Rab10 in insulin-stimulated GLUT4 translocation


*Department of Biochemistry, 7200 Vail, Dartmouth Medical School, Hanover, NH 03755, U.S.A., and †Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, Miyagi 980-8587, Japan

In fat and muscle cells, insulin stimulates the movement to and fusion of intracellular vesicles containing GLUT4 with the plasma membrane, a process referred to as GLUT4 translocation. Previous studies have indicated that Akt [also known as PKB (protein kinase B)] phosphorylation of AS160, a GAP (GTPase-activating protein) for Rabs, is required for GLUT4 translocation. The results suggest that this phosphorylation suppresses the GAP activity and leads to the elevation of the GTP form of one or more Rabs required for GLUT4 translocation. Based on their presence in GLUT4 vesicles and activity as AS160 GAP substrates, Rabs 8A, 8B, 10 and 14 are candidate Rabs. Here, we provide further evidence that Rab10 participates in GLUT4 translocation in 3T3-L1 adipocytes. Among Rabs 8A, 8B, 10 and 14, only the knockdown of Rab10 inhibited GLUT4 translocation. In addition, we describe the subcellular distribution of Rab10 and estimate the fraction of Rab10 in the active GTP form in vivo. Approx. 5% of the total Rab10 was present in GLUT4 vesicles isolated from the low-density microsomes. In both the basal and the insulin state, 90% of the total Rab10 was in the inactive GDP state. Thus, if insulin increases the GTP form of Rab10, the increase is limited to a small portion of the total Rab10. Finally, we report that the Rab10 mutant considered to be constitutively active (Rab10 Q68L) is a substrate for the AS160 GAP domain and, hence, cannot be used to deduce rigorously the function of Rab10 in its GTP form.

Key words: adipocyte, AS160, GLUT4, GTPase-activating protein (GAP), insulin, Rab10.

INTRODUCTION

Insulin rapidly stimulates glucose transport in fat and muscle cells. The basis for this effect is the insulin-stimulated fusion of intracellular vesicles containing GLUT4 with the plasma membrane, a process referred to as insulin-stimulated GLUT4 translocation [1].

The pathway(s) of signal transduction from the insulin receptor to GLUT4 translocation is not completely understood. Insulin activates the protein kinase Akt [also known as PKB (protein kinase B)] by a well-established pathway, and signalling to GLUT4 translocation is, at least in part, downstream of Akt [1]. In recent years, we and others have characterized an Akt substrate, known as AS160, that participates in GLUT4 translocation [2–4]. AS160 is a GAP (GTPase-activating protein) for Rabs. Rabs are small G-proteins that, in their GTP form, function in vesicle movement by linking vesicles to microtubule and microfilament motors and in vesicle fusion to target membranes through interactions with the docking and fusion machinery [5]. Considerable evidence supports the following hypothesis [2–4]; in the absence of insulin, AS160 maintains one or more Rabs in their inactive GDP form; insulin elicits Akt phosphorylation of AS160, which thereby suppresses its GAP activity and so leads to the elevation of the GTP form of the critical Rab(s); elevation of the GTP form of the Rab(s) triggers GLUT4 vesicle movement to and/or fusion with the plasma membrane.

A challenge for this hypothesis is to identify the Rab(s) that participate downstream of AS160 in GLUT4 translocation from among the 60 mammalian Rabs. We have taken the approach of identifying the Rabs that are present in GLUT4 vesicles by MS and then determining which of these are substrates for the GAP domain of AS160. This approach has yielded Rabs 2A, 8A, 8B, 10 and 14 as candidates. Among this group of Rabs, Rab2A seems unlikely to act in GLUT4 translocation, since Rab2A is known to participate in trafficking between the ER (endoplasmic reticulum) and the Golgi [6]. In a recent study, we presented evidence that Rab10, but not Rab8A, is required for GLUT4 translocation in 3T3-L1 adipocytes [7]. Notably, knockdown of Rab10, but not Rab8A, partially inhibited GLUT4 translocation. The present study examines the potential role of Rab8B and Rab14 in this process in 3T3-L1 adipocytes. In addition, we describe the subcellular distribution of Rab10 and the effect of insulin on GTP loading of Rab10 in vivo.

EXPERIMENTAL

Plasmids, antibodies and recombinant proteins

The pSiren-RetroQ shRNA (small-hairpin RNA) plasmids for knockdown of mouse Rabs 8A and 10 (plasmid KD251) and the plasmid for control shRNA were those described in [7]. The pSiren-RetroQ shRNA plasmids for mouse Rabs 8B and 14 were prepared according to the manufacturer’s instructions (Clontech). The sequences used were AATGTGATATGAACGACAA for Rab8B and TGATTGTCTCCACACATT for Rab14. The plasmids for the following proteins were those described previously: FLAG-tagged Rab10 wild-type, the Q68L mutant (Rab10Q/L) and the Rab10 with silent mutations for rescue of the knockdown [7]; HA–GLUT4–GFP, the non-phosphorylatable form of FLAG-tagged AS160 (AS160 4P) and the catalytically inactive form of AS160 (AS160 R/K) [2]; GST (glutathione transferase)-fusion proteins with the GAP domain of wild-type and catalytically inactive (R/K mutant) AS160 and with Rab10 [8]. The plasmids for FLAG-tagged Rab10 S18V and for GST–Rab10 Q68L were generated by mutation of the corresponding plasmids for wild-type proteins with the QuikChange® kit (Stratagene) and verified by DNA sequencing. The plasmid for the GST-fusion protein with the C-terminal Rab-binding domain

Abbreviations used: C12E9, nonaethylene glycol dodecyl ether; ER, endoplasmic reticulum; GAP, GTPase-activating protein; GST, glutathione transferase; HDM, high-density microsome; HEK-293F, human embryonic kidney; IRAP, insulin-regulated aminopeptidase; LDM, low-density microsome; p[NH]ppG, guanosine 5′-(β,γ-imido)triphosphate; shRNA, small-hairpin RNA.

1 To whom correspondence should be addressed (email gustav.e.lienhard@dartmouth.edu).
of mouse Mical L2 protein (amino acids 806–1009) [9] was generated by PCR amplification of this region from a larger Mical L2 plasmid and ligated into pGEX-6p (Amersham Biosciences). The larger Mical L2 plasmid was obtained from a mixed mouse testis/embryo cDNA library in a yeast two-hybrid screen with Rab10 as bait (T. Itoh and M. Fukuda, unpublished work).

Antibodies against the following proteins were those previously described: Rabs 8A/B and Rab10 [7]; the cytoplasmic domain of the IRAP (insulin-regulated aminopeptidase) [10]; and the C-terminus of GLUT4 [11]. Anti-FLAG antibody conjugated to horseradish peroxidase was purchased from Sigma. An affinity-purified antibody against Rab14 was generated by immunization of rabbits with a unique peptide from mouse Rab14 (amino acids 167–183), followed by purification of the antibody from the serum on the immobilized peptide, as described in [12]. GST-fusion proteins were prepared in Escherichia coli and isolated on glutathione–agarose as described previously [8].

Cell culture and transfection

3T3-L1 adipocytes were carried as fibroblasts and differentiated into adipocytes as described in [13]. shRNAs were introduced into the fibroblasts by retroviral infection with the pSiren-RetroQ retrovirus that also contains the puromycin resistance gene, and cells stably expressing the shRNA were differentiated into adipocytes. On day 4 of differentiation, the plasmids for HA–GLUT4–GFP and/or FLAG-tagged Rab10 were introduced by electroporation by the method of Sano et al. [2], HEK-293F (human embryonic kidney) cells were transiently transfected with plasmids using the Lipofectamine™ 2000 reagent according to the manufacturer’s instructions (Invitrogen). At 24 h after transfection, cells were analysed by the methods described below. Where immunoblotting was performed, plates of the cells were dissolved in SDS sample buffer containing protease inhibitors.

Assay for cell-surface GLUT4

HA–GLUT4–GFP at the cell surface was assayed by the quantitative single-cell fluorescence assay, which is described in detail in [2]. In this method, adipocytes in the unstimulated or insulin-treated state were fixed with formaldehyde, and the HA–GLUT4–GFP at the cell surface was stained with anti-HA followed by Cy3-conjugated secondary antibody. The Cy3 and GFP fluorescence intensities of approx. 50 transfected cells were measured and corrected for the control values given by untransfected cells. The cell-surface GLUT4 in each cell was then expressed as the ratio of the corrected Cy3 to corrected GFP fluorescence, in order to normalize for different levels of HA–GLUT4–GFP expression.

Rab GAP assay

The activity of the GAP domain of AS160 was measured exactly as described in [8]. In this assay, the GST–Rab was loaded with [γ-32P]GTP and then treated with the GST–GAP domain for various time periods. The percentage of GTP converted into GDP was then determined by separating GDP from GTP by TLC followed by phosphor imaging.

Subcellular fractionation of 3T3-L1 adipocytes and isolation of GLUT4 vesicles

3T3-L1 adipocytes were fractionated into LDMs (low-density microsomes) and HDMs (high-density microsomes), plasma membranes, mitochondria/nuclei and cytosol, by using an established method [14]. GLUT4 vesicles were isolated by immunoadsorption with anti-GLUT4 on immobilized Protein A, from the LDM/cytosol fraction of 3T3-L1 adipocytes, as described in [15].

Assays for GTP content of Rab10 in vivo

The methods here are adaptations of the ones described in [16]. In the radioactive GTP/GDP assay, 35 mm dishes of either 3T3-L1 adipocytes or HEK-293F cells transfected with either FLAG-tagged wild-type Rab10 or Rab10Q/L were incubated for 2 h with 0.75 ml of DMEM (Dulbecco’s modified Eagle’s medium) with P, at 1 m M containing 125 or 20 µCi of [32P]GTP, respectively. The medium was removed, and the cells were washed with ice-cold PBS and lysed in 0.5 ml of 50 mM Tris/HCl, 100 mM NaCl and 5 mM MgCl2 (pH 7.5) containing non-ionic detergent (1.7% C12E9 (nonaethylene glycol dodecylether)) and protease inhibitors (10 µM leupeptin, 10 µM EP475 (trans-epoxysuccinyl-L-leucylamido-3-methylbutanate), 1 µM pepstatin A and 10 µg/ml aprotinin) at 4°C. In the experiments with adipocytes, the lysis buffer also included a phosphatase inhibitor (200 nM calyculin A). The lysate was centrifuged at 16 000 g for 10 min, and the FLAG-tagged Rab10 was immunoadsorbed from the supernatant on 10 µl of anti-FLAG–agarose (Sigma) for 1 h at 4°C. The immunoadsorbate was quickly washed three times with the above buffer containing 0.3% C12E9 and once with a buffer containing 0.03% C12E9. The asorbed Rab10 and its bound guanine nucleotides were released by adding 20 µl of 0.2% SDS, 5 mM GTP, 5 mM GDP and 5 mM EDTA (pH 6.6) and heating at 65°C for 2 min. The radiolabelled GTP and GDP in an aliquot were separated by TLC, and the radioactivity in each was measured by phosphor imaging. A control was performed in which cells not expressing the FLAG-tagged Rab10 were put through the same procedure, and the much smaller amounts of radioactivity found in the GDP and GTP were subtracted as a blank from values found with FLAG-tagged Rab10. The percentage of GTP bound to Rab10 was calculated from the corrected radioactivity values with the assumption that the β- and γ-phosphates of GTP and the β-phosphate of GDP were at the same specific radioactivity as the result of labelling.

For the pull-down assay of Rab10 GTP, we first established that GST–Mical L2 selectively bound to the GTP form of Rab10. A 10-cm plate of HEK-293F cells expressing FLAG-tagged Rab10 was lysed in 2 ml of 1.5% C12E9, 50 mM Tris/HCl, 100 mM NaCl and 1 mM MgCl2 (pH 8.0) containing protease inhibitors. The supernatant from centrifugation of the lysate at 27 000 g for 15 min was divided into equal portions. The Rab10 was loaded with either GDP or p[NH]ppG (guanosine 5′-β,γ-imidotriphosphate) by adding the nucleotide at 0.5 mM followed by 2 mM EDTA and incubation at 4°C for 30 min. Subsequently, the guanine nucleotide was fixed on the Rab10 by addition of 5 mM MgCl2. Portions (0.5 ml) of these solutions were then incubated with 50 µl of glutathione–agarose containing either 100 µg of GST–Mical L2 or 50 µg of GST at 4°C for 1 h. The beads were washed quickly three times with cold 0.5% C12E9, 50 mM Tris/HCl, 100 mM NaCl and 5 mM MgCl2 (pH 8.0). The bound proteins were released with SDS sample buffer, and the samples were immunoblotted with anti-FLAG to detect the bound Rab10.

This procedure was applied to 3T3-L1 adipocytes by treating 10-cm plates of cells with insulin for various time periods. Each plate was washed with PBS and then lysed in 1 ml of 50 mM Tris/HCl, 100 mM NaCl and 5 mM MgCl2 (pH 8.0) containing 3% C12E9, 100 µg/ml GST–Mical L2 and protease and phosphatase inhibitors (as above) at 4°C. The lysate was
Figure 1  Knockdown of Rabs

(A) Immunoblots of 3T3-L1 adipocytes. SDS samples of adipocytes expressing control (Con) shRNA and shRNAs for Rab 8A, 8B, 10 and 14 were immunoblotted for the designated Rabs. (B) HEK-293F cells were co-transfected with FLAG-tagged Rab 8A or 8B and a plasmid expressing the control (Con) shRNA or shRNA for Rab 8A or 8B. SDS samples of the cells were immunoblotted with anti-FLAG. For each blot, the 1× loads contained equal amounts of protein as assessed by the Lowry protein assay [27].

centrifuged at 16000 g for 10 min, and the supernatant was then incubated with 50 µl of glutathione–agarose at 4 °C for 1 h. The beads were then quickly washed three times with the above buffer containing 0.5 % C12E9, and the absorbed Rab10 was released with SDS sample buffer and detected by immunoblotting with anti-Rab10.

RESULTS

Effects of Rab knockdowns on GLUT4 translocation

Previously, we showed that among the Rabs found in GLUT4 vesicles by MS, Rabs 2A, 8A, 8B, 10 and 14 were substrates for the GAP domain of AS160 [8]. Since Rab2 is involved in trafficking from the ER to the Golgi [6], we have concentrated on the possible role of Rabs 8A, 8B, 10 and 14 in GLUT4 translocation. Each of these Rabs was knocked down through retroviral expression of an shRNA (Figure 1A). The knockdowns of Rab10 and 14 were greater than 75 %, and in fact there was no detectable band in the immunoblot (Figure 1A). Measurement of the knockdown of Rab 8A and 8B was complicated by the lack of specific antibodies for each isoform, which are 83 % identical. Immunoblotting with an antibody that reacts with both showed approx. 50 % knockdown of Rab8 with shRNA for Rab8A and approx. 25 % knockdown of Rab8 with the shRNA for Rab8B (Figure 1A). As a measure of the effectiveness of the shRNAs for Rab8, we co-expressed each with the corresponding FLAG-tagged Rab8 in HEK-293F cells. In this assay, each shRNA caused more than 90 % knockdown (Figure 1B). If we assume from this result that the two shRNAs are equally (but less) effective in 3T3-L1 adipocytes, then the distribution of Rab8 in adipocytes is approx. 67 % Rab8A and 33 % Rab8B and each was knocked down by approx. 75 %.

Figure 2 presents the effect of the knockdown of these Rabs on insulin-stimulated GLUT4 translocation. In the control, insulin caused a 10-fold increase in GLUT4 at the cell surface. Knockdown of Rab10 inhibited the insulin-stimulated increase in GLUT4 at the cell surface by approx. 80 %. Knockdown of Rabs 8A, 8B and 14 had no significant effect. To ensure that the effect of the Rab10 shRNA was due to knockdown of Rab10, we showed that expression of a knockdown-resistant mutant of Rab10 fully restored insulin-stimulated GLUT4 translocation (Figure 3A).

Figure 2 Effect of Rab knockdown on insulin-stimulated GLUT4 translocation

HA–GLUT4–GFP at the cell surface in cells expressing the control (Con) shRNA or the shRNA for Rabs 8A, 8B, 10 and 14 in the absence and presence of insulin (160 nM for 30 min) was measured as described in the Experimental section. The results are the means ± S.E.M. for three independent experiments. The values in each experiment have been normalized to a value of 1.0 for the control with insulin.

Figure 3 Effect of knockdown-resistant Rab10 (rRab10) on insulin-stimulated GLUT4 translocation

(A) 3T3-L1 adipocytes expressing control (Con) shRNA or Rab10 shRNA were co-transfected with HA–GLUT4–GFP and either FLAG-tagged rRab10 or the empty Rab vector (V). HA–GLUT–GFP at the cell surface in the absence and presence of insulin (160 nM for 30 min) was measured, as described in the Experimental section. Results are means ± range for two independent experiments. The values in each experiment have been normalized to a value of 1.0 for the control with insulin. (B) Immunoblot of the samples in (A) for Rab10. The endogenous Rab10 is labelled eRab10. The 1× loads contained equal amounts of protein as assessed by the Lowry protein assay [27].
Immunoblotting for Rab10 established that the knockdown-resistant mutant of Rab10 was expressed (Figure 3B). Because of its FLAG tag, it runs with a slightly slower mobility than endogenous Rab10. These results are in qualitative agreement with our previous study on Rabs in GLUT4 translocation [7]. In that study, we examined Rabs 8A and 10, but not 8B and 14, and found that knockdown of Rab10, but not 8A, inhibited GLUT4 translocation. In the present study the effect of Rab10 knockdown is considerably greater than that found in the previous study (approx. 30% inhibition versus 80% here). A possible explanation for the difference is the greater extent of Rab10 knockdown in the present study; in the earlier study, a Rab10 band was detected in the knockdown by immunoblotting under the same conditions where none was detected here. In this previous study, it was established that the Rab10 knockdown had no effect on Akt activation or its phosphorylation of AS160 as assessed by immunoblotting [7], and the same results were obtained here (results not shown). Also, in the previous study the knockdown of Rab10 had no evident effect on the subcellular distribution of HA–GLUT4–GFP in either the basal or insulin-treated state, as assessed by the GFP immunofluorescence (Figure 3A of [7]). For both the normal adipocytes and Rab10 knockdown adipocytes, in both the basal and the insulin state the intracellular HA–GLUT4–GFP was concentrated in a perinuclear location and also present in vesicles throughout the cytosol. The same was true for the distribution of HA–GLUT4–GFP in the normal and Rab10-knockdown adipocytes examined in the present study (results not shown).

GAP activity against Rab10Q/L

If Rab10 is required for GLUT4 translocation, it might be expected that constitutively active Rab10 would cause GLUT4 to translocate to the cell surface in the absence of insulin. Previously, we found that overexpression of a putative constitutively active mutant Rab10 (Rab10Q/L) in 3T3-L1 adipocytes caused a 2-fold increase in GLUT4 at the cell surface [7]. This effect is modest compared with the 10-fold increase seen with insulin. It may be that this modest effect reflects the requirement for additional signalling to cause full GLUT4 translocation. However, it was recently reported [17] that while the Q/L mutation is very effective at blocking the intrinsic GTPase activity of a Rab, it can be less effective at blocking the activity of a GAP towards the Rab. Consequently, we have examined the activity of the GAP domain of AS160 towards Rab10Q/L. GST–Rab10Q/L proved to be a substrate for the GAP domain of AS160 (Figure 4, left panel), although not as an efficient one as GST–Rab10 itself (Figure 4, right panel). From the data in Figure 4 and our previous measurements of GAP activity against GST–Rab10 at various concentrations of the GAP domain (Figure 3 of [8]), we estimate that the Q/L mutant is approx. 1/20 as active a substrate as is the wild-type Rab10. Thus the modest effect on GLUT4 translocation of Rab10Q/L overexpression could reflect in part its conversion into the inactive GDP form by AS160 at the site where AS160 and Rab10 function.

Subcellular distribution of Rab10

We have generated a specific antibody for immunoblotting Rab10 [7]. However, the antibody is not suitable for immunofluorescence, since adipocytes in which Rab10 is knocked down show as much immunofluorescence as do normal adipocytes (results not shown). Hence, in order to determine the subcellular distribution of Rab10, we have fractionated 3T3-L1 adipocytes and immunoblotted the fractions. Rab10 was most concentrated in the HDMs and the plasma membranes, with a lower concentration in the LDMs (Figure 5A). From the relative signal intensities for Rab10 in the LDMs, HDMs and plasma membranes, which were approx. 0.35, 1.0 and 1.0 (Figure 5A), and the relative total amounts of each fraction (see the legend of Figure 5A), the distribution of Rab10 among the three fractions was approx. 25% LDMs, 55% HDMs and 20% plasma membranes. The HDMs consist of larger membranous structures derived from the ER, Golgi, endosomes and plasma membranes, whereas the LDMs contain smaller membranous structures, including endosomal vesicles and specific GLUT4-containing vesicles [18]. Insulin treatment did not lead to redistribution of Rab10. This distribution of Rab10 contrasts with that of the IRAP, an integral membrane protein that is a component of GLUT4 vesicles whose subcellular distribution closely resembles that of GLUT4 [19]. A larger fraction of the IRAP was located in the LDMs, and in response to insulin IRAP translocated from the LDMs to the plasma membrane (Figure 5A).

Previously, vesicles containing GLUT4 have been found to contain Rab10 by MS of their proteins [8]. By immunoblotting, we confirmed the presence of Rab10 in GLUT4 vesicles isolated from the HDMs by immunoadsorption with anti-GLUT4 (Figure 5B). By comparison of the relative signal intensities for Rab10 in the LDMs and the GLUT4 vesicles (Figure 5B) and the relative amounts of each loaded (see the legend of Figure 5B), approx. 10% of the Rab10 was isolated with the GLUT4 vesicles. Only approx. 50% of the GLUT4 vesicles in the LDMs were isolated, as assessed by the relative IRAP signals and the relative loads for the IRAP blot (Figure 5B and legend). Hence, approx. 20% of the Rab10 in the LDM fraction is in GLUT4 vesicles. Since, as noted above, the LDMs contain approx. 25% of the total cellular Rab10, approx. 5% of the total Rab10 was located in GLUT4 vesicles. Insulin treatment did not change the amount of Rab10 found in the GLUT4 vesicles (Figure 5B). This result was unexpected, since insulin stimulates the fusion of GLUT4 vesicles with the plasma membrane, and thereby reduces the amount of GLUT4 vesicles.
approx. 50% decrease in the amount of IRAP in the GLUT4 vesicles (Figure 5B). Rabs in their GDP form are extracted from membranes by the GDP dissociation inhibitor protein and then returned to vesicles in which they function [5]. If insulin causes Rab10 in GLUT4 vesicles to move to the plasma membrane along with the other components, this Rab retrieval process may be functioning rapidly enough so that net transfer of Rab10 to the plasma membrane is not detected. Also, the GLUT4 vesicle preparation is a mixture of specific exocytic GLUT4 vesicles and vesicles containing GLUT4 that are derived from the endosomes and trans-Golgi network. If both types of vesicle contain Rab10, the proportion of Rab10 in the vesicles that moves to the plasma membrane in response to insulin may not be large enough to be detectable.

**Effect of Insulin on GTP loading of Rab10**

Considerable evidence indicates that an insulin-stimulated increase in the GTP form of Rab10 is part of the mechanism of GLUT4 translocation (see the Introduction section and above). It was therefore of interest to assess whether this increase occurs on a sufficient scale to be detected. In order to examine this possibility, we overexpressed FLAG-tagged Rab10 in adipocytes, labelled the nucleotide pools with $^{32}$P,Pi, treated cells with insulin or not, immunoprecipitated the FLAG-tagged Rab10 from non-ionic detergent lysates and analysed its relative content of bound GTP and GDP by TLC. In unstimulated adipocytes, only a small portion (10%) of the FLAG-tagged Rab10 was in the GTP form, and insulin treatment did not detectably increase this percentage (Figure 6, left). To be certain that this method would detect GTP-loaded Rab10, we also examined GTP loading on overexpressed FLAG-tagged Rab10Q/L. As expected, a much larger percentage (50%) of this mutant was in the GTP form. Insulin treatment had little effect on its GTP content (Figure 6, left).

This $^{32}$P-labelling method was also used to assess the effect of AS160 on the GTP loading of FLAG-tagged Rab10 in vitro with HEK-293F cells. We employed a mutant of AS160 in which four of the Akt phosphorylation sites are mutated to alanine (AS160 4P), since earlier studies indicate that the GAP activity of this mutant is not subject to regulation by phosphorylation [2]. For controls we employed the empty FLAG vector for AS160 and also the point mutant of AS160 in which the GAP domain is inactivated (R/K mutant) [2]. As with the adipocytes, only a small portion (10%) of the FLAG-tagged Rab10 was in the GTP form in the controls (Figure 6, right). The 4P mutant of AS160 caused a decrease in the percentage of FLAG-tagged Rab10 in the GTP form compared with the two controls, but because the percentage of GTP form was low, the significance of this effect is uncertain. To overcome this issue, we examined GTP loading of FLAG-tagged Rab10Q/L in this system. In agreement with adipocytes, 55% of the Rab10Q/L was in the GTP form in the vector control. Expression of the 4P mutant reduced this percentage to 20%. Thus the 4P mutant probably acts as a GAP towards Rab10Q/L.

### Figure 5  Subcellular location of Rab10

(A) Basal (B) and insulin-treated (160 nM for 20 min) (I) 3T3-L1 adipocytes were fractionated into LDMs, HDMs, mitochondria/nuclei (M/N), cytosol (CYT) and plasma membrane (PM), as described in the Experimental section. These were immunoblotted for Rab10 and IRAP. The 1× load was 15 µg for Rab10 and 5 µg for IRAP. The average yields of each fraction per 10-cm plate of cells from four preparations were 260 µg of LDMs, 190 µg of HDMs, 1100 µg of M/N, 200 µg of CYT and 75 µg of PM. (B) Vesicles were adsorbed from the LDMs of basal (B) and insulin-treated (160 nM for 30 min) (I) cells with anti-GLUT4 (G) or control antibody (C) as described in the Experimental section. The starting LDMs and the isolated vesicles were immunoblotted for Rab10 and IRAP. For detectable.

### Figure 6  GTP on Rab10 in vivo, measured with $^{32}$P-labelled guanine nucleotides

3T3-L1 adipocytes were transfected with FLAG-tagged Rab10 or Rab10Q/L, labelled with $^{32}$P,Pi, treated with insulin (160 nM for 15 min) (I) or not (B) and lysed in non-ionic detergent; the Rab10 was isolated by immunoprecipitation and the relative contents of $^{32}$P labelled GTP and GDP were determined by TLC, as described in the Experimental section (left). The same procedure was used for HEK-293F cells that were co-transfected with the non-phosphorylatable AS160 4P mutant, the GAP-inactive AS160 R/K mutant, or the control empty vector (V) (right). Results are mean ± range for two independent experiments. WT, wild-type. The Authors Journal compilation © 2008 Biochemical Society.
presence of insulin. Ishikura et al. [21] examined the effect of towards the Rab(s) required for GLUT4 translocation in the stimulated GLUT4 translocation is inhibited by expression of different approach to assess the participation of each Rab. Insulin-in GLUT4 translocation in L6 myoblasts. Their study used a evidence that Rab8A and Rab14, but not Rab10, participate functioning of the residual protein.

be due either to a limited role for the protein or to the continued knockdown studies is that when a partial effect is found, it can larger effect may be (80% knockdown of Rab10 seen here compared with our earlier study the difference is that insulin stimulation of GLUT4 translocation requires a second signalling step in addition to elevation of the GTP form of Rab10. This conclusion is consistent with the finding that the knockdown of AS160, which would be expected to cause elevation of the GTP form of Rab10 and so have the same effect as expression of the Rab10Q/L, causes a 3-fold increase in cell-surface GLUT4 [4]. Nevertheless, because Rab10Q/L is not entirely fixed in the GTP form, the conclusion that a second signalling step is required can only be tentative.

A key prediction of the signalling pathway for GLUT4 translocation is that insulin treatment leads to elevation of the GTP form of Rab10 (see the Introduction section). In an effort to detect this predicted elevation, we employed two methods commonly used to detect the activation of small G-proteins in vitro. Neither method showed a detectable increase in the GTP form of Rab10. We think that the most likely explanation for this result is that elevation of the GTP form of Rab10 occurs, but is localized and so is limited, and hence not detected in the analysis of total cellular Rab10. A reasonable hypothesis is that GLUT4 translocation requires the elevation of the GTP form of the Rab10 located on GLUT4 vesicles. For example, in the yeast secretory system, elevation of the GTP form of a secretory vesicle Rab (Sec4) leads to tethering of the vesicle to the cell membrane via the exocyst complex, followed by its fusion with the membrane [22]. However, we cannot exclude the possibility that the site of Rab10 activation for GLUT4 translocation is the plasma membrane rather than the GLUT4 vesicles. An example in which Rab activation in the target membrane, rather than in the vesicle, leads to vesicle fusion is the fusion of ER-derived vesicles with the Golgi in yeast [23]. The results from subcellular fractionation and isolation of GLUT4 vesicles revealed that approx. 5% of the total cellular Rab10 is located in GLUT4 vesicles derived from the LDVs, which contain most of the insulin-responsive GLUT4 vesicles. Thus, if one assumes that Rab10 activation occurs in the GLUT4 vesicles, it could be limited to this small portion of the Rab10.

In the future, to establish definitively the role of Rab10 in GLUT4 translocation, it will be important to identify the effectors for the GTP form of Rab10, as well as the GEF (guanine-nucleotide-exchange factor) that catalyses the conversion of the GDP form of Rab10 into its GTP form, and to determine whether and how these proteins act in GLUT4 translocation. The protein

DISCUSSION

The present study extends our previous one, in which Rab8A and Rab10 were examined for a potential role in GLUT4 translocation [7], by further examination of these two Rabs as well as Rab8B and Rab14, the other two candidate Rabs. Only knockdown of Rab10 inhibited GLUT4 translocation; thus, among this group of Rabs, most likely only Rab10 is involved. It remains possible that the closely related Rabs 8A and 8B also participate, but have redundant function; in the future, it may be possible to examine this issue through the combined knockdown of both. The substantially larger inhibition of GLUT4 translocation with knockdown of Rab10 seen here compared with our earlier study (80% versus 30% inhibition) indicates that Rab10 plays an essential role in GLUT4 translocation. The larger effect may be due to greater knockdown in the present study. A limitation of all knockdown studies is that when a partial effect is found, it can be due either to a limited role for the protein or to the continued functioning of the residual protein.

In contrast with our results, Ishikura et al. [21] have presented evidence that Rab8A and Rab14, but not Rab10, participate in GLUT4 translocation in L6 myoblasts. Their study used a different approach to assess the participation of each Rab. Insulin-stimulated GLUT4 translocation is inhibited by expression of the non-phosphorylatable mutant of AS160, AS160 4P [2]. This inhibition most likely occurs because the AS160 4P cannot be phosphorylated by Akt and so continues to function as a GAP towards the Rab(s) required for GLUT4 translocation in the presence of insulin. Ishikura et al. [21] examined the effect of overexpressing wild-type and constitutively active (Q/L) forms of Rabs 8A, 10 and 14 on the inhibition of GLUT4 translocation by AS160 4P. Overexpression of any Rab downstream of AS160 4P would be expected to lessen the inhibition. They found that wild-type and constitutively active Rab8A and wild-type Rab14 largely prevented the inhibition by AS160 4P, whereas wild-type and constitutively active Rab10 did not. This surprising difference in results may be due to the difference between adipocytes and myoblasts. A potential complication with the approach in which either wild-type or constitutively active Rabs are overexpressed is that because of the overexpression the Rab may act in a way that it does not normally act at its endogenous concentration.

Our finding that Rab10Q/L is a substrate for both the GAP domain of AS160 in vitro and the full-length AS160 4P in vivo shows that the common assumption that this form of a Rab is fixed in the GTP form is not correct. It indicates that if the cellular concentration of a GAP in the region of its RabQ/L substrate is relatively high, the RabQ/L may be largely in the GDP form. Hence, the employment of a RabQ/L mutant to deduce the function of a Rab in its GTP form is not an entirely reliable approach.

We previously found that overexpression of Rab10Q/L in adipocytes causes an approx. 2-fold increase in the amount of GLUT4 at the cell surface, in contrast with the approx. 10-fold increase in response to insulin [7]. One plausible explanation for the difference is that insulin stimulation of GLUT4 translocation requires a second signalling step in addition to elevation of the GTP form of Rab10. This conclusion is consistent with the finding that the knockdown of AS160, which would be expected to cause elevation of the GTP form of Rab10 and so have the same effect as expression of the Rab10Q/L, causes a 3-fold increase in cell-surface GLUT4 [4]. Nevertheless, because Rab10Q/L is not entirely fixed in the GTP form, the conclusion that a second signalling step is required can only be tentative.

A key prediction of the signalling pathway for GLUT4 translocation is that insulin treatment leads to elevation of the GTP form of Rab10 (see the Introduction section). In an effort to detect this predicted elevation, we employed two methods commonly used to detect the activation of small G-proteins in vitro. Neither method showed a detectable increase in the GTP form of Rab10. We think that the most likely explanation for this result is that elevation of the GTP form of Rab10 occurs, but is localized and so is limited, and hence not detected in the analysis of total cellular Rab10. A reasonable hypothesis is that GLUT4 translocation requires the elevation of the GTP form of the Rab10 located on GLUT4 vesicles. For example, in the yeast secretory system, elevation of the GTP form of a secretory vesicle Rab (Sec4) leads to tethering of the vesicle to the cell membrane via the exocyst complex, followed by its fusion with the membrane [22]. However, we cannot exclude the possibility that the site of Rab10 activation for GLUT4 translocation is the plasma membrane rather than the GLUT4 vesicles. An example in which Rab activation in the target membrane, rather than in the vesicle, leads to vesicle fusion is the fusion of ER-derived vesicles with the Golgi in yeast [23]. The results from subcellular fractionation and isolation of GLUT4 vesicles revealed that approx. 5% of the total cellular Rab10 is located in GLUT4 vesicles derived from the LDVs, which contain most of the insulin-responsive GLUT4 vesicles. Thus, if one assumes that Rab10 activation occurs in the GLUT4 vesicles, it could be limited to this small portion of the Rab10.

In the future, to establish definitively the role of Rab10 in GLUT4 translocation, it will be important to identify the effectors for the GTP form of Rab10, as well as the GEF (guanine-nucleotide-exchange factor) that catalyses the conversion of the GDP form of Rab10 into its GTP form, and to determine whether and how these proteins act in GLUT4 translocation. The protein

© The Authors Journal compilation © 2008 Biochemical Society
Mss4, which is a weak exchange factor for some RabS, also interacts with nucleotide-free Rab10 [24]. In other systems, the GTP form of a Rab on a vesicle has been found to serve as a link to motor proteins attached to microtubules and microfilaments [5]. There is considerable evidence that GLUT4 vesicles move on microtubules, and suggestive evidence that they move on microfilaments [25]. Hence, one type of effector for Rab10 GTP may be a motor protein or a protein that links to a motor protein. In other systems, the GTP form of a Rab on a vesicle interacts with a complex of proteins to tether the vesicle to its target membrane in anticipation of fusion [5]. There is evidence indicating that GLUT4 vesicles tether to the plasma membrane via the mammalian exocyst complex [1]. Thus another type of effector protein for Rab10 GTP may be a component of the exocyst complex. In the yeast, the Rab Sec4 is found with the Sec15 subunit of the yeast exocyst [22]. Drosophila Sec15 has been found to interact with Drosophila RabS 3, 8, 1 and 27 [26]. It is interesting to note that among the 60 mammalian RabS, Rab10 is the one most similar to Rab8, and Rabs 8 and 10 are the ones most most similar to yeast Sec4. Consequently, a potential effector for Rab10 GTP is the Sec15 subunit of the mammalian exocyst.

We are indebted to Dr. Timothy McGraw at Weil Medical College of Cornell University for helpful discussions. We thank Dr. Takashi Itoh (Graduate School of Life Sciences, Tohoku University, Miyagi, Japan) for isolating the Mical L2 plasmid. This research was supported by grant number DK25336 from the NIH (National Institutes of Health).

REFERENCES