Insulin Stimulates Guanine Nucleotide Exchange on Rab4 via a Wortmannin-sensitive Signaling Pathway in Rat Adipocytes*

Hiroshi Shibata‡, Waka Omata, and Itaru Kojima

From the Department of Cell Biology, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371, Japan

Insulin stimulates glucose transport in muscles and adipose cells by promoting translocation of glucose transporter isoform, GLUT4, from intracellular compartment(s) to the plasma membrane (1–3), although the molecular mechanisms of the insulin action are still obscure. Recent kinetic and morphological studies have proposed a three-compartment model for the subcellular trafficking of GLUT4 (3, 4). In the model, GLUT4 molecules are associated with at least two intracellular compartments; one is the early endosome in which GLUT4 molecules seem to appear only in the presence of insulin, and the second one is a more specialized compartment where the glucose transporter molecules are sequestered in the basal state. Although the precise nature of the latter compartment remains indistinct, after insulin stimulation, GLUT4 molecules leave the compartment, are recruited on the plasma membrane, and then enter the endosomal recycling pathway.

Over the past decade, it has become apparent that intracellular vesicle traffic is regulated by a variety of GTP-binding proteins, including Rab and Arf families of Ras-related small GTP-binding proteins (5, 6), as well as trimeric GTP-binding proteins (7) and large GTP-binding proteins such as dynamin (8). In permeabilized adipocytes, nonhydrolyzable GTP analogs induce GLUT4 translocation, suggesting that GTP-binding protein(s) may be involved in insulin-stimulated GLUT4 translocation (9–12). In this regard, Rab3D has been shown to be predominantly expressed in adipocytes and induced during differentiation of 3T3-L1 cells into adipocytes (13), although it remains to be demonstrated whether the GTPase is involved in the insulin action (14). On the other hand, Cormont et al. (15) reported that Rab4 is associated with GLUT4-containing vesicles in rat adipocytes, and insulin stimulation resulted in redistribution of the protein from the vesicle to the cytosol. Rab4, a member of the Rab family, has been demonstrated to be associated with GLUT4-containing vesicles and implicated in the insulin-induced GLUT4 translocation. It remains to be clarified, however, whether or not Rab4 lies downstream of the insulin receptor and functions as a signaling component of insulin activation of glucose transport. In the light of the observations that insulin stimulates exocytosis of GLUT1 (20, 21) and transferrin receptor (22, 23), both constitutively recycling cell surface proteins, Rab4 might be a possible target of insulin action on intracellular trafficking of these proteins. In the present study, we examined this possibility and demonstrated that guanine nucleotide exchange on Rab4 was stimulated by insulin in a wortmannin-sensitive manner, providing evidence for the first time that Rab4 lies downstream of the insulin-stimulated phosphatidylinositol (PI) 3-kinase.

EXPERIMENTAL PROCEDURES

Materials—

125I-Labeled protein A and [35S]GTPyS were from Du Pont NEN. GTP-S was purchased from Boehringer Mannheim. Wortmannin was obtained from Sigma and dissolved in dimethyl sulfoxide at 10 mM (stock solution). LY294002 was purchased from Calbiochem and dissolved in Me3SO at 5 mM (stock solution). Protein G-Sepharose was from Pharmacia Biotech Inc.

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‡ To whom correspondence should be addressed. Tel.: 81-27-220-8839; Fax: 81-27-220–8893; E-mail: hshibata@sb.gunma-u.ac.jp.

† The abbreviations used are: GTPyS, guanosine 5’-O-(3-thiotriphosphate); PI, phosphatidylinositol; GAP, GTPase-activating protein.
Antibodies—Polyclonal antibodies against GLUT4 were raised in this laboratory as described previously (24). Polyclonal antibodies against rat Rab4 were obtained by immunizing a rabbit with peptide, (C)QLRSPRTQAPSAQE conjugated with bovine serum albumin. The anti-Rab4 antiserum was purified with SulfoLink coupling gel column (Fierce) coupled with the peptide according to the manufacturer’s instructions. When immunoblot was performed using total homogenate of rat adipocytes, a single band with a molecular size of 25 kDa was recognized with the anti-Rab4 antibodies.

Preparation of Rat Adipose Cells—Isolated adipocytes were prepared by the collagenase method from epididymal adipose tissue of Harlan Sprague Dawley rats (from Charles-River, approximately 170–220 g) (25). Unless otherwise specified, isolated cells were suspended in buffer A (25 mM Kreb-Henseleit Hepes buffer supplemented with 20 mg/ml bovine serum albumin (fraction V) and 3 mM pyruvate, pH 7.4). Insulin Stimulation of Rab4—To measure the binding of [35S]GTPyS to Rab4 in permeabilized cells—[35S]GTPyS binding to Rab4 was measured as described by Ullrich et al. (26) with a modification. The isolated cells were washed and resuspended in high K+ low Ca2+ 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, 23.4 mM Hepes/KOH, 20 mg/ml bovine serum albumin, 3 mM pyruvate, pH 7.4) (27), and incubated for 30 min at 37 °C. Then, the electroporation was carried out four times in a Gene-Pulser (from Bio-Rad) set at 250 V/cm and 1.8 kV/cm. After incubation for 15 min at 37 °C without or with 100 nM insulin, the permeabilized cells were incubated for an additional 30 min in the presence of 50 nM [35S]GTPyS. At the end of the incubation, the cells were washed and 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% (w/v). One half of the infranatant was incubated with 15 μl of affinity-purified anti-Rab4 antibodies and 20 μl (bed volume) of protein G-Sepharose (from Pharmacia) for 40 min on a rocking platform at 4 °C. The other half was incubated with protein G-Sepharose alone. The Sepharose beads were spun down at 4 °C for 1 min at 3,000 × g and washed twice in 1 ml of washing buffer containing 1% Nonidet P-40. The beads were filtered through 25-mm nitrocellulose filter, and the filter was washed three times with 3 ml of ice-cold filtration buffer (25 mM MgCl2, 100 mM NaCl, 20 mM Tris/Cl, pH 7.5). The filter was dried and counted in 10 ml of scintillation fluid in a scintillation counter. The amount of [35S]GTPyS bound to Rab4 immunoprecipitated with anti-Rab4 antibodies was determined after subtraction of the radioactivity obtained with protein G-Sepharose alone.

Measurement of 3-O-Methyl-d-glucose Uptake—The cellular glucose transport activity was estimated by measuring the rate of 0.1 mM 3-O-methyl-d-glucose uptake by the oil-flotation method as described previously (27).

Preparation of Subcellular Fractions—The microsomal and soluble fractions were prepared by differential centrifugation as described previously (24). The cells were washed and homogenized in STE buffer (250 mM sucrose, 10 mM Tris/HCl, and 1 mM EDTA/Na, pH 7.4). The homogenate was centrifuged for 2 min at 3,000 × g. The supernatant (S-1) was saved as the microsomal fraction and pelleted by centrifugation for 60 min at 150,000 × g. The supernatant (S-2) was saved as the soluble fraction.

Electrophoresis and Immunoblotting—Immunodetection of Rab4 was carried out as described previously (24). Briefly, proteins in the microsomal and soluble fractions were separated on SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gels, as described by Laemmli (28), and transferred to a polyvinylidene difluoride membrane (from Millipore) at 120 mA for 4 h. The polynylidened difluoride membrane was blocked with solution containing 5% bovine serum albumin, 10 mM Tris/HCl (pH 7.4), and 154 mM NaCl for 1 h at room temperature. The blocked membrane was incubated in anti-Rab4 antibodies (1:500 dilution) overnight at 4 °C. The membrane was washed and incubated with [125I]-protein A (0.2 μCi/ml) for 1 h at room temperature. Following extensive washes, the membrane was dried, and the blots were visualized by using FUJIX BAS2000 bio-imaging analyzer (Fuji Photo Film, Tokyo, Japan).

Statistical analysis was done by Student’s t test. All the results reported herein were confirmed by repeating the experiments with different batches of adipocytes on different occasions.

RESULTS

In the present study, we first examined the amount of [35S]GTPyS specifically bound to Rab4 in electrically permeabilized rat adipocytes. As reported previously, exogenously added nucleotides such as cyclic AMP and GTPyS can enter the cell interior and stimulate lipolysis (27) and glucose transport (11), respectively, in electrically permeabilized cells. In addition, insulin considerably stimulates glucose transport as well as translocation of GLUT4 in these cells (24). To measure the amount of [35S]GTPyS bound to Rab4, the GTPase was immunoprecipitated with anti-Rab4 antibodies and protein G-Sepharose beads after the cells were incubated with [35S]GTPyS. As shown in Fig. 1, the radioactivity associated with the pellet in the presence of the antibodies decreased by the addition of the antigen peptide, (C)QLRSPRTQAPSAQE, in a concentration-dependent manner. At 1 mg/ml of the peptide, the radioactivity associated with the pellet was not significantly different from that obtained in the absence of the antibodies. Those blank values were essentially unchanged either in the absence or the presence of insulin (data not shown). In the following experiments, the amount of [35S]GTPyS bound to Rab4 was determined by subtraction of the radioactivity obtained with protein G-Sepharose beads alone. Under the conditions with anti-Rab4 antibodies, nearly all Rab4 molecules were immunoprecipitated, since no Rab4 was detected by immunoblotting in the supernatant after immunoprecipitation either in the basal or insulin-stimulated cells (data not shown).

Next, we incubated the cells with [35S]GTPyS for increasing periods of time in the absence and the presence of insulin. As shown in Fig. 2, the amount of [35S]GTPyS bound to immunoprecipitated Rab4 increased in a time-dependent manner during 45 min of the incubation period in the absence of insulin. Addition of 100 nM insulin resulted in about 2-fold stimulation of the binding of [35S]GTPyS to Rab4. The stimulatory effect of insulin on [35S]GTPyS binding was detected at as early as 5 min after the addition of the hormone. These results suggested that insulin acutely stimulates guanine nucleotide exchange on Rab4 GTPase in rat adipocytes.

Since our previous report indicated that Rab4 may play a
critical role in the insulin-induced exocytotic fusion of GLUT4-containing vesicles (16), we compared, in the next set of experiments, the effects of insulin on \( ^{35} \text{S} \)GTP\( \gamma \)S binding to Rab4 and cellular glucose transport activity. As illustrated in Fig. 3, the stimulatory effects of insulin of \( ^{35} \text{S} \)GTP\( \gamma \)S binding to Rab4 were concentration-dependent and in good correlation with those of glucose transport activity. Several lines of recent experimental evidence suggest that PI 3-kinase is an indispensable signaling component of insulin stimulation of GLUT4 translocation. Thus, pharmacological attenuation of the kinase with wortmannin or LY294002, both potent and specific inhibitors of PI 3-kinase, markedly decreases the insulin effects on glucose transport and GLUT4 translocation (29–32). In addition, microinjection or overexpression of a mutant p85 regulatory subunit of PI 3-kinase lacking the ability to bind and activate the p110 catalytic subunit inhibited insulin-stimulated GLUT4 translocation (33, 34). These observations led us to investigate whether the activation of Rab4 by insulin is dependent on PI 3-kinase activity. As depicted in Fig. 4, pretreatment of the cells with 100 nM wortmannin or 50 \( \mu \)M LY294002 resulted in marked inhibition of the insulin effects on \( ^{35} \text{S} \)GTP\( \gamma \)S binding to Rab4 as well as glucose transport activity, suggesting that insulin activate guanine nucleotide exchange on the GTPase via a PI 3-kinase-dependent signaling pathway.

Fig. 5 shows the effects of wortmannin on \( ^{35} \text{S} \)GTP\( \gamma \)S binding to Rab4 in the basal and insulin-stimulated cells. Wortmannin inhibited the nucleotide binding to Rab4 stimulated by insulin in a concentration-dependent manner with a half-maximal concentration in the low nanomolar range. Intriguingly, wortmannin also attenuated the \( ^{35} \text{S} \)GTP\( \gamma \)S binding to Rab4 in the basal cells, raising a possibility that PI 3-kinase activity may be necessary for guanine nucleotide exchange on Rab4 even in the absence of insulin and that insulin may up-regulate the guanine nucleotide exchange activity by stimulating the lipid kinase.

Finally, we examined the effect of wortmannin on insulin-induced subcellular redistribution of Rab4 in the electrically permeabilized adipocytes. As illustrated in Fig. 6, insulin induced a subcellular shift of Rab4 from the microsomal fraction to the soluble fraction. Pretreatment of the cells with 100 nM wortmannin completely abolished the effect of insulin on the subcellular shift of Rab4 (Fig. 6). These results confirm the observation in intact adipocytes by Le Marchand-Brustel et al. (35) and suggest that activation of Rab4 may be accompanied with dissociation of the GTPase from the intracellular membrane.

**DISCUSSION**

The Rab family of GTP-binding proteins is believed to function as regulators of intracellular membrane traffic (5). Among those, Rab4 has been shown to be associated with the early endosomes and to control recycling of cell surface receptors from the early endosomes to the plasma membrane (17, 18). In addition, Rab4 has been recently demonstrated to be associated with GLUT4-containing vesicles in adipocytes (15) and muscles (35, 36), and insulin stimulation leads to subcellular redistri-
stimulates \(^{35}\text{S}\text{GTP}^\gamma\text{S}\) clearly demonstrated for the first time that insulin acutely makes it possible to study the action of insulin under more binding to endogenous Rab4 instead of overexpressed protein, for 30 min with 50 \(\mu\text{M}^{35}\text{S}\text{GTP}^\gamma\text{S}\) in the absence (○) or presence (●) of 100 nM insulin. The amount of \(^{35}\text{S}\text{GTP}^\gamma\text{S}\) bound to Rab4 was measured as described under “ Experimental Procedures.” The results are the means ± S.D. of three determinations.

### Graphs

#### FIG. 5. Effects of wortmannin on \(^{35}\text{S}\text{GTP}^\gamma\text{S}\) binding to Rab4 in basal or insulin-stimulated cells.

Adipocytes in buffer X were incubated for 30 min and then permeabilized as described under “Experimental Procedures.” The permeabilized cells were incubated for 10 min with the indicated concentrations of wortmannin, then incubated for 30 min with 50 \(\mu\text{M}^{35}\text{S}\text{GTP}^\gamma\text{S}\) in the absence (○) or presence (●) of 100 nM insulin. The amount of \(^{35}\text{S}\text{GTP}^\gamma\text{S}\) bound to Rab4 was measured as described under “Experimental Procedures.” The results are the means ± S.D. of three determinations.

#### FIG. 6. Effects of wortmannin on insulin-induced Rab4 redistribution.

Adipocytes in buffer X were incubated for 60 min at 37 °C and then permeabilized as described under “Experimental Procedures.” The cells were incubated for 10 min without or with 100 nM wortmannin, then incubated for 20 min in the absence or presence of 100 nM insulin. At the end of the incubation, the cells were washed, homogenized, and subjected to subcellular fractionation and immunoblotting as described under “Experimental Procedures.” A, representative immunoblot data. B, relative amounts of Rab4. The relative intensities of Rab4 bands were quantified by using FUJIX BAS2000 bio-imaging analyzer. a, microsomal fractions. b, soluble fractions. The results are the means ± S.D. of three determinations. * \(p < 0.05\) versus basal value; ** \(p < 0.01\) versus basal value

The stimulation by insulin of \(^{35}\text{S}\text{GTP}^\gamma\text{S}\) binding to Rab4 well correlates with that of cellular glucose transport activity. First, the concentration-effect relationship of insulin stimulation of the nucleotide binding was in good accordance with that of glucose transport activity (Fig. 3). Second, the insulin-stimulated \(^{35}\text{S}\text{GTP}^\gamma\text{S}\) binding to Rab4 was inhibited with wortmannin with a half-maximal concentration in the low nanomolar range (Fig. 5), similar to that of the inhibition by wortmannin of glucose transport activity (29, 30). These results, together with our previous observations (16), provide further evidence that activation of Rab4 may be one of possible mechanism(s) by which insulin promotes GLUT4 translocation.

Interestingly, the binding of \(^{35}\text{S}\text{GTP}^\gamma\text{S}\) to Rab4 in the basal cells was also inhibited considerably with wortmannin (Fig. 5), which has little effect on the basal glucose transport activity (29). Previous reports indicate that PI 3-kinase may be essential for the constitutive recycling of cell surface proteins, including transferrin receptor, insulin-like growth factor-2 receptor, and GLUT1 from the early endosomes to the plasma membrane (40–42). The inhibition with wortmannin of \(^{35}\text{S}\text{GTP}^\gamma\text{S}\) binding to Rab4 in the basal cells (Fig. 5) suggests that, even in the absence of insulin, PI 3-kinase may be a critical regulator of guanine nucleotide exchange on Rab4, which is necessary for the constitutive recycling of the cell surface proteins, and that insulin may up-regulate the exchange activity of Rab4 by activation of PI 3-kinase. We cannot rule out the possibility, however, that the insulin-stimulated PI 3-kinase(s) may be distinct from other wortmannin-sensitive PI 3-kinase(s) regulating the constitutive endosomal recycling. Further studies will be necessary to elucidate this point. The discrepancy of the effects of wortmannin on Rab4 activity and glucose transport in the basal cells seems in good agreement with our previous report (24) that, in the absence of insulin, most of GLUT4 molecules may be in the compartment(s) outside of the constitutive endosomal recycling pathway regulated by Rab4 and also suggests that GLUT4-containing vesicles may
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pause en route to the cell surface by unknown mechanism despite the presence of Rab4 on the vesicles.

At present, the precise molecular mechanism of the insulin stimulation of guanine nucleotide exchange on Rab4 is unclear. The activity of Rab family GTPase is regulated primarily by two regulatory proteins (43); one is guanine nucleotide exchange factor(s) which positively regulates Rab proteins by accelerating GDP dissociation and subsequent GTP binding and another is GTPase-activating protein (GAP) which inactivates Rab by stimulating GTP hydrolysis on Rab GTPase. Recently, Bortoluzzi et al. (44) reported GAP activity for Rab4 in the membrane fraction of 3T3-L1 cells, although insulin had no effect on the Rab4-GAP activity or its subcellular localization. Our present data provides the first evidence that insulin may activate as yet unidentified guanine nucleotide exchange factor(s) for Rab4 and an important insight as to the molecular mechanism(s) of insulin stimulation of glucose transport.

In summary, the present study clearly demonstrated for the first time that insulin activates Rab4 by accelerating guanine nucleotide exchange on the GTPase in rat adipocytes. The activation by insulin of Rab4 was wortmannin- and LY294002-sensitive, suggesting Rab4 lies downstream of the insulin-stimulated PI 3-kinase. Our results indicate that Rab4 is one of the intracellular targets of insulin action on the vesicle traffic including GLUT4 translocation.

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