusion between the trans-

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1 The abbreviations used are: GTPγS, guanosine 5’-O-(3-thiotriphosphate); PI 3-kinase, phosphoinositide 3-kinase; GDPβS, guanosine 5’-O-(2-thiodiphosphate); SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein; GST, glutathione S-transferase; GAP, GTPase-activating protein; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; DTTP, dithiothyretol; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride.
port vesicle and the target membrane (19–21). By analogy with synaptic vesicle exocytosis, two vesicle membrane (v)-SNARE proteins, vesicle-associated membrane protein (VAMP)-2 and VAMP-3, were found on the immunopurified GLUT4-containing vesicles from adipocytes (22, 23). Subsequent studies revealed that their cognate target membrane (t)-SNAREs are Syntaxin 4 and SNAP-23 (a non-neuronal homologue of SNAP-25, synaptosomal protein with a molecular mass of 25 kDa) (24–26). One v-SNARE (VAMP-2 or VAMP-3) and the two t-SNARE proteins form a ternary SDS-resistant SNARE complex (Refs. 24 and 27; for review, see Ref. 28). Selective cleavage by botulinum or tetanus toxins of these SNARE proteins (29, 30), microinjection of antibodies for them (31, 32), or introduction into the cell of the cytoplasmic domain or synthetic peptides derived from these SNARE proteins (33) resulted in a marked inhibition of insulin-induced GLUT4 translocation, providing evidence that the SNARE complex formation is indispensable for fusion of the GLUT4-containing vesicles with the plasma membrane.

Although Rab4 and the SNARE proteins thus play essential roles in exocytosis of the GLUT4-containing vesicles, there has been little direct evidence for a functional link between these two systems in adipocytes and muscles. An intriguing question would be whether Rab4 interacts with any of the SNARE proteins and participates in the SNARE complex assembly. We here examined the possible interaction of Rab4 with syntaxin 4 since this t-SNARE protein seems to play a pivotal role in the formation of the SNARE complex. Munc-18c and Synip are both recently identified syntaxin 4-binding proteins (34, 35). Munc-18c is a ubiquitously expressed isoform of n-sec1/munc-18, a neural-specific homolog of the yeast Sec1p, regulating synaptic vesicle exocytosis in neuronal cells (36). Although rat adipocytes and 3T3-L1 adipocytes express syntaxin 2, 3, and 4, munc-18c predominantly binds to syntaxin 4 with a high affinity, preventing the binding of VAMP-2 or SNAP-23 to syntaxin 4 (24, 32, 37–39). Overexpression of munc-18c inhibited insulin-evoked GLUT4 translocation (32, 38, 39), whereas insulin stimulation caused dissociation of munc-18c from syntaxin 4 (39). Thus, by binding to syntaxin 4, munc-18c negatively regulates the SNARE complex assembly and the subsequent fusion of the GLUT4-containing vesicles with the plasma membrane. On the other hand, Synip, which is expressed exclusively in adipocyte and muscle, specifically interacts with syntaxin 4 in a competitive manner with VAMP-2 (35). Insulin stimulation causes a dissociation of Synip from syntaxin 4 whereas the C-terminal domain of Synip inhibits insulin-induced GLUT4 translocation. Thus, these syntaxin 4-binding proteins are directly involved in the “activation” of the t-SNARE (40) and subsequent assembly of the ternary SNARE complex, although the mechanisms of insulin activation of syntaxin 4 are still unknown.

In the present study, in an attempt to elucidate the mechanism of Rab4 action in GLUT4 translocation, we investigated the in vivo and in vitro interaction between Rab4 and syntaxin 4 by three methods: a communoprecipitation assay, a pull-down assay, and an in vitro binding assay. The results of our study indicated that Rab4 directly interacts with syntaxin 4 and the interaction is regulated by the guanine nucleotide status of Rab4 as well as the conformational status of syntaxin 4.

**EXPERIMENTAL PROCEDURES**

**Materials**—The polyclonal antibody directed to Rab4 was obtained by immunizing a rabbit with peptide, (CQLRSLPRTRQTPAQSE, conjugated with bovine serum albumin and affinity-purified as described previously (10). The anti-syntaxin 4 sheep polyclonal antibody and anti-munc-18c rabbit antibody were generous gifts from Dr. Jeffrey E. Pessin (University of Iowa, Iowa City, IA). The antibodies directed to Syntaxin 1A and VAMP-2 were from Upstate Biotechnology, Inc. and Wako Chemical (Kyoto, Japan), respectively. GTPyS and GDPyS were purchased from Roche Molecular Biochemicals. ^32P^-Labeled protein A, ^32P^-labeled protein G, ^[alpha]-[gamma]TPGTP, and ^[alpha]-[gamma]TPGTP were from DuPont. Protein G-Sepharose was from Amersham Pharmacia Biotech. The cDNAs for syntaxin 1A and 4 were gifts from Dr. Richard H. Scheller (Stanford University, Stanford, CA). The Rab4-(191–210) peptide (DAALRQLRPRTPAQSE), derived from the C-terminal domains of rat Rab4 (10), was synthesized and purified by high performance liquid chromatography to 85–95% homogeneity.

**Preparation of Rat Adipocytes and Permeabilization**—Isolated rat adipocytes were prepared from the epididymal adipose tissue of Harlan Sprague-Dawley rats (from Charles River, ~170–220 g) (41). Unless otherwise specified, isolated cells were suspended in Buffer A (25 mM K5H2PO4, 10 mM Tris/Cl (pH 7.4), and 3 mM pyruvate, pH 7.4). The cells to be permeabilized by electroperoration were suspended in high K"/low Ca" buffer designated as Buffer X (118.0 mM KCl, 4.74 mM NaCl, 0.38 mM CaCl2, 1.0 mM EGTA, 1.18 mM MgSO4, 1.18 mM KH2PO4, 25.4 mM Heps/ROH, 20 mg/ml bovine serum albumin, 3 mm pyruvate, pH 7.4). The electroperoration was carried out four times in a Gene-Pulsor (Bio-Rad) set at 25 microfarads and 2 kV/cm as described previously (42).

**Immunoprecipitation and Immunoblotting**—After incubation with or without 100 nM insulin or 1 mM glycine for 15 min at 37°C, the permeabilized adipocytes were washed and homogenized in STE buffer (250 mM sucrose, 10 mM Tris/Cl, and 1 mM EDTA/Na, pH 7.4). The homogenate was centrifuged for 2 min at 3,000 g × g. The pellet and the fat fraction were discarded, and Nonidet P-40 was added to the infranatant solution to a final concentration of 1% (v/v). For immunoprecipitation of Rab4 or syntaxin 4, the infranatant was incubated with 15 μl of affinity-purified anti-Rab4 antibody and 20 μl (bed volume) of protein G-Sepharose (Amersham Pharmacia Biotech) or anti-syntaxin 4 antibody conjugated to SulfoLink Coupling Gel (Pierce) for 2 h on a rocking platform at 4°C. At the end of incubation, the beads were spun down at 3,000 g × g for 1 min at 4°C and washed three times with 1 ml of STE buffer containing 1% Nonidet P-40.

**Immunodetection of syntaxin 4 or Rab4** was carried out as described previously (10). Briefly, the immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gels, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) at 120 mA for 4 h. The PVDF membrane was blocked with solution containing 5% bovine serum albumin, 10 mM Tris/Cl (pH 7.4) and 154 mM NaCl for 1 h at room temperature. The blocked membranes were incubated in anti-Rab4 antibody (1:500 dilution) or anti-syntaxin 4 antibody (1:100 dilution) overnight at 4°C. The membrane was washed and incubated with ^[gamma]TP-protein A (0.2 μCi/ml) or ^[gamma]TP-protein G (0.1 μCi/ml) for 1 h at room temperature. Following extensive washing, the membrane was dried and the blots were visualized and quantified by using Fujix BAS2000 bio-imaging analyzer (Fujix Photo Film, Tokyo, Japan).

**Subcellular Distribution of Rab4**—The subcellular distribution of Rab4 in rat adipocytes was examined by immunoblotting. The subcellular membrane and soluble fractions were prepared by differential and sucrose density gradient centrifugation as described previously (9). Briefly, the cells were washed and homogenized in STE buffer. The homogenate was centrifuged for 2 min at 3,000 g × g. The pellet (P-1) and membrane fraction was discarded, and the infranatant solution (S-1) was centrifuged for 15 min at 20,000 g × g. The supernatant (S-2) was further centrifuged as described below, and the pellet (P-2) was suspended in 0.5 ml of STE buffer and layered on top of a linear (15–32.5%, w/w) sucrose density gradient (1.14 × 8.5 cm in size) and centrifuged for 40 min at 160,000 g × s; the sucrose solution was supplemented with 1 mM EDTA/Na and buffered with 10 mM Tris/HCl (pH 7.5). After the centrifugation, the plasma membrane fraction (1–3.5 cm from the bottom of the tube) was collected and kept in ice. The 20,000 g × g supernatant (S-2) was centrifuged for 30 min at 23,700 g × g, and the resulting supernatant (S-3) and pellet (P-3) were saved as the low density and high density microsomal fractions, respectively. The plasma membrane and the low density microsomal fractions were pelleted by centrifugation for 60 min at 150,000 g × g.

**[^32P]GTP Binding to Rab4 in Permeabilized Cells**—[^32P]GTP binding to Rab4 in rat adipocytes was measured as described previously (15) with a slight modification. In brief, the isolated cells in Buffer X were electroperorated as described above. After incubation for 15 min at 37°C, the permeabilized cells were incubated with or without 100 nM insulin in the presence of 50 μM[^32P]GTP (0 for the radiolabel was added after homogenization), 2, 5, or 15 min. At the end of incubation,
the cells were homogenized in washing buffer (25 mM MgCl₂, 100 mM NaCl, 1 mM GTP, 50 mM Tris/Cl, pH 7.5) and the homogenate was centrifuged for 2 min at 3,000 × g. The pellet and the fat fraction were discarded, and Nonidet P-40 was added to the infranatant solution to a final concentration of 1% (v/v). The infranatant was incubated with 10 μl of the rabbit anti-Rab4 antibodies and 20 μl (bed volume) of protein G-Sepharose for 60 min on a rocking platform at 4 °C. The Sepharose beads were washed four times with 1 ml of washing buffer at 4 °C, and the amount of [γ-³²P]GTP bound to Rab4 was measured by liquid scintillation counting.

Expression Constructs—The cDNA encoding rat Rab4 was obtained from the mRNA of AR42J cells by reverse transcription-PCR using the primers 5’-GGGACC-ACCCTGCTGCTGCTCTGGC-3’ and 5’-CCCTCGAGCTTTGTTTGGGAGCTCC-3’ (for Rab4ΔC115), respectively. The sequences of the Rab4 mutants were analyzed by ABI 373 DNA sequencer. The cDNA constructs for two Rab4 mutants, Rab4Q67L and Rab4S22N;5 were prepared by using QuickChange site-directed mutagenesis kit (Toyobo, Japan) with the primers 5’-GGGAC-ACCCCTGCTGCTGCTCTGGC-3’ and 5’-CCCTCGAGCTTTGTTTGGGAGCTCC-3’ (for Rab4ΔC115), respectively. The sequences of the Rab4 mutants were analyzed by ABI 373 DNA sequencer.

The coding region of the cytoplasmic domains of syntaxin 1A (amino acids 4–267) and syntaxin 4 (amino acids 1–237) were amplified by PCR from the full-length cDNAs for syntaxin 1A and syntaxin 4, respectively, with the primers 5’-CCCTCGAGCTTTGTTTGGGAGCTCC-3’ and 5’-CCCTCGAGCTTTGTTTGGGAGCTCC-3’ (for syntaxin 4). The cytoplasmic domain of VAMP-2 (amino acids 1–94) was prepared by reverse transcription-PCR from the mRNA of AR42J cells using the primers 5’-CCCTCGAGCTTTGTTTGGGAGCTCC-3’ and 5’-CCCTCGAGCTTTGTTTGGGAGCTCC-3’ (for syntaxin 4). The coding region of syntaxin 4 by using bacterially expressed recombinant protein G-Sepharose for 60 min on a rocking platform at 4 °C. The Sepharose beads were washed four times with 1 ml of washing buffer at 4 °C, and the amount of [γ-³²P]GTP bound to Rab4 was measured by liquid scintillation counting.

Purification of GST Fusion and Hexahistidine-tagged Proteins—GST fusion and His6-tagged Rab4 proteins were expressed in BL21-CodonPlus (DE3) cells (Stratagene) by induction with 1 mM isopropyl-1-thio-

The GST fusion proteins were expressed in BL21-CodonPlus (DE3) cells (Stratagene) by induction with 1 mM isopropyl-1-thio-

Purification of GST Fusion and Hexahistidine-tagged Proteins—GST fusion and His6-tagged Rab4 proteins were expressed in BL21-CodonPlus (DE3) cells (Stratagene) by induction with 1 mM isopropyl-1-thio-

Pull-down Assay with GST Fusion Protein—After incubation with or without 100 mM insulin, 1 mM GTP*S, or 1 mM GDP*S for 15 min at 37 °C, electrically permeabilized adipocytes were washed three times and homogenized in STE buffer. The homogenate was centrifuged for 2 min at 3,000 × g. The pellet and the fat fraction were discarded, and Nonidet P-40 was added to the infranatant solution to a final concentration of 1% (v/v). The cell lysate was incubated with GST-syntaxin 4-(1–273) (10 μg) in the presence of 1 mg/ml BSA for 1 h at 4 °C, followed by glutathione-Sepharose beads (80 μl) for an additional 1 h. The beads were washed four times with 1 ml HNTG buffer (50 mM NaCl, 1% Triton X-100, 10% glycerol, and 1 mM EDTA/Na) and then two times with distilled water. The retained proteins were eluted and separated by 12% SDS-polyacrylamide gel electrophoresis, and then were immunoblotted with the anti-Rab4 antibodies.

RESULTS

To investigate the possible interaction between Rab4 and syntaxin 4, we first examined whether the two proteins were coimmunoprecipitated from the lysates of rat adipocytes. As shown in Fig. 1, Rab4 and syntaxin 4 were coimmunoprecipitated from the lysates of electrically permeabilized cells either with anti-Rab4 or anti-syntaxin 4 antibodies. The association between the two proteins was reduced by insulin stimulation, whereas it was significantly increased by the addition of GTP*S. These findings indicated that Rab4 directly or indirectly interacts with syntaxin 4 in rat adipocytes and that the interaction is regulated by insulin and GTP*S. Previous studies, however, indicated that syntaxin 4 is largely confined to the plasma membrane (24, 43), whereas the majority of Rab4 is found in the internal membrane compartments including the early endosomes and the GLUT4-containing vesicles (4, 5, 44). Those observations are inconsistent with our data that Rab4 and syntaxin 4 were coimmunoprecipitated in the basal state. Nevertheless, more recent studies have shown the localization of Rab4 at the plasma membrane in 3T3-L1 adipocytes (14, 46).

Thus, we examined the subcellular distribution of Rab4 by membrane fractionation and immunoblotting. As illustrated in Fig. 2, Rab4 was localized to the plasma membrane fraction as well as to the high and low density microsomal and the soluble fractions. The relative amounts of Rab4 in the plasma membrane, high density microsomal, low density microsomal, and soluble fractions were 4%, 20%, 38%, and 38%, respectively. Insulin reduced the amount of Rab4 by ~60% in the high and low density microsomal fractions, whereas it increased it 2-fold in the soluble fraction. Wortmannin markedly blocked the insulin-induced changes in Rab4 in these fractions. On the other hand, whereas insulin caused a slight decrease in Rab4 in the plasma membrane fraction, wortmannin treatment resulted in an additional decrease in Rab4 in this fraction. These results suggested that both pathways from the plasma membrane complex and association with the plasma membrane of Rab4 is PI 3-kinase-dependent, whereas dissociation from the latter may not require the kinase activity.

In Vitro Binding Assay—Unless otherwise described, bacterially expressed His₄₆-tagged Rab4 (1 μg) was incubated with GTP (1 mM) in the binding buffer (20 mM Heps/NaOH, pH 8.0, 2 mM EDTA, 1 mM DTT) for 30 min at 37 °C. The GST-syntaxin 1A (4–267), GST-syntaxin 4 (1–273), or GST-VAMP-2 (1–94) fusion proteins (10 μg each) were incubated in 1 ml of Buffer B (150 mM potassium acetate, 20 mM Heps/ KOH, 0.5 mM dithiothreitol, 0.05% Tween 20, pH 7.0) supplemented with 1% BSA for 1 h at 4 °C on a seasshaker. Then, GTP-loaded His₄₆-tagged Rab4 was added to the tube, and the incubation was continued for an additional 2 h at 4 °C. At the end of incubation, the tube was centrifuged at 15,000 × g for 15 min, and the supernatant was incubated with glutathione-Sepharose beads (80 μl) for 1 h at 4 °C. The beads were washed four times with 1 ml of Buffer B at room temperature, and the bound proteins were eluted with 80 μl of the elution buffer (10 mM Heps/NaOH, 1% Triton X-100, 10% glycerol, and 1 mM EDTA/Na). The eluted proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting as described above.

Purification of GST Fusion Protein—After incubation with or without 100 mM insulin, 1 mM GTP*S, or 1 mM GDP*S for 15 min at 37 °C, electrically permeabilized adipocytes were washed three times and homogenized in STE buffer. The homogenate was centrifuged for 2 min at 3,000 × g. The pellet and the fat fraction were discarded, and Nonidet P-40 was added to the infranatant solution to a final concentration of 1% (v/v). The cell lysate was incubated with GST-syntaxin 4-(1–273) (10 μg) in the presence of 1 mg/ml BSA for 1 h at 4 °C, followed by glutathione-Sepharose beads (80 μl) for an additional 1 h. The beads were washed four times with 1 ml HNTG buffer (50 mM NaCl, 1% Triton X-100, 10% glycerol, and 1 mM EDTA/Na) and then two times with distilled water. The retained proteins were eluted and separated by 12% SDS-polyacrylamide gel electrophoresis, and then were immunoblotted with the anti-Rab4 antibodies.

To investigate the possible interaction between Rab4 and syntaxin 4, we first examined whether the two proteins were coimmunoprecipitated from the lysates of rat adipocytes. As shown in Fig. 1, Rab4 and syntaxin 4 were coimmunoprecipitated from the lysates of electrically permeabilized cells either with anti-Rab4 or anti-syntaxin 4 antibodies. The association between the two proteins was reduced by insulin stimulation, whereas it was significantly increased by the addition of GTP*S. These findings indicated that Rab4 directly or indirectly interacts with syntaxin 4 in rat adipocytes and that the interaction is regulated by insulin and GTP*S. Previous studies, however, indicated that syntaxin 4 is largely confined to the plasma membrane (24, 43), whereas the majority of Rab4 is found in the internal membrane compartments including the early endosomes and the GLUT4-containing vesicles (4, 5, 44). Those observations are inconsistent with our data that Rab4 and syntaxin 4 were coimmunoprecipitated in the basal state. Nevertheless, more recent studies have shown the localization of Rab4 at the plasma membrane in 3T3-L1 adipocytes (14, 46).

Thus, we examined the subcellular distribution of Rab4 by membrane fractionation and immunoblotting. As illustrated in Fig. 2, Rab4 was localized to the plasma membrane fraction as well as to the high and low density microsomal and the soluble fractions. The relative amounts of Rab4 in the plasma membrane, high density microsomal, low density microsomal, and soluble fractions were 4%, 20%, 38%, and 38%, respectively. Insulin reduced the amount of Rab4 by ~60% in the high and low density microsomal fractions, whereas it increased it 2-fold in the soluble fraction. Wortmannin markedly blocked the insulin-induced changes in Rab4 in these fractions. On the other hand, whereas insulin caused a slight decrease in Rab4 in the plasma membrane fraction, wortmannin treatment resulted in an additional decrease in Rab4 in this fraction. These results suggested that both pathways from the plasma membrane complex and association with the plasma membrane of Rab4 is PI 3-kinase-dependent, whereas dissociation from the latter may not require the kinase activity.

To clarify whether Rab4 interacts with syntaxin 4 directly or not, we examined the in vitro interaction between Rab4 and syntaxin 4 by using bacterially expressed recombinant pro-
In vitro binding of Rab4 to GST-syntaxin 4. Bacterially expressed His₆-tagged Rab4 (1 μg) was incubated with GTP (1 mM) in the binding buffer (20 mM Hepes/NaOH, pH 8.0, 2 mM EDTA, 1 mM DTT) for 30 min at 37 °C, followed by GST-syntaxin 4-(1–273), GST-syntaxin 1A-(4–267), or GST-VAMP-2-(1–94) fusion proteins (10 μg each) for 1 h at 4 °C. At the end of incubation, glutathione-Sepharose beads were added to the mixture and the incubation was continued for an additional 1 h. The beads were washed extensively, and the retained proteins were eluted and subjected to immunoblotting for Rab4. As illustrated in Fig. 3, His₆-tagged Rab4 preincubated with GTP was bound to the GST fusion protein containing the cytoplasmic domain of syntaxin 4 (GST-syntaxin 4-(1–273)) but not to that containing the cytoplasmic domain of syntaxin 1A (GST-syntaxin 1A-(4–267)) or VAMP-2 (GST-VAMP-2-(1–94)). Consistent with this, Rab4 was pulled down from the cell lysates with GST-syntaxin 4-(1–273) but not with GST-syntaxin 1A-(4–267) or GST-VAMP-2-(1–94) (Fig. 4). These results suggest that Rab4 interacts with the cytoplasmic domain of syntaxin 4 in a direct and specific manner.

We next investigated the mechanisms of insulin- or GTP₇S-induced alterations of the interaction between Rab4 and syntaxin 4. The closer interaction between Rab4 and syntaxin 4 in GTP₇S-treated cells (Fig. 1) raised a possibility that the affinity of Rab4 to syntaxin 4 is regulated upward by GTP binding to Rab4. Alternatively, GTP₇S treatment may cause a conformational change in syntaxin 4 or a release of an inhibitor(s) from the t-SNARE, facilitating Rab4 binding to syntaxin 4, regardless of the guanine nucleotide status of Rab4. To test these possibilities, we treated electrically permeabilized cells with insulin, GTP₇S, or GDP₇S, and then pulled down endogenous Rab4 with GST-syntaxin 4-(1–273) from the cell lysates. As depicted in Fig. 5, GTP₇S treatment increased, but insulin or GDP₇S treatment decreased the amount of Rab4 precipitated with GST-syntaxin 4-(1–273) compared with the basal state. Although the data are not shown, we did not find any significant changes in the association of endogenous Rab4 with GST-syntaxin 1A-(4–267) or GST-VAMP-2-(1–94). These results indicate that the affinity of Rab4 to syntaxin 4 is regulated by the conformational change of Rab4 because it is unlikely that GST-syntaxin 4-(1–273) added to the lysates would be altered by treatment of the cells with insulin or guanine nucleotides. Furthermore, an in vitro binding assay also demonstrated that incubation of His₆-tagged Rab4 with GTP₇S greatly increased its affinity to GST-syntaxin 4-(1–273), whereas GDP₇S was with little effect (Fig. 6). These results suggest that GTP loading on Rab4 increases its affinity to syntaxin 4, whereas GDP-bound Rab4 has less affinity to syntaxin 4.

On the other hand, the disruption by insulin of the interaction between Rab4 and syntaxin 4 (Fig. 1) is apparently paradoxical. Previous studies showed that both insulin and GTP₇S cause GLUT4 translocation (9, 47–49) and that insulin stimulates GTP loading on Rab4 in rat adipocytes (15). In addition, Rab4 seems to be involved in both insulin- and GTP₇S-induced GLUT4 translocation because the Rab4-derived peptide, Rab4-(191–210) blocked glucose transport induced by either stimulant (10). Taking account of these observations, insulin was
1% (v/v). The infranatant fractions were incubated with GST (GST-VAMP-2-(1–94) and GST-syntaxin 4-(1–273)) for 1 h at 4 °C, followed by glutathione-agarose beads for an additional 1 h. The beads were washed extensively, and the retained proteins were eluted and subjected to immunoblotting for Rab4.

**Rab4 Interaction with Syntaxin 4**

![Fig. 4. Pull-down of Rab4 with GST fusion proteins from the cell lysates.](image)

Electrically permeabilized rat adipocytes in Buffer X were incubated for 20 min at 37 °C, and then the cells were washed three times with STE buffer, homogenized, and centrifuged for 2 min at 3,000 × g. The pellet and the fat fraction were discarded, and Nonidet P-40 was added to the infranatant solution to a final concentration of 1% (v/v). The infranatant fractions were incubated with GST (lane 1), GST-syntaxin 4-(1–273) (lane 2), GST-syntaxin 1A-(4–267) (lane 3), or GST-VAMP-2-(1–94) (lane 4) (10 μg each) for 1 h at 4 °C, followed by glutathione-agarose beads for an additional 1 h. The beads were washed extensively, and the retained proteins were eluted and subjected to immunoblotting for Rab4.

**Fig. 5. Effects of insulin and guanine nucleotides on pull-down of Rab4 with GST-syntaxin 4.** Electrically permeabilized rat adipocytes in Buffer X were incubated with nothing (control), insulin (100 nm), GTPgS (1 μM), or GDPgS (1 μM) for 20 min. At the end of the incubation, the cells were washed and homogenized as described under “Experimental Procedures.” After centrifugation for 2 min at 3,000 × g, the infranatant fractions were incubated with GST-syntaxin 4-(1–273) for 1 h at 4 °C, followed by glutathione-agarose beads for 1 h. The beads were washed extensively, and the retained proteins were eluted and subjected to immunoblotting for Rab4.

**Fig. 6. Effects of guanine nucleotides on the in vitro binding of Rab4 to GST-syntaxin 4.** Bacterially expressed His<sub>6</sub>-tagged wild-type Rab4 (1 μg) were incubated for 30 min at 30 °C alone, with 1 mM GTPgS or with 1 mM GDPgS, then mixed with GST-syntaxin 4-(1–273) (10 μg) and incubated for 1 h at 4 °C. After the addition of glutathione-Sepharose beads, the mixture was incubated for an additional 1 h. After four washes, the beads were spun down and the proteins bound to the beads were separated on SDS-PAGE and subjected to immunoblotting for Rab4.

The amount of Rab4 coimmunoprecipitated with syntaxin 4 rapidly increased and reached a maximum 2 min after stimulation, then gradually decreased to reach the steady state level within 15 min. In the presence of wortmannin, these insulin-induced changes were markedly inhibited. The initial increase in Rab4 binding to syntaxin 4 was caused presumably by insulin-stimulated GTP loading. However, this insulin effect did not account for Rab4 dissociation from syntaxin 4 in the second phase. Bortuluzzi et al. (13) have shown that the GAP activity for Rab4 is present at the plasma membrane in 3T3-L1 adipocytes. Thus, one possible explanation is that syntaxin 4-bound Rab4 dissociates from the t-SNARE as a result of GTP hydrolysis by the action of Rab4-GAP, the activity of which may overcome the insulin-stimulated Rab4 binding to syntaxin 4. This notion is supported by the observation that nonhydrolyzable GTPgS increased Rab4 binding to syntaxin 4 (Fig. 1). However, we could not rule out other possibilities for Rab4 dissociation from syntaxin 4 because the subcellular localization of Rab4 may or may not correlate with its guanine nucleotide status (see “Discussion” below).

To investigate whether the guanine nucleotide status of Rab4 correlates with its interaction with syntaxin 4, we measured [γ-<sup>32</sup>P]GTP binding to Rab4 in electrically permeabilized cells. Although we previously reported that insulin stimulates [γ<sup>35</sup>S]GTPγS loading on Rab4 (15), the assay did not take account of GTP hydrolysis because Rab4-bound GTPγS was not hydrolyzed. Thus, we conducted the GTP binding assay using hydrolyzable [γ-<sup>32</sup>P]GTP instead of [γ<sup>35</sup>S]GTPγS in an attempt to examine if insulin shows a biphasic effect on GTP binding to Rab4. Unfortunately, however, insulin increased [γ-<sup>32</sup>P]GTP binding to Rab4 in a simple time-dependent manner during the 15 min of incubation (Fig. 8). Although these results confirmed our previous observations, they do not account for the mechanism of the biphasic effect of insulin on Rab4 association with syntaxin 4. One reason may be that the present assay did not reflect the ratio of GDP to GTP bound to Rab4 in the cells. Because of technical difficulties in metabolically labeling the guanine nucleotide pool of isolated adipocytes, we were unable to measure the ratio of GDP to GTP on Rab4. To further confirm the notion that the interaction between Rab4 and syntaxin 4 is regulated by the guanine nucleotide status of Rab4, we examined the in vitro binding to GST-syntaxin 4-(1–273) of two Rab4 mutants. We first characterized the biochemical properties of the mutant proteins. In the GTP binding assays, [γ-<sup>32</sup>P]GTP was bound to the wild-type (Rab4<sub>WT</sub>) and GTPase-deficient (Rab4<sub>Q67L</sub>) Rab4 but not to the GTP binding-defective mutant (Rab4<sub>S22N</sub>) (Fig. 9A), indicating that Rab4<sub>S22N</sub> lacks the ability to bind GTP. On the other hand, Rab4-bound [γ-<sup>32</sup>P]GTP was hydrolyzed by the wild-type protein but not by the Rab4<sub>Q67L</sub> mutant (Fig. 9, B and C), indicating that Rab4<sub>S22N</sub> is defective in the GTPase activity. These features of the Rab4 mutants are consistent with previous observations (11, 12). We then carried out an in vitro binding assay using these Rab4 mutants. The results clearly demon-
Rab4Q67L to GST-syntaxin 4-(1–273) was markedly inhibited in the presence of munc-18c. As shown in Fig. 11A, the t-SNARE, we conducted the 4-munc-18c complex causes displacement of munc-18c and displacement of the negative regulator Sly1. Thus, it is also plasmic reticulum-to-Golgi transport in the yeast, resulting in the inhibition of Ypt1 with Sed5, the t-SNARE implicated in the endo-

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Second, GTP

First, the coimmunoprecipitation of the lysates of rat adipocytes (Fig. 10). Final, an in vitro binding assay revealed that bacterially expressed His
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tagged Rab4 was bound to GST-syntaxin 4-(1–273) but not to GST-syntaxin 1A-(4–267) or GST-VAMP-2-(1–94) (Fig. 3). Although some earlier studies failed to detect Rab4 in the plasma membrane fraction, our fractionation study revealed the presence of Rab4 in the plasma membrane fraction of rat adipocytes although the amount was not large (4% of total Rab4) (Fig. 2). These results are in agreement with recent works by other investigators (14, 45) and rationalize the coimmunoprecipitation of Rab4 with syntaxin 4 in the basal state (Fig. 1). However, although a large portion is confined to the plasma membrane, syntaxin 4 is also localized to the microsomal fractions (24, 43) although the physiological significance of this is unknown. Thus, we could not exclude the possibility that the coimmunoprecipitated proteins are derived from the microsomal fractions.

The second important finding in this study is that the affinity of Rab4 to syntaxin 4 seems to be regulated by the guanine nucleotide status of Rab4. First, the coimmunoprecipitation study showed that the association between Rab4 and syntaxin 4 was increased by treatment of the cells with GTPyS (Fig. 1). Second, GTPyS treatment of the cells significantly increased, but GDPβS treatment decreased, the amount of endogenous Rab4 precipitated with GST-syntaxin 4-(1–273) from the cell lysates (Fig. 5). Third, GTPyS loading on His
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Although these results indicate that the conformational change of Rab4 by GTP loading is critical for its interaction with syntaxin 4, we also showed in the present study that GTP

FIG. 8. Effect of insulin on [γ-32P]GTP binding to Rab4 in permeabilized cells. Adipocytes in Buffer X were incubated for 30 min at 37°C and permeabilized as described under “Experimental Procedures.” The cells were incubated with 50 μM [γ-32P]GTP in the absence (○) or presence (●) of 100 nM insulin for the indicated period. At the end of incubation, the cells were homogenized and Rab4 was immunoprecipitated as described under “Experimental Procedures.” The amount of [γ-32P]GTP bound to Rab4 was measured by liquid scintillation counting. The results are the mean ± S.D. of three determinations.

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A significant higher affinity to GST-syntaxin 4-(1–273) of Rab4Q67L than Rab4S22N (Fig. 10), providing further evidence that GTP loading would increase the affinity of Rab4 to syntaxin 4.

In the next set of experiments, we examined whether the interaction between GTP-bound Rab4 and syntaxin 4 is affected by munc-18c. Previous studies have shown that insulin stimulates GTP loading on Rab4 as well as causing dissociation of munc-18c from syntaxin 4. In the absence of insulin, interaction between Rab4 and syntaxin 4 is associated with syntaxin 4 and that syntaxin 4 is not the target molecule of Rab4 function in still unclear.

We here demonstrated that Rab4 can directly and specifically interact with syntaxin 4. First, Rab4 and syntaxin 4 were coimmunoprecipitated from the plasma, whereas the precise molecular mechanism of Rab4 function is still unclear.

We investigated by three methods the interaction between Rab4 and syntaxin 4, both of which play critical roles in insulin-induced GLUT4 translocation. The role of syntaxin 4 in insulin-induced GLUT4 translocation is rather defined as a component of the ternary SNARE complex, the formation of which is indispensable for fusion of the GLUT4-containing vesicle with the plasma membrane, whereas the precise molecular mechanism of Rab4 function is still unclear.

Rab and SNARE proteins are both implicated in the late step(s) of vectorial transport of vesicles from one membrane compartment to another, although the relationship between the two systems remains obscure. In the present study, we investigated by three methods the interaction between Rab4 and syntaxin 4, both of which play critical roles in insulin-induced GLUT4 translocation. The role of syntaxin 4 in insulin-induced GLUT4 translocation is rather defined as a component of the ternary SNARE complex, the formation of which is indispensable for fusion of the GLUT4-containing vesicle with the plasma membrane, whereas the precise molecular mechanism of Rab4 function is still unclear.

We here demonstrated that Rab4 can directly and specifically interact with syntaxin 4. First, Rab4 and syntaxin 4 were coimmunoprecipitated from the lysates of rat adipocytes (Fig. 1). Second, endogenous Rab4 was pulled down from the cell lysates with GST-syntaxin 4-(1–273) but not with GST-syntaxin 1A-(4–267) or GST-VAMP-2-(1–94) (Fig. 4). Third, an in vitro binding assay revealed that bacterially expressed His
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loading on Rab4 may not be sufficient for its binding to syn-3taxin 4, especially under in vivo conditions. The results of in vitro binding assay in the presence of munc-18c, which binds to and stabilizes syntaxin 4 in a closed conformation, revealed the inability of GTP-loaded Rab4 to interact with the syntaxin 4-munc-18c complex (Fig. 11). Given that GST-syntaxin 4-(1-273) takes an open conformation in the absence of munc-18c, these results suggest that the interaction between Rab4 and syntaxin 4 is also regulated by the conformational status of syntaxin 4. Alternatively, Rab4 and munc-18c may simply compete for the binding site of syntaxin 4. In any case, our data indicate that dissociation of munc-18c from syntaxin 4 is required for Rab4 to bind syntaxin 4 under in vivo conditions.

These results are also consistent with the observation by Cormont et al. (12) that overexpression of constitutively active Rab4 failed to stimulate GLUT4 translocation.

The opposite effects of insulin and GTPγS on the interaction between Rab4 and syntaxin 4 (Figs. 1 and 5) were apparently paradoxical given previous observations that, 1) both insulin and GTPγS induce GLUT4 translocation (9, 46–48), 2) Rab4 is involved in the effects of both stimulants (10), and 3) insulin promotes [35S]GTPγS binding to Rab4 in a PI 3-kinase-dependent manner (15). However, the time course of insulin-regulated Rab4 interaction with syntaxin 4 revealed a biphasic effect of insulin (Fig. 7); insulin initially caused a rapid increase in Rab4 associated with syntaxin 4 with a peak at 2 min, followed by the dissociation of Rab4 from syntaxin 4. In contrast, insulin stimulated [γ-32P]GTP binding to Rab4 in a simple time-dependent manner (Fig. 8), consistent with our previous study (15). These results suggest that insulin presumably promotes Rab4 binding to syntaxin 4 by GTP loading, which may be overcome by subsequent GTP hydrolysis by the action of Rab4-GAP, resulting in a shift of the GTPase from the membrane to the cytosol. Consistent with this notion, Millar et al. (14) have shown that a nonhydrolyzable GTP analogue, GTPγS, causes an accumulation of Rab4 at the plasma membrane, whereas Cormont et al. (4) have indicated that insulin increases the cytosolic fraction of Rab4. Thus, although both insulin and
GTP-γS induce GLUT4 translocation and stimulate glucose transport in a Rab4-dependent manner (9, 10), their effects on the subcellular localization of Rab4 are quite different. On the other hand, however, Gerez et al. (53) have recently demonstrated that phosphorylated GTP-bound Rab4 is associated with a peptidyl-prolyl isomerase Pin1 and accumulates in the cytosol during mitosis. Their study indicated that under certain conditions the guanine nucleotide status of Rab4 does not necessarily correlate with its subcellular distribution. Thus, it is also possible that Rab4 may dissociate from syntaxin 4 by mechanisms other than GTP hydrolysis. To clarify this point, it is necessary to measure the GTP to GDP ratio on Rab4 by metabolically labeling the guanine nucleotide pool of isolated adipocytes, which was not conducted in the present study because of technical difficulties. In regard to the mechanism of Rab4 dissociation, we also examined whether syntaxin 4 has GAP activity for Rab4. However, \([\gamma-32P]GTP\) hydrolysis on Rab4 was not affected in the presence of GST-syntaxin 4-(1–273).2

The physiological implication of the interaction of Rab4 with syntaxin 4 in insulin-induced GLUT4 translocation is unclear at present. Taking into account the previous observation that a GTP-binding-defective mutant of Rab4 inhibits insulin-induced GLUT4 translocation (11), it is possible that syntaxin 4 is one of the targets for Rab4 and the interaction of GTP-bound Rab4 with syntaxin 4 may be critical for docking and/or fusion of the GLUT4-containing vesicles with the plasma membrane. However, because GTP-bound Rab4 was unable to displace munc-18c from syntaxin 4 (Fig. 11), the Rab4-syntaxin 4 interaction may not be directly involved in the activation of syntaxin 4. In addition, whereas the Rab4 C-terminal domain-derided peptide, Rab4-(191–210), inhibits insulin- or GTP-γS-stimulated GLUT4 translocation (10), it did not interfere with the Rab4 binding to syntaxin 4, suggesting that some target(s) other than syntaxin 4 may be present. On the other hand, recent studies have demonstrated that the interaction between SNARE proteins is not selective (50, 54). In contrast, Rab family GTPases are localized to distinct subcellular membrane compartments. Our data showed that Rab4 was bound to GST-syntaxin 4-(1–273) but not to GST-syntaxin 1A-(4–267) or GST-VAMP-2-(1–94) (Figs. 3 and 4). Thus, an alternative possibility is that the interaction of Rab4 with syntaxin 4 participates in the selectivity in targeting of the GLUT4-containing vesicles to the plasma membrane. Further work will be necessary to clarify this point.

In summary, the present study provides important insights into the molecular mechanism of Rab4 action in insulin-induced GLUT4 translocation. Our data clearly showed that Rab4 directly interacts with syntaxin 4, which may be one of the targets for Rab4 action. In addition, it was indicated that the interaction between the two proteins is regulated by both the guanine nucleotide status of Rab4 and the conformational status of syntaxin 4. The immediate upstream insulin signals for the activation of Rab4 and syntaxin 4 may be different and are to be elucidated.

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