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Rab4, but Not the Transferrin Receptor, Is Colocalized with GLUT4 in an Insulin-Sensitive Intracellular Compartment in Rat Skeletal Muscle

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The role of Rab4, a small molecular weight GTP binding protein implicated in endosomal/plasma membrane (PM) recycling, in the translocation of the GLUT4 transporter in rat skeletal muscle was studied. Muscle membranes, prepared by subcellular fractionation of control and insulin treated rat skeletal muscle, were subjected to SDS/PAGE and immunoblot analyses. Insulin treatment caused an increase in GLUT4 in a plasma membrane (PM) enriched fraction from an intracellular membrane (IM) fraction. Immunoprecipitation of GLUT4 vesicles from the IM compartment revealed that Rab4 could be coprecipitated. Importantly, however, and unlike in adipocytes, immunoisolated GLUT4 vesicles from rat skeletal muscle contained no detectable immunoreactivity towards the transferrin receptor, suggesting that Rab4 was present in a GLUT4 IM pool that was not characteristic of early endosomes. The involvement of Rab4 in GLUT4 translocation was further supported by the finding that insulin treatment resulted in a significant (43%) reduction in Rab4 in the IM compartment. Our results suggest (i) that insulin induces the loss of both GLUT4 and Rab4 from the same IM compartment, (ii) that Rab4 may be involved in GLUT4 translocation based on its coprecipitation with the transporter from the insulin-sensitive pool and (iii) that Rab4 can be localized to intracellular membranes which appear not to be of early endosome origin.

It is now well accepted that in both fat and skeletal muscle the principal mechanism by which insulin stimulates glucose transport involves the mobilization, from an intracellular membrane (IM) store, of GLUT4 glucose transporters to the plasma membrane (PM) (1-4). The mechanism(s) by which GLUT4 is recruited to the PM in response to insulin treatment still remains poorly understood, but considerable interest has recently focused on the potential involvement of GTP-binding proteins in the insulin-induced translocation of GLUT4 (5,6). In particular, members of the Rab protein family, which are localized to distinct cellular organelles or membrane compartments, have been implicated in various aspects of vesicular trafficking, such as vesicle docking, uncoating or fusion, reviewed in (7). Of the many Rab proteins that have now been identified the Rab4 GTPase, which is thought to be localized to the early

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endosome compartment and which has been suggested to play a role in the early endosome/PM recycling pathway (7,8), is thought to be the most likely candidate protein involved in GLUT4 translocation. Evidence for this proposition has emerged from the finding that this protein is associated with GLUT4 in adipocytes where it may be involved in GLUT4 translocation to the PM (6). Uphues *et al* also recently reported the presence of small GTP binding proteins in GLUT4-containing vesicles isolated from rat hearts and proposed that they may be involved in GLUT4 translocation in cardiac muscle. However, unlike adipocytes, GLUT4-containing vesicles from rat heart contained no detectable Rab4 even when extremely sensitive bio-imaging methods were used (9). The precise reason for the apparent discrepancy in Rab4 distribution in adipocytes and cardiac muscle is unclear and whether this GTPase has a role to play in GLUT4 translocation in skeletal muscle remains unknown at present. The present study had two major objectives. First to assess whether, in rat skeletal muscle, Rab4 could be colocalized with the insulin-sensitive pool of GLUT4 and, if so, whether the IM pool of Rab4 was also acutely sensitive to insulin treatment. Second, based on the finding that in adipocytes the GLUT4 pool isolated from the light microsomes contains Rab4 and other early endosome proteins such as the transferrin receptor (6,10), to assess whether the insulin-sensitive IM pool of GLUT4 in muscle also represents an early endosome compartment.

Materials and Methods

Animals - Male Sprague Dawley rats (250g) were placed under terminal anaesthesia using a combination of Hipnorm (Fentanyl Citrate, 0.2 mg/kg; Fluanisone, 7 mg/kg i.p) and Hipnoval (a muscle relaxant, Midazolam Hydrochloride, 1.5 mg/kg i.p). Animals were separated into two groups. One group received a supramaximal intravenous dose of human insulin (1.5 units) prepared in 0.9% saline solution whilst the second group was given saline alone. Thirty minutes after administration of insulin or saline animals were killed and hindlimb muscles rapidly excised, frozen in liquid N_2 and subsequently stored at -80°C until required for study. Blood glucose was monitored in rats prior to treatment with insulin and at time of death. Insulin treatment resulted in a significant reduction in blood glucose from 5.1 ± 0.45 mM to 1.9 ± 0.47 mM (values are mean ± SEM n = 4 - 6 rats).

Subcellular fractionation of rat skeletal muscle - The procedure for isolating rat muscle membrane fractions enriched with plasma membranes (PM) and intracellular membranes endowed with the insulin sensitive pool of GLUT4 has previously been reported in detail (11-13). Briefly, the procedure involves the homogenisation of hindlimb muscle from control and insulin treated rats. The homogenate is then carried through a number of differential centrifugation steps to isolate a crude membrane fraction that is applied to a discontinuous sucrose gradient (25, 30 and 35% sucrose wt/wt). The gradient is then centrifuged at 190,000 *g* for 16 h. Extensive membrane marker analyses has shown that membranes isolated on top of the 25% sucrose fraction are largely of PM origin (14), whereas, those recovered on top of the 35% sucrose fraction are largely intracellular based on the finding that they contain a store of GLUT4 transporters which becomes depleted upon insulin treatment. Protein content in isolated membrane fractions was determined using the Bradford method (15). The intracellular

membranes isolated from the top of the 35% sucrose fraction were either used immediately for immunoprecipitation analyses or stored at - 80°C until required for further study.

Western-blot analyses - Muscle membrane fractions (10 μg) were subjected to SDS-PAGE on either 9% or 12% resolving polyacrylamide gels (16). Separated proteins were electrophoretically transferred onto PVDF (polyvinylidene difluoride) sheets, blocked and incubated overnight at 4°C with isoform-specific antibodies to either GLUT4 (1:500, East Acres Biologicals, Southbridge, MA, USA) or to anti-Rab4 (17)(1:300, the antisera was generously provided by Dr Bruno Goud (Curie Institute, Paris, France). Following primary antibody incubation, PVDF membranes were washed prior to incubation with 0.1 μCi/ml of ^{125}I-protein A. PVDF membranes were subsequently washed, air dried and autoradiography performed by exposure to XAR-5 Kodak film at - 80 °C. Autoradiographs were quantitated using a Molecular Dynamics laser scanner with Image Quant 3 software.

Immunoisolation of intracellular GLUT4-containing vesicles - Cyanogen-bromide activated Sepharose beads (90 μm diameter, Pharmacia, Sweden) were used for immunoadsorption studies. Beads were conjugated to Protein A as per manufacturers instructions. Beads were finally resuspended in PBS containing 0.4% BSA. Membranes isolated on top of the 35% sucrose band (representing the IM fraction enriched with insulin-sensitive pool of GLUT4 transporters) were used to immunoisolate GLUT4-containing vesicles. 100 μg of IM protein was treated with anti-GLUT4 (10 μl in PBS/0.4% BSA) with mixing by rotation. Immunoprecipitated GLUT4 vesicles were complexed by the addition of Protein A-Sepharose beads and pelleted by centrifugation at 12,000 *g* for 15 sec. The resulting supernatant was carefully removed and retained. The pelleted beads were washed four times by successive resuspension and recentrifugation in PBS. The supernatant from each step was pooled and combined with the original supernatant that had been retained and finally centrifuged at 190,000 *g* for 30 min. The pellet from this step represented the immune supernatant. The immune pellet and supernatant were resuspended in Laemmli buffer (16). Control samples were treated in an identical manner except that IM membranes were reacted with an irrelevant serum for the non-immune serum control. Immunblotting was performed with antisera specific for GLUT4, Rab4 and the transferrin receptor (Hybrid Tech, CA, at a dilution of 1:1000) as described above.

Results

Effects of insulin on the subcellular distribution of GLUT4 and intracellular Rab4 abundance.

We immunoblotted PM and IM fractions prepared from control and insulin treated skeletal muscle to assess whether insulin treatment resulted in changes in the subcellular distribution of GLUT4. Figure 1 shows that acute insulin treatment caused an increase in the immunoreactivity of the PM fraction which appeared to take place at the expense of GLUT4 transporters present in the IM fraction. Densitometric analyses of four muscle preparations

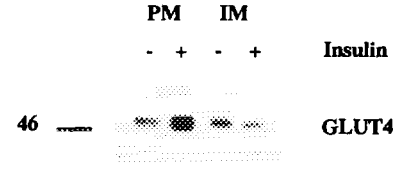

Figure 1. Western blot showing GLUT4 distribution in equal amounts of PM and IM (10 μg protein) prepared from control and insulin treated rat skeletal muscle.

revealed that the increase in GLUT4 in the PM was on average ~ 2-fold (not shown) When the total protein recoveries of each fraction were taken into account (PM, 28 + 2 μg/g muscle; IM, 148 + 22 μg/g muscle) the decrease in IM GLUT4 could fully account for the net increase in PM GLUT4 content. When crude, unfractionated, membranes were immunoblotted for GLUT4 insulin had no effect on GLUT4 abundance suggesting that the observed changes in PM and IM GLUT4 content were unlikely to have arisen as a result of changes in protein synthesis (data not shown).

The IM fraction donating GLUT4 to the PM was also immunoblotted using Rab4 antibodies. This GTP binding protein was detected as a 24 kDa protein band and its specific abundance in this fraction was reduced following acute insulin treatment (Figure 2a). Quantitative densitometry of four muscle preparations revealed that relative to the IM fraction isolated from non-insulin treated muscle insulin caused a fall in Rab4 content in the IM fraction by 43 + 6% (Figure 2b).

Co-localization of Rab4 with intracellular vesicles containing GLUT4 glucose transporters.

To test whether the observed insulin-induced decrease in GLUT4 and Rab4 content in the IM fraction were linked we screened immunoisolated GLUT4 vesicles from the IM

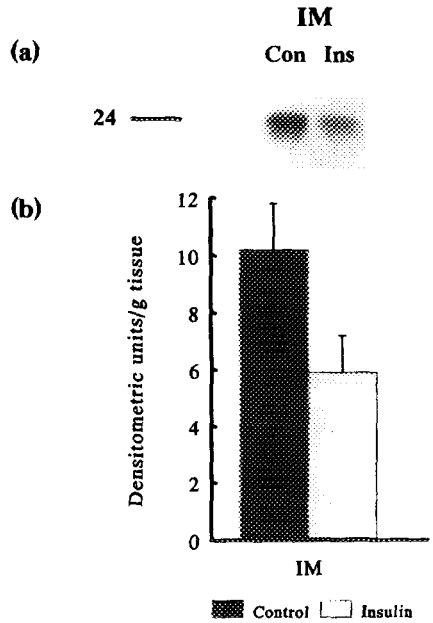

Figure 2.(a) Representative Western blot showing Rab4 abundance in the IM fraction isolated from control and insulin-treated rat skeletal muscle. **(b)** Densitometric scanning data showing the effects of insulin treatment on Rab4 abundance in the IM fraction. Bars represent Rab4 recovery in the IM fraction expressed in arbitary units/g of muscle. Data is mean + SEM from four separate muscle preparations.

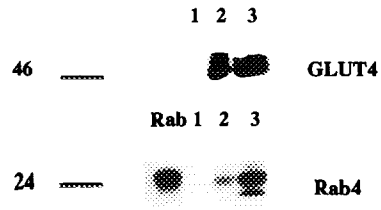

Figure 3. Immunoscreening of GLUT4 containing vesicles with Rab4 antibodies. Lane 1 represents the immune pellet obtained using an irrelevant nonimmune serum control. Lane 2 represents the immune pellet obtained using a GLUT4-specific antibody and Lane 3 is the resulting immune supernatant from the GLUT4 vesicle precipitation. Recombinant Rab4 protein (0.1 μg, Rab) was used as a positive Rab4 control.

compartment with antisera specific for Rab4. Figure 3 shows that immunoprecipitation of GLUT4 vesicles results in the coprecipitation of Rab4 suggesting that the latter may have a role to play in GLUT4 translocation to the PM. GLUT4 vesicles were also screened using an antibody to the transferrin receptor which is generally accepted as being a good marker of the recyclying endosomal/PM compartment. Figure 4 shows a quantitative scanning analyses of transferrin receptor (Tfr) immunoreactivity in GLUT4 immunoprecipitated vesicles and the immune supernatant. The figure shows that Tfr immunoreactivity was solely localized to the immune supernatant (IS), whereas, GLUT4 vesicles were devoid of Tfr.

Discussion

In an attempt to understand the molecular basis of the insulin-induced recruitment of GLUT4 glucose transporters there has been growing interest towards the analyses and identification of proteins associated with intracellular vesicles enriched with GLUT4 in fat and skeletal muscle (18,19). In particular small molecular weight GTP binding proteins belonging to the Rab family of GTPases, which belong to the larger superfamily of Ras-related proteins (6) have been implicated in the hormonal regulation of glucose transport. Recent studies in adipocytes have shown that Rab4 is associated with GLUT4 vesicles in an intracellular microsomal fraction and that insulin redistributes Rab4 from this fraction to the cytosolic compartment (6). No such information is available for skeletal muscle which, by virtue of its total body mass, is the principal site for glucose utilisation and disposal in the fed state and which also represents a primary insulin target tissue. Some evidence does exist showing that in skeletal muscle insulin causes the recruitment of proteins, capable of binding [^{32}P]GTP, from a light microsomal fraction to the PM in a manner analogous to that of GLUT4 translocation (5). However, in that particular study the identity of the GTP binding proteins was not assessed and, moreover, no definite

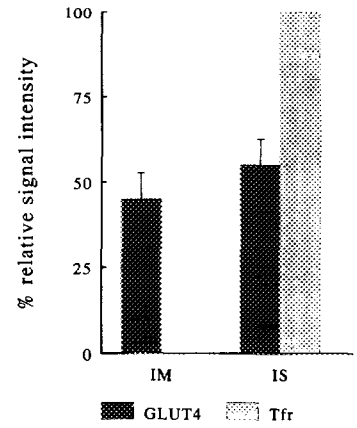

Figure 4. Densitometric scanning data showing the absence of the transferrin receptor from GLUT4 vesicles immunoisolated from the IM fraction. The figure shows that all the transferrin receptor reactivity is localized in the immune supernatant (IS). Data represents the mean (+ SD) observations from two separate immunoprecipitation experiments.

conclusion was reached as to whether the observed subcellular movement of GTP-binding proteins and GLUT4 were linked processes (5).

Our findings suggest that insulin induces the loss of Rab4 from the same subcellular fraction that donates GLUT4 to the PM. Moreover, given that Rab4 can be coprecipitated with GLUT4 raises the possibility that this GTPase may play a part in the mobilization of GLUT4 from the insulin-sensitive IM pool. These findings are in broad agreement with the observations of Cormont *et al* who have shown that Rab4 is colocalized with intracellular GLUT4 in adipocytes and that insulin causes Rab4 to redistribute from the 'light' microsomal fraction to the cytosol (6). However, the finding that GLUT4 vesicles do not possess Tfr would indicate, that unlike in adipocytes (10), the IM pool housing GLUT4 and Rab4 in skeletal muscle is unlikely to be of early endosome origin. Moreover, the presence of Rab4 in immunoisolated GLUT4 vesicles devoid of Tfr would signify that the expression of this GTP binding protein is not confined solely to the early endosome/PM recycling pathway as has generally been thought (7).

In contrast to what we have shown here, and the observations made in adipocytes (6), Rab4 does not appear to be a component of the insulin-sensitive GLUT4 pool present in rat heart. Interestingly, however, insulin was found to mediate the re-distribution of Rab4 to the heart PM from the cytosol rather than from an IM fraction leading to the suggestion that in cardiac muscle Rab4 may be involved in the endocytic/PM recycling of GLUT4 in the heart (9). Moreover, GLUT4 vesicles isolated from rat heart contained another, as yet, unidentified 24 kDa GTP binding protein whose abundance in these vesicles fell after insulin-treatment suggesting that in rat heart at least two GTP binding proteins may be involved in GLUT4 translocation. The reason

for the apparent discrepancy between our results and those of Uphues *et al* (1994) are presently unclear. However, it is not unreasonable to assume that the disparity may, in part, be due to a tissue-specific effect originating from variations in the protein composition of GLUT4-containing vesicles isolated from heart and skeletal muscle. This latter proposition is supported by the finding that the GLUT1 glucose transporter does not colocalize with intracellular GLUT4 in rat skeletal muscle but that it has been shown to do so in rat cardiac muscle (20). Since GLUT1 has been implicated in playing an important role in the maintenance of basal glucose uptake in the heart (20) and the observation that it is, to some extent, stored intracellularly with GLUT4 may indicate that signals other than insulin could also activate their recruitment to the PM by mechanisms distinct from those operating in skeletal muscle and fat.

Exactly how Rab4 may facilitate GLUT4 translocation in fat and skeletal muscle remains poorly understood at present but it is thought directionality in vesicular transport may, in part, be provided by GTP hydrolysis. However, the initial event triggering the movement of GLUT4/Rab4 from the IM compartment may involve phosphorylation of Rab4 since of the known family of Rab proteins, Rab1 and Rab4 are known to contain within their carboxy-terminal region consensus phosphorylation sites for $p34^{cdc2}$ kinase (which plays an integral role in cell cycle events)(8,21) and the insulin-activated extracellular-signal regulated kinase (ERK1) (22). Both kinases are thought to phosphorylate the same Ser196 residue (22) and in and doing may initiate the cycle of events that lead to hydrolysis of GTP and disassociation of Rab4 from membranous structures. Whether defects in muscle Rab4 expression or phosphorylation affect the magnitude of GLUT4 translocation, and whether Rab4 facilitates GLUT4 delivery directly to the PM or to an endosomal/PM recycling compartment is unknown at present, but such information would clearly be of value in helping to understand the physiological and pathological regulation of glucose transport in insulin-sensitive tissues.

Acknowledgments

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