Insulin-induced translocation of the glucose transporter GLUT4 in cardiac muscle: studies on the role of small-molecular-mass GTP-binding proteins

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Subcellular fractions obtained from rat cardiac ventricular tissue were used to elucidate a possible functional relationship between small-molecular-mass G-proteins and the insulin-responsive glucose transporter GLUT4. Proteins were separated by SDS/PAGE and transferred to nitrocellulose membranes. Incubation with [α-^{32}P]GTP revealed the presence of two major distinct GTP-binding protein bands of 24 and 26 kDa in both plasma and microsomal membranes. Immunoadsorption of microsomal membranes to anti-GLUT4 antibodies was used to isolate GLUT4-enriched membrane vesicles. This material was found to contain a much decreased amount of small G-proteins, with the exclusive presence of the 24 kDa species. Insulin treatment *in vivo* had no effect on the microsomal membrane content of small GTP-binding proteins, but significantly decreased the 24 kDa species in GLUT4-enriched vesicles by $36 \pm 5\%$ ($n = 3$). This correlated with a decreased (30–40%) recovery of GLUT4-enriched vesicles from insulin-treated animals. Western-blot analysis of microsomal membranes with a panel of antisera against rab GTP-binding proteins indicated the presence of rab4A, with a molecular mass of 24 kDa, whereas rab1A, rab2 and rab6 were not observed. rab4A was barely detectable in GLUT4-enriched vesicles; however, insulin produced an extensive shift of rab4A from the cytosol and the microsomal fraction to the plasma membrane with a parallel increase in GLUT4. These data show that a small GTP-binding protein is co-localized with GLUT4 in an insulin-responsive intracellular compartment, and strongly suggest that this protein is involved in the exocytosis of GLUT4 in cardiac muscle. Furthermore, the observed translocation of rab4A is compatible with insulin-induced endosome recycling processes, possibly including the glucose transporters.

INTRODUCTION

One of the most prominent actions of insulin on muscle and adipose tissue consists of a rapid and large stimulation of glucose uptake (for reviews, see [1,2]). A major mechanism mediating this acute hormonal effect is known to be translocation of glucose-transporter proteins from an intracellular storage site to the plasma membrane [3–5]. Extensive investigations have established that muscle and fat express two isoforms of glucose transporters, named GLUT1 and GLUT4 (for reviews, see [6,7]). GLUT1 is more abundant in the plasma membrane, but can be translocated in response to insulin, thereby mediating a certain amount of hormonal action [8]. On the other hand, immunocytochemical and biochemical evidence obtained with adipose tissue [9] and both cardiac [10] and skeletal muscle [11] indicates that in the basal state GLUT4 is almost completely sequestered in intracellular tubulo-vesicular structures found in the *trans*-Golgi region. When stimulated with insulin, GLUT4 is shifted to the plasma membrane, and may also enter a recycling pathway [12]. Thus, exocytosis of GLUT4 is thought to represent the major site of insulin action [9,12,13]. Unfortunately, the components mediating the specificity and directionality of this event and the mechanisms of insulin signalling to the translocation machinery remain unknown.

In mammalian cells a family of ras-like rab GTP-binding proteins has been implicated in the control of cellular traffic (for reviews, see [14,15]). Members of the rab family have been localized to distinct intracellular compartments, suggesting specialized transport functions of the different rab proteins. Thus, rab1 has been found in the endoplasmic reticulum and Golgi apparatus [16], rab2 has been detected in the *cis*-Golgi region [17], rab6 is associated with medial and *trans*-Golgi cisternae [18], as well as with the trans-Golgi network [19], and rab4A is associated with early endosomes and recycling vesicles [20]. By using the expression of mutant rab proteins and specific antibodies, direct evidence for the control of endocytic [21,22] and secretory pathways [16] by rab proteins has been obtained.

Several lines of evidence suggest that small G-proteins, possibly rab proteins, could also be involved in the exocytic translocation of GLUT4. Thus, Baldini et al. [23] reported that guanosine 5′-[γ-thio]triphosphate (GTP[S]) stimulates the translocation of GLUT4 in permeabilized adipocytes. The same group observed an increased expression of a new rab3 isotype in differentiating 3T3-L1 cells, temporally coincident with the expression of GLUT4 [24]. Furthermore, several small G-proteins in the 21–27 kDa range could be identified as components of GLUT4-containing vesicles isolated from adipocyte low-density microsomal fractions [25]. However, the significance of these proteins for GLUT4 translocation remains unclear. In addition, Schürmann et al. [26] reported that in adipocytes the intracellular GTP-binding proteins are not tightly associated with the vesicles containing the insulin-responsive glucose transporter.

In the present investigation we have addressed the question of a possible functional relationship between small G-proteins and GLUT4 in rat cardiac muscle, using two different approaches. First, attempts have been made to demonstrate a co-localization of small G-proteins and GLUT4 in the intracellular insulin-sensitive glucose-transporter compartment. Second, we studied the subcellular distribution of rab GTP-binding proteins under basal and insulin-stimulated conditions. The data suggest that a

Abbreviation used: GTP[S], guanosine 5′-[γ-thio]triphosphate.

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24 kDa G-protein represents one component of the GLUT4 translocation machinery. Further, rab4A could function as an additional component of this and other insulin-dependent cellular trafficking events.

MATERIALS AND METHODS

Chemicals

[α-^{32}P]GTP (3000 Ci/mmol) was purchased from New England Nuclear (Dreieich, Germany). ^{125}I-labelled Protein A (30 mCi/mg) was from Amersham (Braunschweig, Germany). Reagents for SDS/PAGE were supplied by Pharmacia and Sigma (München, Germany). DTT and GTP[S] were from Sigma. Insulin (Actrapid HM; 100 units/ml) was supplied by Novo (Mainz, Germany). Immunobead goat anti-rabbit immunoglobulin was purchased from Bio-Rad (München, Germany). The polyclonal anti-GLUT4 antiserum was a product of Calbiochem (Bad Soden, Germany). All other chemicals were of the highest grade commercially available. Polyclonal rabbit antisera were generated against recombinant rab1A, rab2 and rab6 as detailed previously [27]. Rabbit antiserum against recombinant human rab4A was generated by immunization with baculovirus-expressed purified protein (M. McCaffrey and B. Goud, unpublished work).

Membrane preparation and characterization

Lean male Zucker rats fed *ad libitum* and weighing 280–320 g were used in all experiments. Plasma and microsomal membranes from cardiac ventricle were prepared as recently described by us [28]. Briefly, ventricular tissue was removed and homogenized in a buffer containing 10 mM Tris/HCl, 0.1 mM phenylmethanesulphonyl fluoride and 2.6 mM dithiothreitol by using an Ultra-Turrax (Ika, Neu-Isenburg, Germany) for 60 s. Homogenization was continued by 10 strokes in a glass–Teflon homogenizer, followed by 3×3 strokes in a tight-fitting Potter–Elvehjem homogenizer. After centrifugation at 3000 *g* for 10 min, the supernatant was centrifuged at 200000 *g* for 90 min to pellet the crude membrane fraction and to obtain the cytosol (supernatant). Further purification was achieved by applying this fraction to a discontinuous gradient consisting of 0.57, 0.72, 1.07 and 1.43 M sucrose in the above buffer and centrifugation at 40000 *g* for 16 h. Membranes were harvested from each sucrose layer and stored at −70 °C. Protein was determined by a modification of the Bio-Rad protein assay with BSA as a standard. Ouabain-sensitive Na^+/K^+-ATPase was used as the sarcolemma marker enzyme and assayed as described [28]. Ca^{2+}/K^+-ATPase was used as the microsomal-membrane marker enzyme and was determined as described by Hidalgo et al. [29]. Membranes recovered from the 0.72 M sucrose layer were enriched 5-fold in the activity of the Na^+/K^+-ATPase and considered as a plasma-membrane fraction, whereas membranes obtained in the 1.07 M sucrose layer showed a 0.5-fold activity of this marker enzyme, compared with the homogenate. Furthermore, Ca^{2+}/K^+-ATPase increased 4-fold in this fraction. The 1.07 M sucrose membrane fraction was hence termed microsomal membranes. For the insulin-stimulation studies, rats received a tail-vein injection of regular insulin (4 units/100 g body wt.) and hearts were removed 20 min later.

Immunoadsorption of GLUT4-containing vesicles

Immunobeads with goat anti-rabbit immunoglobulin were used for adsorption of GLUT4-containing membrane vesicles. Microsomal membranes (0.5–1 μg/μl) were incubated for 14 h with immunobeads at 4 °C in PBS, pH 7.4, containing phenylmethanesulphonyl fluoride (0.1 mM), dithiothreitol (2.6 mM), EDTA (1 mM) and BSA (0.4%) in a final volume of 1 ml. After centrifugation, 3 μl of anti-GLUT4 antiserum was added to the supernatant, and the membranes were sonicated for 15 s. Control membranes were treated identically, except that the antiserum was not added. After a 2 h incubation at 4 °C the membranes were pelleted, resuspended and incubated for an additional 2 h with immunobeads. After centrifugation, the beads were washed three times with PBS, and the vesicle proteins were eluted with Laemmli sample buffer [30].

Labelling of small GTP-binding proteins

Protein samples were subjected to one-dimensional SDS/PAGE using 12% horizontal gels. Two-dimensional PAGE was performed as described by O'Farrell [31] with modifications. Isoelectric focusing was performed on Immobiline DryStrips (Pharmacia, Germany), which exhibit an immobilized pH gradient of 3.0–10.5. Electrophoresis was carried out for 8 h at 300 V and for 10 h at 2 kV. After focusing was complete, strips were applied to horizontal SDS/PAGE as outlined above. Proteins were transferred to nitrocellulose filters in a semi-dry blotting apparatus, and GTP binding to proteins was determined as previously described [32]. Blots were washed for 10 min in buffer containing 50 mM Tris/HCl, pH 7.5, 0.3% Tween 20 and 2 μM $MgCl_2$. Incubation was then continued for 30 min at room temperature in the same buffer in the presence of [α-^{32}P]GTP (1 μCi/ml), 1 nM GTP and 10 μM ATP. After several washing steps in the same buffer without [α-^{32}P]GTP, blots were air-dried and subjected to autoradiography by using Hyperfilm-MP and intensifying screens. In addition, blots were detected on a FUJIX BAS 1000 bio-imaging analyser (Fuji, Tokyo, Japan). Quantification was performed on a SPARCstation (Sun Microsystems, U.S.A.) by using image-analysis software. Significance of reported differences was evaluated by using the null hypothesis and *t*-statistics for unpaired data.

Immunoblotting

Protein samples were separated by SDS/PAGE using 10% horizontal gels and transferred to nitrocellulose filters as described above. Filters were blocked for 90 min in PBS, pH 7.4, containing 0.05% Tween and 10% milk powder. Thereafter, filters were incubated for 16 h at 4 °C with a 1:500 dilution of the anti-GLUT4 antiserum or the different anti-rab antisera, respectively. After extensive washing with PBS containing 0.05% Tween, filters were incubated for 2 h with ^{125}I-Protein A (0.3 μCi/ml). Filters were again extensively washed, air-dried and exposed to Hyperfilm-MP films by using intensifying screens. Autoradiograms were analysed by using laser scanning densitometry. Additionally, blots were detected on a FUJIX BAS 1000 bio-imaging analyser and quantified as outlined above.

RESULTS

Subcellular distribution of small G-proteins

In order to characterize the pattern of cardiac small G-proteins, plasma and microsomal membranes were resolved in SDS/PAGE, transferred to nitrocellulose and labelled with [α-^{32}P]-GTP. As illustrated in Figure 1a, two major GTP-binding proteins, with molecular masses of 24 and 26 kDa, could be detected in microsomal membranes. On over-exposure of the autoradiograms, a third species of 29 kDa became visible (not shown in Figure 1). These small G-proteins are enriched in the

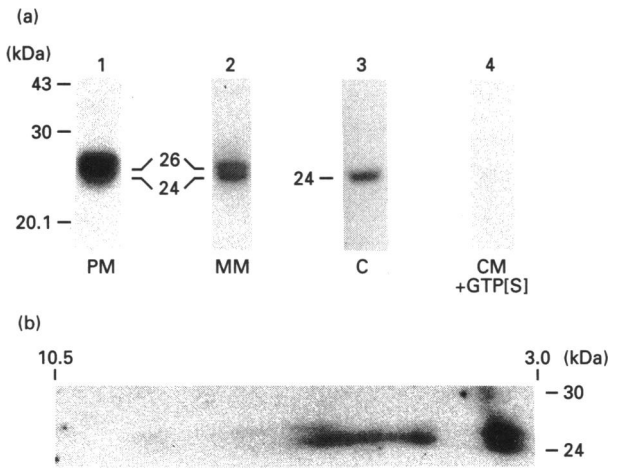

Figure 1 Labelling of small GTP-binding proteins with [α-^{32}P]GTP in subcellular fractions from cardiac muscle

(**a**) Portions (20 μg) of plasma membranes (PM) and microsomal membranes (MM), and 30 μg portions of cytosol (C) and crude membranes (CM), were electrophoresed and transferred to a nitrocellulose sheet. Each nitrocellulose strip was then incubated with [α-^{32}P]GTP (1 μCi/ml) in the absence or presence (lane 4) of 10 μM GTP[S]. Blots were then processed for autoradiography as described in the Materials and methods section. Exposure time was 60 h without intensifying screens (lanes 1 and 2), or 24 h with screens (lanes 3 and 4). (**b**) Microsomal membranes (120 μg) were subjected to isoelectric focusing followed by SDS/PAGE as detailed in the Materials and methods section. Protein was applied to the low-pH end of the gel. After blotting, the nitrocellulose sheet was incubated with [α-^{32}P]GTP, washed and autoradiographed. The numbers on the right side of the gel represent molecular-mass markers (kDa); the numbers above the gel represent the pH.

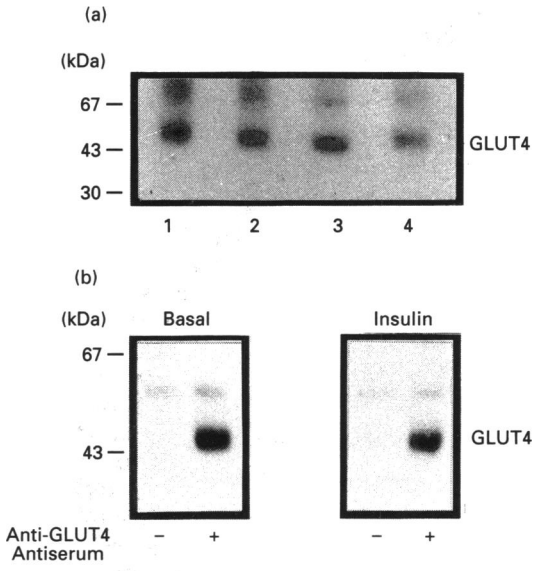

Figure 2 Immunoadsorption of GLUT4 from cardiac microsomal membranes

(**a**) Microsomal membranes (0.5–1 mg) were resuspended in PBS in a final volume of 1 ml (lane 1) and incubated with immunobeads as described in the Materials and methods section. The supernatant (lane 2) was then incubated in the absence (lane 3) or presence (lane 4) of a polyclonal anti-GLUT4 antiserum, followed by addition of immunoglobulin-coated immunobeads and centrifugation. Samples of the starting membrane suspension (lane 1) and the supernatants (lanes 2, 3 and 4) were subjected to SDS/PAGE and immunoblotted with antiserum against GLUT4. The nitrocellulose sheets were incubated with ^{125}I-Protein A and submitted to autoradiography. (**b**) GLUT4-containing vesicles were immunoprecipitated from cardiac microsomal membranes of basal and insulin-stimulated rats, as described above. Vesicle proteins were eluted, analysed by SDS/PAGE on a 10% polyacrylamide gel and immunoblotted with an anti-GLUT4 antiserum. Autoradiograms were analysed by laser scanning densitometry.

plasma membrane and are much less abundant in the cytosolic fraction, requiring larger amounts of protein and intensifying screens for detection (Figure 1a). The specificity of [α-^{32}P]GTP binding was demonstrated by addition of GTP[S] (10 μM), which completely inhibited the binding reaction. It is noteworthy that an identical pattern of small G-proteins was also observed in subcellular fractions obtained from adult ventricular cardiomyocytes (I. Uphues and J. Eckel, unpublished work). Two-dimensional electrophoresis of microsomal membranes showed that at least the 24 kDa G-protein could be resolved into several spots, indicating the presence of multiple 24 kDa G-protein species in cardiac muscle (Figure 1b).

Co-localization of small G-proteins with GLUT4

Intracellular GLUT4 is known to be present in a unique population of membrane vesicles [9–11], which are thought to contain the proteins responsible for GLUT4 translocation [33]. In order to assess co-localization of GLUT4 and the small G-proteins described above, microsomal membranes were immunoadsorbed to a polyclonal anti-GLUT4 antibody directed against the C-terminal portion of the glucose transporter. Pre-adsorption of the membranes to immunobeads slightly (10%) decreased the amount of GLUT4 in the microsomal-membranes fraction (Figure 2a, lane 2 versus lane 1), but was included since it greatly decreased the amount of non-specific adsorption in the final immunoprecipitate. Quantification of GLUT4 in the supernatant of control incubations and immunoprecipitates indicated that about 30% of the GLUT4-containing vesicles were removed by this procedure (Figure 2a, lanes 3 and 4). These results were obtained by using saturating concentrations of both primary and secondary antibodies. In order to verify that the immunoprecipitated material is representative of the intracellular compartment of GLUT4, microsomal membranes were prepared from hearts of basal and insulin-stimulated rats and subjected to the immunoadsorption protocol. As illustrated in Figure 2(b), with insulin the level of GLUT4 in the immunopellets decreased by 30–40%. This agrees with the effect of insulin on the GLUT4 pool in cardiac microsomal membranes [28], and confirms that our preparation of GLUT4-containing vesicles is derived from the intracellular insulin-sensitive compartment of GLUT4.

When GLUT4-containing membrane vesicles were subjected to ligand blotting with [α-^{32}P]GTP, a significant and specific labelling of a 24 kDa small G-protein could be detected (Figure 3a). The data further show that the major amount of microsomal small G-proteins remains in the non-adsorbed supernatant (about 95%). Taking into account that this is paralleled by the adsorption of 30% of GLUT4, it can be estimated that 10–15% of the small G-proteins present in the microsomal-membranes fraction are co-localized with GLUT4. A comparison of GLUT4-containing vesicles from basal and insulin-stimulated hearts showed that the hormone significantly ($P = 0.022$) decreased the 24 kDa species by $36 \pm 5\%$ ($n = 3$) (Figure 3b). This insulin-sensitivity may reflect the decreased recovery of GLUT4-containing vesicles from insulin-treated animals (Figure 2b). The data show, however, that the 24 kDa G-protein co-localizes with the insulin-responsive intracellular compartment of GLUT4. Insulin is unable to alter the distribution of small G-proteins in the whole microsomal-membranes fraction (Figure 3b), indicating that the selectivity of insulin action most probably involves a specific subset of small G-proteins.

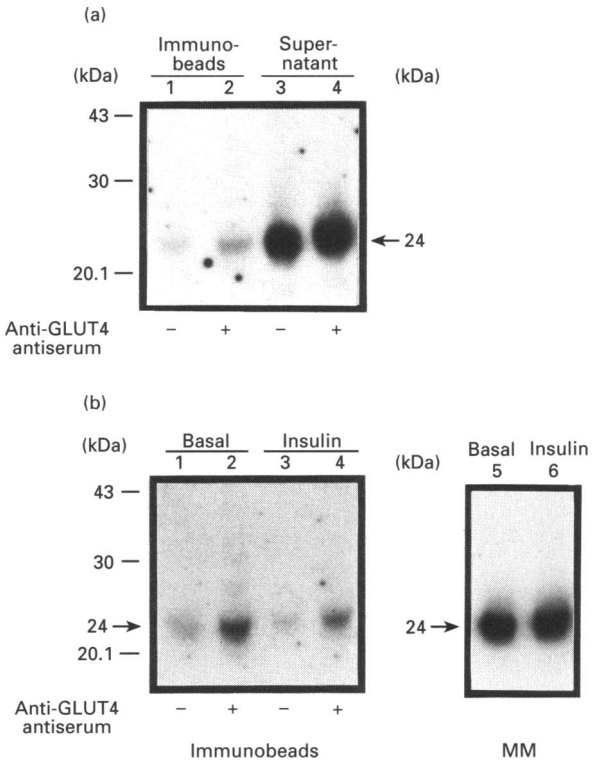

Figure 3 Co-localization of small GTP-binding proteins with GLUT4

(**a**) Microsomal membranes (0.5–1 $\mu g/\mu l$) were pre-adsorbed to immunobeads, followed by incubation in the absence or presence of anti-GLUT4 antiserum. Immunoprecipitation was then performed by addition of immunoglobulin-coated immunobeads, as detailed in Figure 2. Samples of the eluted vesicle proteins (lanes 1 and 2) and the supernatant (lanes 3 and 4) were analysed by SDS/PAGE on a 12% polyacrylamide gel and subjected to ligand blotting with [α-^{32}P]GTP, as outlined in Figure 1. (**b**) GLUT4-containing vesicles (lanes 1–4) and microsomal membranes (MM; lanes 5 and 6) were isolated from cardiac ventricular tissue of basal and insulin-stimulated rats. Electrophoresis and ligand blotting using [α-^{32}P]GTP were carried out as described above. Quantification was performed with a bio-imaging analyser.

Subcellular distribution of rab GTP-binding proteins

In light of the co-localization of a 24 kDa G-protein with GLUT4 and the importance of rab proteins for cellular traffic [14,15], we performed an analysis of the expression of rab proteins in cardiac microsomal membranes, using antisera against recombinant rab1A, rab2, rab4A and rab6. The specificity of these antisera has been reported previously [27]. As illustrated in Figure 4, only rab4A, with a molecular mass of 24 kDa, could be detected in the microsomal-membranes fraction. Antisera against rab1A, rab2 and rab6, which are highly reactive against the recombinant proteins, failed to recognize these proteins in cardiac microsomes (Figure 4).

We then hypothesized that rab4A could be the small G-protein found to be co-localized with GLUT4. This assumption was evaluated by immunoblotting GLUT4-containing vesicles and microsomal membranes with anti-rab4A antiserum. As shown in Figure 5, near-equal amounts of GLUT4 were applied to the gel, to enable an estimation of the sensitivity of rab4A detection. The data show that under these conditions rab4A was barely detectable in GLUT4-containing vesicles, despite being clearly visible in the microsomal-membranes fraction. It should be noted that this result was obtained with the extremely sensitive bio-imaging system, which quantified a very faint rab4A signal in GLUT4-containing vesicles (less than 2% of microsomal rab4A).

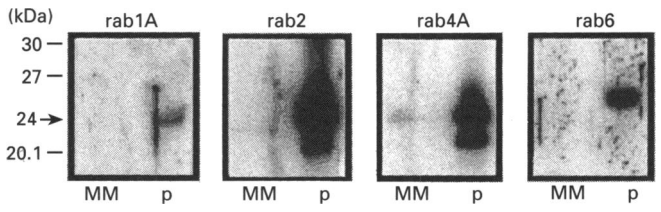

Figure 4 Immunoblot analysis of rab proteins in cardiac microsomal membranes

Microsomal membranes (MM; 30 μg) and recombinant rab1A, rab2, rab4A and rab6 (50 ng) (p) were analysed by SDS/PAGE on a 10% polyacrylamide gel, transferred to nitrocellulose and immunoblotted with specific antisera against rab1A, rab2, rab4A and rab6. After incubation with ^{125}I-Protein A, the bands were observed with a bio-imaging analyser.

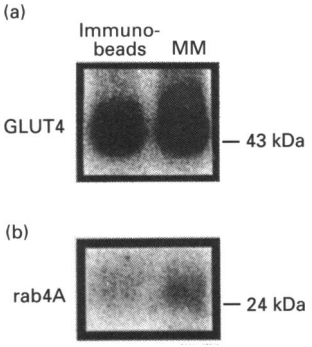

Figure 5 Immunodetection of GLUT4 and rab4A in GLUT4-containing vesicles and microsomal membranes

GLUT4-containing vesicles and microsomal membranes were obtained from cardiac ventricular tissue of control rats. A portion of eluted vesicle proteins (lane 1) and 15 μg of microsomal membranes (lane 2) were separated by SDS/PAGE, transferred to nitrocellulose and immunodetected with antisera against GLUT4 (**a**) and rab4A (**b**). Bands were detected by using ^{125}I-Protein A and a bio-imaging analyser.

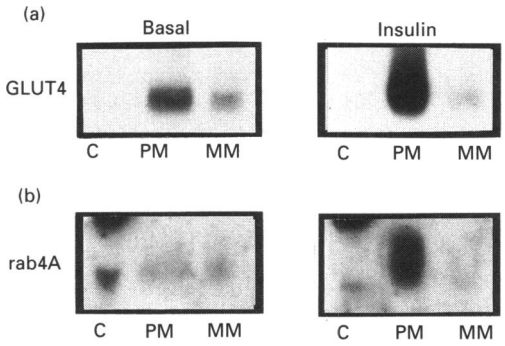

Figure 6 Insulin-induced translocation of GLUT4 and rab4A

Subcellular fractions were prepared from cardiac ventricle of basal and insulin-stimulated rats. Portions (30 μg) of cytosol (C), plasma membranes (PM) and microsomal membranes (MM) were analysed by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with antisera against GLUT4 (**a**) and rab4A (**b**). After incubation with ^{125}I-Protein A, the nitrocellulose sheets were subjected to autoradiography.

On the other hand, rab4A could not be detected in GLUT4-containing vesicles by using conventional autoradiography. This indicates that rab4A, if present at all, does not represent a major component of GLUT4-containing vesicles and that the 24 kDa

species found to be co-localized with GLUT4 must be different from rab4A.

Although not present in GLUT4-containing vesicles in substantial amounts, rab4A might redistribute upon insulin stimulation and possibly mediate additional steps of GLUT4 exocytosis. We therefore determined the translocation of GLUT4 after stimulation *in vivo* with insulin, and analysed a putative simultaneous translocation of rab4A (Figure 6). With insulin the GLUT4 content in the plasma-membranes fraction increased about 4-fold, with a parallel 30–40 % decrease in the microsomal-membranes fraction. No GLUT4 could be detected in the cytosol in either the basal or the insulin-stimulated state (Figure 6). On the other hand, in the basal state rab4A was most abundant in the cytosol. Administration of insulin induced an extensive shift of rab4A to the plasma membrane from the cytosol and the microsomal fraction (Figure 6). Thus, under identical experimental conditions both GLUT4 and rab4A are translocated to the plasma membrane in a co-ordinated and similar fashion.

DISCUSSION

The results of the present investigation indicate the existence of three small G-proteins (24, 26 and 29 kDa) in cardiac tissue, which could be identified by the binding of [α-^{32}P]GTP to subcellular fractions resolved in SDS/PAGE and transferred to nitrocellulose membranes. As discussed by Bhullar [34], this method preferentially detects the protein products of genes related to rab and ral, whereas other small G-proteins exhibit a very low affinity towards GTP under these assay conditions. Cardiac small G-proteins were found to be enriched in plasma membranes, and were much less abundant in the soluble cytoplasmic fraction. Our data are in complete agreement with a recent study by Doucet et al. [35] on rat cardiac ventricular tissue, confirming this pattern of small G-proteins in the heart.

A putative function of certain cardiac small G-proteins could be related to the control of cellular traffic, as shown in a variety of other tissues [14–16,21,22]. In the heart, the exocytic translocation of the insulin-responsive glucose transporter GLUT4 is of major importance for glucose metabolism, since this rate-limiting step is controlled by both insulin [10,28,36] and contraction of the cardiomyocyte [28]. Intracellular GLUT4 is located in a specific population of membrane vesicles [9–11], and this compartment should also contain the proteins participating in vesicular trafficking. As shown in the present study, at least 30 % of GLUT4-containing vesicles could be removed from cardiac microsomes by an immunoadsorption technique. In previous studies an efficiency of 30–100 % was reported for adipocytes [25,33,37,38] and skeletal muscle [11], most probably depending on the type of tissue and the degree of homogenization [11]. Recently, Kraegen et al. [36] showed that in cardiac tissue about 50 % of GLUT4-containing vesicles could be immunoisolated, in good agreement with our observations. Most importantly, we demonstrate here the insulin-sensitivity of the GLUT4-containing vesicles (Figure 2), confirming that this material is representative of the hormone-responsive intracellular compartment of GLUT4.

A major finding of the present investigation consists of the observation that a specific amount of the 24 kDa small G-proteins co-purifies with the GLUT4-containing vesicles, amounting to about 10–15 % of total small G-proteins present in the microsomal fraction. The absence of the 26 kDa species from GLUT4-containing vesicles indicates that this is not a general interaction between microsomal components; instead, it points to a selective, most probably functionally related, co-localization of GLUT4 and the 24 kDa G-protein. This assumption is strongly supported by our additional observation showing that in GLUT4-containing vesicles from insulin-treated animals this G-protein decreases to the same extent (30–40 %) as the GLUT4 protein itself. Thus, in response to insulin both GLUT4 and the 24 kDa G-protein are removed from the intracellular glucose transporter compartment in a co-ordinated fashion. Given the functional significance of small G-proteins for mediating exocytic cellular traffic [15,16], we conclude that the 24 kDa G-protein could be involved in the translocation of cardiac GLUT4.

Concerning this relationship, controversial findings have been obtained in rat adipocytes, most probably due to methodological differences [25,26]. Using sucrose-gradient fractionation, Schürmann et al. [26] were unable to detect a specific association of GLUT4-containing vesicles and small G-proteins. On the other hand, Cormont et al. [25] described the co-localization of several small G-proteins with GLUT4, using the immunoadsorption approach. However, a large amount of non-specific binding precluded the investigation of insulin-sensitivity of these proteins. In the present study this problem was eliminated by pre-adsorption of the microsomal membranes, leading to the detection of the 24 kDa insulin-sensitive G-protein in cardiac GLUT4-containing vesicles. Our data obtained with cardiac muscle support the original hypothesis by Baldini et al. [23] of a G-protein-mediated recruitment of GLUT4 and identify such a G-protein as a component of the intracellular GLUT4 compartment. Additional components of GLUT4 vesicles have recently been identified in rat adipocytes: these include the vesicle-associated membrane proteins (VAMPs) of molecular mass 17–18 kDa [39] and the secretory carrier membrane proteins (SCAMPs, 35–40 kDa) [40]. The latter proteins are present in a wide variety of cells and are considered as markers of a general recycling system [40]. The precise function of VAMPs and SCAMPs for GLUT4 translocation is at present unclear; it has been suggested, however, that they mediate the trafficking process itself [39,40]. In this scenario the role of a small G-protein could be the control of GLUT4 translocation, possibly including the initiation of vesicle transport and the targeting and fusion with the plasma membrane [15]. Additional work will be needed to dissect the different steps of GLUT4 translocation and to elucidate the specific function of the 24 kDa G-protein in this process.

In order to identify this G-protein as a member of the rab family of GTP-binding proteins, we used antisera against rab1A, rab2, rab4A and rab6, which, due to their cellular localization [16–20], could be involved in the translocation of GLUT4. In microsomal membranes only rab4A was found to be present in significant amounts. However, we failed to detect rab4A in our GLUT4 membrane-vesicle preparation, using conventional autoradiography. Results obtained with the highly sensitive bioimaging technique suggest that at least very small amounts of rab4A could be present in GLUT4-containing vesicles. We therefore conclude that the 24 kDa GLUT4-vesicle G-protein, which can easily be detected by ligand blotting, must be different from rab4A. It may be speculated that it is related to rab3D, which is thought to mediate the insulin-dependent exocytosis of GLUT4 in adipocytes and is also expressed in muscle and heart [24]. Alternatively, the 24 kDa G-protein could be another member of the large and growing family of rab GTP-binding proteins, or be even different from that [15]. In contrast with our findings, in a recent report Cormont et al. [41] showed that a substantial amount of rab4A co-localizes with GLUT4-containing vesicles immunoprecipitated from unstimulated rat adipocytes. The reason for this discrepancy is at present unclear. However, earlier observations [9,20] concerning the subcellular

distribution of rab4A and GLUT4 argue against a co-localization of these proteins in the intracellular glucose-transporter compartment. Thus, it has been demonstrated that at least 70% of rab4A is associated with early endosomes and endocytic vesicles [20]. On the other hand, in the absence of insulin early endosomes contain less than 2% of GLUT4, as shown by immunocytochemical techniques [9]. Therefore, immunoadsorption of GLUT4 from intracellular membranes should co-adsorb only minor amounts of rab4A, in agreement with the findings of the present investigation. Thus, at least in the heart the intracellular localization of rab4A appears to be distinct from the pool of the insulin-responsive glucose transporter GLUT4.

The second key finding of this study is related to the observed redistribution of rab4A in response to insulin. Specifically, rab4A was found to accumulate in the plasma membrane, with a major decrease in the cytosol. The implications of this rab4A translocation for the function of the cardiomyocyte are at present unknown. In other cellular systems, rab4A was localized to transferrin-receptor-containing early endosomes [20], and was therefore suggested to control the pathway of receptor recycling. Most interestingly, rab4A can be phosphorylated on serine, resulting in a translocation of rab4A into the cytosol [42]. It has been hypothesized that in mitotic cells this is responsible for arrest of endocytic processes [20]. We show here that insulin is able to redistribute rab4A from the cytosol to the plasma membrane, possibly by a dephosphorylation reaction involving the activation of protein phosphatase-1 [43]. Furthermore, it is well established that insulin induces a translocation of transferrin receptors to the plasma membrane [44]. Taking into account that the plasma-membrane fraction may also contain endocytic vesicles and early endosomes, it seems likely that insulin is able to increase the amount of rab4A in these vesicles, leading to an enhanced recycling activity.

Indirect evidence suggests that a similar process could also take place for GLUT4. First, after insulin stimulation an increased amount of GLUT4 was found in early endosomes [9]. Second, as discussed by Satoh et al. [13], the subcellular trafficking kinetics of GLUT4 are compatible with a model of two transporter pools, an endosomal recycling pool and a tubulo-vesicular compartment. Third, as shown here, GLUT4 and rab4A are redistributed under the same experimental conditions. Based on these considerations, it may be hypothesized that rab4A mediates the recycling of GLUT4, by analogy to the transferrin receptor. Additional work will be needed to evaluate this important hypothesis. Our data contrast with the results of Le Marchand-Brustel's group [41], showing that in adipocytes insulin shifts rab4 to the cytosol. However, based on the known functions of rab4 (see above), it seems unlikely that this redistribution of the G-protein in response to insulin could mediate cellular recycling processes, which would be opposite to the reported effects of insulin [9,12,13,44].

In summary, our data suggest that at least two small G-proteins could be involved in the insulin-mediated translocation of cardiac GLUT4. A still unidentified 24 kDa G-protein is a component of the intracellular GLUT4 pool and may control the transfer to the plasma membrane. At a second stage, rab4A could regulate the recycling of GLUT4, keeping up a high plasma-membrane concentration of the transporter in the presence of insulin.

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