

Insulin-dependent translocation of the small GTP-binding protein rab3C in cardiac muscle: studies on insulin-resistant Zucker rats

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Abstract The failure of insulin-regulated recruitment of the GLUT4 glucose transporter in cardiac muscle of obese Zucker rats is associated with alterations of the subcellular distribution of the small-molecular-mass GTP-binding protein rab4A. Here, we show by subcellular fractionation and Western blotting a translocation of the small-molecular-mass GTP-binding protein rab3C from microsomal membranes to plasma membranes in lean control rats following in vivo insulin stimulation. However, in cardiac muscle of obese animals no significant effect of the hormone on the subcellular distribution of rab3C was observed. In GLUT4-enriched membrane vesicles, obtained from cardiac microsomes of the obese group as well as of lean controls, rab3C was not detectable. It is suggested that the altered behaviour of rab3C may contribute to an impaired trafficking of GLUT4 in the insulin-resistant state.

Key words: Small GTP-binding protein; Rab3; Cardiac muscle; Insulin resistance; Zucker rat

1. Introduction

The genetically obese (*fafa*) Zucker rat is a well-documented animal model for insulin resistance in skeletal [1,2] and cardiac muscle [3,4]. It has been suggested that a defect of the glucose uptake itself may contribute besides other defects to this insulin resistance [5,6]. Insulin action on glucose uptake is mediated by the glucose transporter isoform GLUT4, which is translocated from intracellular vesicular structures to the plasma membrane [7,8]. In cardiac muscle of obese Zucker rats, a reduced GLUT4 protein expression and a failure of insulin-regulated recruitment of GLUT4 was observed [9]. Similar results for skeletal muscle were recently reported by Handberg et al. [6] and King et al. [5].

GTP-binding proteins seem to be involved in GLUT4 translocation because guanosine 5'-[γ-thio]triphosphate and other non-hydrolyzable GTP analogs mimic insulin action in inducing a shift of GLUT4 to the plasma membrane in permeabilized fat cells [10]. Recently, we reported the presence of an insulin-sensitive 24 kDa GTP-binding protein in GLUT4 vesicles and the insulin-induced translocation of rab4A to the plasma membrane in cardiac tissue [11]. Further, we found an altered distribution of rab4A in cardiac muscle of obese Zucker rats and the lack of the 24 kDa GTP-binding protein in GLUT4 vesicles [9], suggesting the putative involvement of small G-proteins in the GLUT4-recycling and translocation machinery and alterations of this process in the insulin-resistant state.

The rab3 subgroup of GTP-binding proteins, specifically rab3A and rab3C, has been suggested to be involved in exocytosis and vesicle docking [12,13]. Interestingly, rab3D showed an increased expression in 3T3-L1 adipocytes, temporally coincident with the appearance of the insulin-sensitive glucose transporter GLUT4 [14]. Recently, Su et al. [15] reported the presence of the small GTP-binding protein rab3C in cardiac tissue and it was shown [13] that rab3C is a vesicle protein that dissociates after stimulation of exocytosis. Using isoform-specific antibodies against rab3C, we have now analysed the insulin-dependent subcellular distribution of rab3C in cardiac muscle of lean and obese Zucker rats under in vivo conditions, in order to elucidate the potential role of this GTP-binding protein for insulin-regulated vesicle traffic. Furthermore, attempts have been made to examine a possible co-localization of rab3C and GLUT4 in the intracellular insulin-sensitive glucose transporter compartment. The data show an insulin-regulated translocation of rab3C in a parallel way to the translocation of GLUT4, but no co-localization of rab3C and GLUT4. Alterations of this process in obese Zucker rats suggest a possible involvement in the pathogenesis of insulin resistance.

2. Materials and methods

2.1. Chemicals

[¹²⁵I]Protein A (30 mCi/mg) was purchased from Amersham (Germany). Reagents for SDS-PAGE were supplied by Pharmacia and Sigma (Germany). Insulin (Actrapid HM, 100 U/ml) was supplied by Novo (Germany). Immunobead goat anti-(rabbit immunoglobulin) was purchased from Bio-Rad (Germany). The polyclonal GLUT4 antiserum was a product of Calbiochem (Germany). All other chemicals were of the highest grade commercially available. Rabbit antiserum against the C-terminus of rab3C was generated as previously described [15]. Polyclonal antisera against rab3A and rab3D were generously provided by F. Darchen (Paris, France) and G. Baldini (New York, NY), respectively.

2.2. Membrane preparation and characterization

Genetically obese (*fafa*) male Zucker rats (12–16 weeks old, 480–520 g) were kindly provided by L. Herberg (Düsseldorf, Germany). Blood samples were collected for determinations of glucose and insulin as outlined previously [4]. Plasma and microsomal membranes from cardiac ventricles were prepared as recently described by us [16]. Briefly, ventricular tissue was removed and homogenized in a buffer containing 10 mM Tris-HCl, 0.1 mM PMSF and 2.6 mM dithiothreitol (DTT) by application of an Ultraturrax 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, the supernatant was centrifuged at 200,000 × g for 90 min to pellet the crude membrane fraction and to obtain the cytosol (supernatant). Further purification was achieved by applying the pellet to a discontinuous gradient consisting of 0.57, 0.72, 1.07 and 1.43 M sucrose buffer and centrifugation at 40,000 × g for 16 h. Membranes were harvested from each sucrose layer and stored at –70°C. Protein was determined using a modification of the Bio-Rad protein assay with BSA as

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a standard. Ouabain-sensitive Na^+/K^+ -ATPase and $\text{Ca}^{2+}/\text{K}^+$ -ATPase were used as marker enzymes for sarcolemma and microsomal membranes, respectively, and determined as described [16]. Membranes recovered from the 0.72 M sucrose layer were enriched 5–7-fold in the activity of the Na^+/K^+ -ATPase and considered as a plasma membrane fraction (PM), whereas membranes obtained in the 1.07 M sucrose layer showed a 0.5-fold activity of this marker enzyme, when compared with the homogenate. Furthermore, $\text{Ca}^{2+}/\text{K}^+$ -ATPase increased 4-fold in this fraction. The 1.07 M membrane fraction was hence termed microsomal membrane fraction (MM). For the insulin-stimulated studies, rats received a tail vein injection of regular insulin (4 U/100 g) and hearts were removed 20 min later.

2.3. Immunoadsorption of GLUT4-containing vesicles

GLUT4-enriched membrane vesicles were prepared essentially as previously described [11]. Briefly, microsomal membranes were incubated for 14 h with immunobeads at 4°C in PBS, pH 7.4, containing PMSF (0.1 mM), DTT (2.6 mM), EDTA (1 mM) and BSA (0.4%). After centrifugation, 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 3 times with PBS and the vesicle proteins were eluted with Laemmli sample buffer [34].

2.4. Immunoblotting

Protein samples were separated by SDS-PAGE using 8–18% gradient gels and transferred to nitrocellulose filters. 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 rab3C antiserum. After extensive washing with PBS containing 0.05% Tween, filters were incubated for 2 h with [125 I]Protein A (0.3 $\mu\text{Ci}/\text{ml}$). Filters were again extensively washed, air-dried and exposed to Hyperfilm-MP films using intensifying screens. Autoradiograms were analysed using laser scanning densitometry. In addition, blots were visualized on a FUJIX BAS 1000 bio-imaging analyser (Fuji, Japan). Quantification was performed on a SPARCstation (Sun Microsystems, USA) using image analysis software. Significance of reported differences was evaluated using the null hypothesis and *t*-statistics for unpaired data.

3. Results

Initial experiments were performed in order to check the presence of different rab3-species in our cardiac tissue preparation. Therefore, subcellular fractions of cardiac muscle were subjected to Western blotting using specific antisera against rab3A and rab3D, but neither rab3A nor rab3D could be detected (data not shown). It should be noted that the functionality of these antisera was verified using synaptosomal membranes [12] and lung tissue (Guerre-Millo, pers. commun.) as

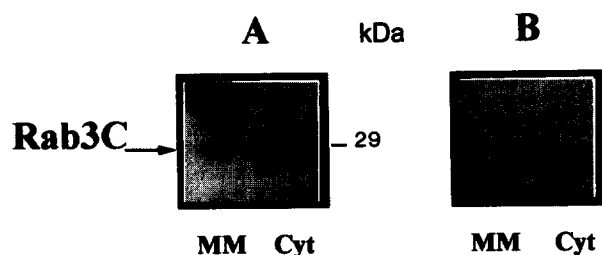
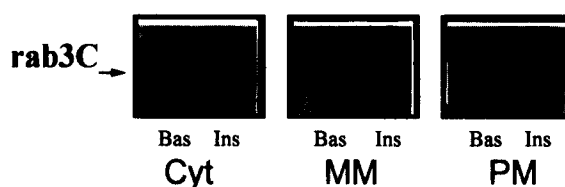


Fig. 1. Immunodetection of rab3C in subcellular fractions of cardiac muscle microsomal membranes (MM; 45 μg) and cytosol (Cyt; 10 μg) were analysed by SDS-PAGE on a 8–18% polyacrylamide gel, transferred to nitrocellulose and immunoblotted with specific antiserum against rab3C in the absence (A) or presence (B) of the immunization peptide. After incubation with [125 I]Protein A, the nitrocellulose sheets were subjected to autoradiography.

lean



obese

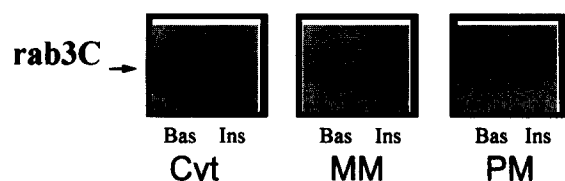
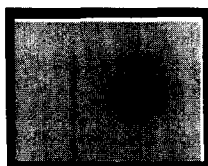


Fig. 2. Insulin-induced translocation of rab3C in lean and obese Zucker rats. Subcellular fractions were prepared from cardiac tissue of basal and insulin-treated lean and obese Zucker rats. 30 μg of cytosol (Cyt), microsomal membranes (MM) and plasma membranes (PM) were analysed by SDS-PAGE (8–18% gradient gel), transferred to nitrocellulose and immunoblotted with antiserum against rab3C. After incubation with [125 I]Protein A, the nitrocellulose sheets were subjected to autoradiography. Representative autoradiograms out of five separate experiments are presented.

a positive control for rab3A and rab3D, respectively. Recently, the protein expression of rab3C has been analysed in several tissues, showing the most prominent abundance in rat heart [15]. We, therefore, performed the analysis of rab3C protein expression in subcellular fractions of cardiac muscle using antiserum against rab3C (Fig. 1). As shown in Fig. 1, the rab3C antiserum intensively labelled a 29 kDa protein band, which is most abundant in the cytosol. This signal was completely abolished in competition experiments with the peptide used for immunization (Fig. 1). Quantification of the subcellular distribution revealed that about 5% of total rab3C exists in a membrane-associated form.

Next, we tested the effect of insulin on the redistribution of rab3C in cardiac muscle of lean and obese Zucker rats. These animals exhibited the well-known pattern of hyperinsulinaemia, normoglycaemia and a profound resistance of insulin-stimulated GLUT4 translocation [9]. Western blot analysis of various subcellular fractions using rab3C antiserum is presented in Fig. 2. In both basal and insulin-stimulated lean controls, the 29 kDa rab3C (see arrow) was most abundant in the cytosol and remained unaffected by the hormone, however, insulin produced a translocation of rab3C from the microsomal membranes to the plasma membranes. In the obese group, rab3C was also most abundant in the cytosolic fraction. Under these conditions insulin was unable to shift rab3C from the microsomal membranes to the plasma membranes (Fig. 2). The quantification of five independent experiments is summarized in Table 1. Insulin was found to induce a reduction of rab3C in the microsomal membranes of lean control rats by about 30%, with a concomitant increase to 240% of control in the plasma membrane fraction. No significant effect of insulin on the redistribution of rab3C in obese rats could be detected (Table 1).

Rab3C



Immuno- MM beads

Fig. 3. Immunodetection of rab3C in GLUT4-containing vesicles and microsomal membranes. GLUT4-containing vesicles and microsomal membranes were obtained from cardiac ventricular tissue of control rats as described in section 2. A portion of eluted vesicle proteins (lane 1) and 15 μ g of microsomal membranes (lane 2) were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-rab3C antiserum. Using our previously published procedure [11], a GLUT4 vesicle preparation was obtained, which is derived from the insulin-sensitive compartment and contains about 30% of total GLUT4 [11]. Near-equal amounts of GLUT4 were applied to the gel as previously published [11] to enable an estimation of the sensitivity of rab3C detection. As shown in Fig. 3, rab3C was not detectable in GLUT4-containing vesicles under these conditions, despite being clearly visible in the microsomal membrane fraction. Similar results were obtained using GLUT4-containing vesicles prepared from obese Zucker rats (data not shown).

In light of the co-localization of a small GTP-binding protein with GLUT4 [11] and the importance of rab proteins for intracellular vesicular traffic [17,18], we performed an immunoblot analysis of GLUT4-containing vesicles with anti-rab3C antiserum. Using our previously published procedure [11], a GLUT4 vesicle preparation was obtained, which is derived from the insulin-sensitive compartment and contains about 30% of total GLUT4 [11]. Near-equal amounts of GLUT4 were applied to the gel as previously published [11] to enable an estimation of the sensitivity of rab3C detection. As shown in Fig. 3, rab3C was not detectable in GLUT4-containing vesicles under these conditions, despite being clearly visible in the microsomal membrane fraction. Similar results were obtained using GLUT4-containing vesicles prepared from obese Zucker rats (data not shown).

4. Discussion

In this report, we analysed the protein expression of the small GTP-binding proteins rab3A, rab3C and rab3D in subcellular fractions of cardiac tissue. It has been suggested that the members of this GTPase subgroup called rab3 are functionally implicated in regulated exocytotic pathways [13,14,19,20]. Rab3A is predominantly expressed in brain and neuroendocrine cells [12], but it was recently also found in rat kidney [23], rat pancreatic islet cells [19] and in adult mouse fat cells and 3T3-L1 adipocytes [22]. Rab3D mRNA and protein is most abundant in adipocytes [14], but it is also expressed in cells of the neuroendocrine AtT-20 cell line [21]. In cardiac tissue, neither rab3A nor rab3D protein could be detected. This also indicates that our cardiac tissue preparation is substantially free of neuronal cells. In contrast, Western blot analysis of subcellular fractions from this cardiac tissue preparation showed the existence of the rab3C-isoform, which is most abundant in the cytosol. This finding is in good agreement with the observations of Su et al. [15] and Cormont et al. [24], who found rab3C mainly in the supernatant fraction of rat heart and rat adipocytes, respectively. Interestingly, an inverse distribution of rab3C was found in neuroendocrine cells [15], suggesting specific functions of this protein in both soluble and membrane-associated states.

One key finding of the present investigation consists in the observation that *in vivo* insulin stimulation induces the translocation of rab3C from the microsomal fraction to the plasma

membranes in cardiac tissue of lean control animals. This translocation of a small GTP-binding protein parallels the insulin-induced translocation of the glucose transporter isoform GLUT4 [9]. In a previous study [11], we reported the insulin-induced translocation of rab4A from the cytosolic fraction to the plasma membrane in cardiac tissue. However, rab4A is associated with the endocytotic part of vesicle-recycling pathways [17,18,25,26] and the rab3 isoforms are involved in exocytosis and vesicle docking [13,14,19,20]. The work of several groups [27,28] on the characterization of GLUT4 trafficking in target cells has led to the concept that the majority of GLUT4 is localized in specific membrane vesicles, which cycle to and from the cell surface in an insulin-dependent manner. Very recent investigations [29] suggest that both exocytosis and endocytosis of GLUT4 are regulated by GTP-binding proteins. In that scenario, rab3C, which is shifted by insulin to the cell surface, could be part of the docking mechanism of GLUT4 vesicles, whereas rab4A is possibly involved in the endocytotic part of the GLUT4-recycling process. It may be argued that the majority of rab3C protein remains in the cytosol upon stimulation with insulin, suggesting that only a small fraction of this protein appears to be insulin-sensitive. However, several lines of evidence support the assumption of a functional significance of rab3C redistribution: First, the doubling of rab3C in the plasma membrane in response to insulin compares favourably well with our recent report on rab4A [9], a small G-protein known to be involved in GLUT4 translocation [24]. Second, the increase in the plasma membrane is paralleled by a decrease in the microsomal fraction, suggesting a redistribution of membrane-associated rab3C in parallel to GLUT4. Third, it is well-established that soluble and membrane-associated rab proteins represent different functional states [36], making it likely that the microsomal rab3C could be relevant for GLUT4 translocation, whereas the large pool of soluble rab3C is not directly involved in this process. At present, we can only correlate the parallel movement of rab3C and GLUT4 and the existence of alternative mechanisms that mediate GLUT4 vesicle docking cannot be excluded [37]. Nevertheless, our data suggest that rab3C must be considered as a good candidate for certain steps of the GLUT4 translocation process.

The second key finding of this study is related to the observed failure of the insulin-stimulated translocation of rab3C in cardiac muscle of obese Zucker rats, which is paralleled by a failure of insulin-regulated recruitment of the glucose trans-

Table 1
Quantification of insulin-induced rab3C redistribution in lean and obese Zucker rats

	Relative abundance of rab3C (insulin/basal)	
	Lean	Obese
Microsomal membranes	0.71 \pm 0.12 <i>P</i> = 0.037	1.23 \pm 0.08 <i>P</i> = 0.046
Plasma membranes	2.4 \pm 0.5 <i>P</i> = 0.049	1.25 \pm 0.2 <i>P</i> = 0.277

Rab3C was immunodetected in microsomal membranes and plasma membranes of basal and insulin-stimulated Zucker rats, as outlined in Fig. 2. Quantification was performed with a bio-imaging analyser and the effect of insulin is expressed by the relative decrease or increase of abundance of rab3C signal intensity (insulin/basal). Data presented are the mean \pm S.E.M. of five experiments.

porter GLUT4 from the intracellular storage site [9]. This observation strongly supports our assumption of a functional relationship between rab3C and GLUT4. Interestingly, a comparable defect has also been observed for the insulin-induced translocation of rab4A in heart muscle of obese Zucker rats [9] and in 3T3-L1 adipocytes rendered insulin-resistant by prolonged insulin treatment [35]. It may be speculated that the altered insulin-induced redistribution of rab3C in obese animals may prevent docking and/or fusion of GLUT4 vesicles with the cell surface after insulin stimulation, whereas the reported defects [9] of rab4A could limit GLUT4-recycling [11], two processes possibly contributing to insulin resistance of cardiac glucose uptake.

The concept of insulin-sensitive GLUT4-containing vesicles gave reason to examine these vesicles for proteins involved in the GLUT4 translocation process. Beside some components which could be considered as part of a general recycling system, e.g. the secretory carrier membrane proteins (SCAMPs, 35–40 kDa) [30] or cellubrevin (17 kDa) [31], also GTP-binding proteins could be detected in GLUT4 vesicles [11,32]. Our present investigation shows that rab3C is not identical with the 24 kDa GTP-binding protein found in GLUT4 vesicles [11]. It may be speculated that this protein is related to another species of the large and growing family of rab GTP-binding proteins. However, rab3C is shifted to the plasma membrane by insulin in parallel to the glucose transporter GLUT4 and could play a role in docking and/or fusion processes of GLUT4 vesicles. This function of a small GTP-binding protein would be consistent with the hypothesis of a stepwise activation of GLUT4 at the cell surface [27,33]. Further investigations are now required to evaluate functional relationship of this small GTP-binding protein to stages of the GLUT4-exocytotic and -recycling pathway.

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