Altered GLUT4 trafficking in adipocytes in the absence of the GTPase Arfrp1

Deike Hesse a,1, Angela Hommel a,1, Alexander Jaschke b, Markus Moser b, Ulrike Bernhardt a, Claudia Zahn a, Reinhart Kluge a, Petra Wittschen c, Achim D. Gruber c, Hadi Al-Hasani a, Hans-Georg Joost d, Annette Schürmann a,*

a Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbruecke, Arthur-Scheunert-Allee 114-116, D-14558 Nuthetal, Germany
b Department of Molecular Medicine, Max Planck Institute of Biochemistry, D-82152 Martinsried, Germany
c Department of Veterinary Pathology, Freie Universität Berlin, D-14163 Berlin, Germany

ARTICLE INFO

Article history:
Received 10 March 2010
Available online 15 March 2010

Keywords:
ArF-proteins
Glucose transporter GLUT4
Golgi apparatus
Golgin proteins

ABSTRACT

The GTPase ADP-ribosylation factor related protein 1 (ARFRP1) controls the recruitment of proteins such as golgin-245 to the trans-Golgi. ARFRP1 is highly expressed in adipose tissues in which the insulin-sensitive glucose transporter GLUT4 is processed through the Golgi to a specialized endosomal compartment, the insulin-responsive storage compartment from which it is translocated to the plasma membrane in response to a stimulation of cells by insulin. In order to examine the role of ARFRP1 for GLUT4 targeting, subcellular distribution of GLUT4 was investigated in adipose tissue specific Arfrp1 knockout (Arfrp1 ad−/−) mice. Immunohistochemical and ultrastructural studies of brown adipocytes demonstrated an abnormal trans-Golgi in Arfrp1 ad−/− adipocytes. In addition, in Arfrp1 ad−/− adipocytes GLUT4 protein accumulated at the plasma membrane rather than being sequestered in an intracellular compartment. A similar missorting of GLUT4 was produced by siRNA-mediated knockdown of Arfrp1 in 3T3-L1 adipocytes which was associated with significantly elevated uptake of deoxyglucose under basal conditions. Thus, Arfrp1 appears to be involved in sorting of GLUT4.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

The GTPase ARFRP1 (ARF-related protein 1) [1] is a member of the family of ADP-ribosylation factors (ARFs) that operate as GTP-dependent molecular switches in the regulation of intracellular protein traffic and in Golgi function [2,3]. ARFRP1 is ubiquitously expressed with higher levels in white and brown adipose tissue, liver, kidney, and intestine [1] and is associated with trans-Golgi membranes [4]. ARFRP1 recruits a second GT-Pase, ARL1, and its effector the GRIP protein golgin-245 to the trans-Golgi network [4–6]. Conventional Arfp1−/− embryos died during early gastrulation between day 6 and 6.5 [7] due to adhesion defects [8]. The adipocyte-specific deletion of Arfp1 resulted in a lipo-odystrophic phenotype due to a defective lipid droplet growth and an elevated lipolysis [9]. We found SNAP23 (synaptosomal-associated protein of 23 kDa) associated with small lipid droplets of control adipocytes as described in the literature [10]. In contrast, SNAP23 was predominantly located in the cytosol and at the cell surface of Arfp1 ad−/− adipocytes [9].

SNARE proteins (VAMP2, syntaxin-4, and SNAP23) have been implicated in the insulin-induced translocation of vesicles containing the GLUT4 glucose transporter to the plasma membrane of adipocytes [11–16] indicating that GLUT4 translocation follows typical membrane fusion rules. Because SNAP23 distribution was altered in adipocytes of Arfp1 ad−/− mice [9] we reasoned that GLUT4 targeting might be modified in adipocytes lacking Arfp1, thereby allowing further dissection of the different vesicular GLUT4 compartments. Since Arfp1 ad−/− mice die early due to the lack of white adipose tissue [9] we studied GLUT4 localization in adipocytes from 18.5 days old Arfp1 ad−/− embryos which did not show impaired growth or survival. In addition, we used 3T3-L1 adipocytes in which expression of Arfp1 was depleted by siRNA.

2. Materials and methods

2.1. Arfp1 ad−/− mice

Generation of Arfp1 ad−/− mice and the fat cell-specific deletion of Arfp1 was described previously [8,9]. Animals were housed in a controlled environment (20 ± 2 °C, 12 h/12 h light/dark cycle) and had free access to water and standard chow diet. All animal experiments were approved by the ethics committee of the Ministry of Agriculture, Nutrition and Forestry (State of Brandenburg, Germany).

2.2. Antibodies

We used the polyclonal antiserum against recombinant GST-ARFRP1 [1,4]. Antiserum against GLUT4 [9] was used for immuno-
histochemistry and for Western blotting in a dilution of 1:1000, anti-GLUT1 [20] 1:1000. Anti-TGN38 antisera (Serotec, Oxford, UK) was used in a dilution of 1:100 (immunohistochemistry) or 1:1000 (Western blotting) and anti-GM130 antisera (BD Transduction Laboratories, NJ, USA) in a 1:100 dilution for immunohistochemistry. For analysis of activation status of Akt and FoxO1 we used the following antibodies for Western blotting in a dilution of 1:1000: anti-Akt (# 9272) and anti-phospho-AKT (Ser473; # 9271), anti-FoxO1 (C29H4; # 2880), and anti-phospho-FoxO1 (Thr24)/FoxO3a (Thr32) antibody (#9464, Cell Signaling), Alexa Fluor® 456 F(ab)2 fragment of goat anti-rabbit IgG (H + L) or Alexa Fluor® 488 F(ab)2 fragment of goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, Oregon, USA) were used in a dilution of 1:800 as secondary antibodies. Antibody against glyceraldehyde phosphate dehydrogenase (GAPDH, Ambion) was used as loading control.

2.3. Immunohistochemistry and immunocytochemistry

Paraffin sections of Arfrp1flox/flox and Arfrp1ad−/− animals at the age of ED18.5 were prepared as described earlier [9]. The indicated primary antibodies were applied overnight at 4 °C in a humid chamber. Subcellular distribution of GLUT4, TGN38, GM130, p115, syntaxin-6 and ARL1 was visualized by fluorescence-conjugated secondary antibodies in a confocal microscope as described [8].

2.4. Quantitative real-time PCR

Quantitative real-time PCR analysis (qRT-PCR) was performed using the Applied Biosystems 7300 Real-time PCR System as described previously [17]. For the determination of the Arfrp1 mRNA levels and to analyze expression of ap2 and GLUT4, the following TaqMan gene expression assays were used: Arfrp1 E2_E3 (Mm01220415_g1), Slc2ad/GLUT4 (Mm00436615_m1), ap2 (Mm00445880_m1). Data were normalized as described (5) whereas a β-actin expression assay with the probe 5'-TTG AGA CCT TCA ACA CCC CAG CCA-3' and the forward primer 5'-GCC AAC CGT GAA AAG ATG AC-3' and reverse primer 5'-TAC GAC CAG AGG CAT ACA G-3' was used as endogenous control.

2.5. Electron microscopy

Brown adipose tissue of Arfrp1flox/flox and Arfrp1ad−/− embryos (ED 18.5) was sectioned into fragments of approximately 1–2 mm². These pieces were immersed in a fixative consisting of 7.5% glutaraldehyde and 3% paraformaldehyde (pH 7.4). The specimens were post fixed in 1% OsO₄ dehydrated in ethanol, and embedded in Epon-Araldite. Block staining of specimens was performed with 1% uranyl acetate and 0.5% phosphotungstic acid. Ultra thin sections (50–60 nm) were obtained with an ultramicrotome, stained with lead citrate, and examined with a Zeiss EM10 electron microscope.

2.6. Cell culture and siRNA-mediated knockdown of Arfrp1 in 3T3-L1 cells

Murine 3T3-L1 fibroblasts were purchased from the American Type Tissue Culture repository. Cells were grown to confluence and differentiated to mature adipocytes for up to 8 days [18] prior to electroporation. For downregulation of Arfrp1 mature adipocytes (5 × 10⁶ cells/electroporation) were electroporated using the Bio-rad Gene Pulser II with settings of 170 V and 960 microfarads with 200 μmol Arfrp1-specific siRNA (5′-GACUGUACGUGAUAGUUGU-3′; also corresponding to si-Arfrp1-a). For control experiments cells were electroporated with 200 μmol scrambled siRNA (5′-CAGUGACGUACGUAGUUGU-3′; also corresponding to si-scrambled-a). We used an additional set of an Arfrp1-specific siRNA (si-Arfrp1-b: 5′-GUAUGUAUCCACUGAUGU-3′) and its corresponding scrambled siRNA (si-scrambled-b: 5′-CUAGUGUAUCCACUGAUGU-3′). After electroporation, cells were immediately mixed with fresh medium before being reseeded onto multiple-well plates or plated on glass coverslips for immunocytochemical detection of GLUT4.

2.7. Detection of glucose transport

Seventy-two hours after electroporation of 3T3-L1 adipocytes with siRNA, basal and insulin-stimulated glucose uptake was assayed as described previously [18].

2.8. Preparation of plasma membrane sheets

Preparation of plasma membrane sheets from differentiated 3T3-L1 adipocytes was performed by the method of Olson et al. [19] with minor modifications as described by von der Crone et al. [20].

3. Results

3.1. Specific alteration of the trans-Golgi in brown Arfrp1ad−/− adipocytes

Our previous studies have shown that activated, GTP-bound ARFRP1 associates with the trans-Golgi and recruits other proteins such as AR1L and its effector, golgin-245 to this compartment [4]. In order to test whether the Golgi is also altered in vivo in the absence of ARFRP1 we stained brown adipocytes of control and Arfrp1ad−/− embryos (ED 18.5) with specific markers for the trans-Golgi (TGN38) and for the cis-Golgi (GM130). Expression of Arfrp1 in BAT of Arfrp1ad−/− embryos was significantly and specifically reduced (Fig. 1A). In contrast to postnatal mice the knockout embryos did not show a growth retardation and a reduced survival [9]; they were therefore the appropriate model to study the influence of Arfrp1 depletion on Golgi organization and GLUT4 localization because secondary alterations due to growth retardation were avoided. As shown in Fig. 1B (left panel), Arfrp1flox/flox cells exhibited distinct distribution of TGN38 in a perinuclear region whereas it was nearly undetectable in Arfrp1ad−/− cells. In order to test whether a perturbation of TGN38 trafficking might result in its degradation we analyzed TGN38 levels but did not detect differences (Fig. 1B, right panel). Syntaxin-6, an additional TGN protein was still membrane associated in brown adipocytes of Arfrp1ad−/− embryos but displaced a broader staining than in control cells (Fig. 1B, lower panel). However, staining of the cis-Golgi with the anti-GM130 antibody was essentially identical in both genotypes, supporting our previous finding that organization of the trans-Golgi is specifically disrupted by in vitro knockdown of Arfrp1.

In addition, the ultrastructure of brown adipocytes was studied by electron microscopy at ED18.5. Fat cells of control embryos showed typical features such as lipid droplets and numerous mitochondria (Fig. 1C, upper panel). In brown fat cells of Arfrp1ad−/− embryos lipid droplets were smaller [9] but nuclei, cell–cell contact and structure of mitochondria were normal. The major difference we observed was striking abnormal vacuolization of the Golgi apparatus from Arfrp1ad−/− adipocytes (Fig. 1C, middle and lower panel).

3.2. Altered targeting of GLUT4 in adipocytes lacking Arfrp1

Since important biosynthesis processing steps of the insulin-regulated glucose transporter GLUT4 are believed to be initiated in the sorting compartments of the TGN [21] where ARFRP1 regu-
lates recruitment of proteins such as ARL1 we examined the subcellular distribution of GLUT4 in BAT at ED18.5 by immunohistochemistry (Fig. 2, left panel). The subcellular distribution of GLUT4 in brown adipocytes differed between Arfrp1^flox/flox and Arfrp1^ad^-/- embryos. Arfrp1^flox/flox adipocytes exhibited a punctuate intracellular distribution of GLUT4, whereas Arfrp1^ad^-/- fat cells showed a distinct concentration of GLUT4 at the plasma membrane (Fig. 2, arrows). As shown in the right panel of Fig. 2, Slc2a4/GLUT4 mRNA was not altered in Arfrp1^ad^-/- adipocytes. In addition, no differences in the amount of GLUT4 were visible in Western blot analysis of lysates from BAT of two days old control and Arfrp1^ad^-/- mice (Fig. 2, lower right panel). We therefore can exclude that an increased expression of GLUT4 in Arfrp1^ad^-/- adipocytes might be responsible for a stronger staining of GLUT4 at the cell surface.
To examine the GLUT4 distribution in adipocytes in the absence of ARFRP1 in cultured adipose cells we suppressed Arfrp1 expression in 3T3-L1 adipocytes by siRNA (Fig. 3A) and analyzed GLUT4 distribution under basal and insulin-stimulated conditions. In the absence of insulin, GLUT4 was detected in intracellular membranes in control 3T3-L1 adipocytes which were electroporated with scrambled siRNA, whereas GLUT4 appeared at the cell surface in the presence of insulin (Fig. 3B). In contrast, knockdown of Arfrp1...
resulted in plasma membrane localization of GLUT4 already under basal conditions (Fig. 3B, arrows), confirming the observation obtained in brown adipocytes of Arfrp1<sup>1<sup>−/−</sup></sup> embryos (Fig. 2, left panel). This finding was verified by a second Arfrp1-specific siRNA (siArfrp1-b) and the corresponding scrambled control (si scram-b). Both sets of Arfrp1-specific siRNAs result in a cell surface localization of GLUT4 in the basal state (Supplementary Fig. 1). To further verify the necessary role of ARFRP1 function in the intracellular retention of endogenous GLUT4, we examined the effect of Arfrp1 knockdown on glucose transport by measuring uptake of labeled deoxyglucose. Scrambled siRNA-transfected adipocytes displayed a 5.6-fold insulin-stimulated increase in the rate of glucose uptake. In comparison, knockdown of Arfrp1 resulted in a higher basal transport rate (3-fold higher than basal scrambled siRNA-transfected cells) with only an approximate 1.6-fold stimulation by insulin (Fig. 3C). These data strongly support a requirement for ARFRP1 in the sorting of proteins such as GLUT4. However, the total insulin-stimulated deoxyglucose transport did not differ between scrambled and siArfrp1 transfected cells indicating that a recruitment of GLUT4 occurs from a similar intracellular compartment. In order to test whether elevated GLUT4 levels at the plasma membrane are the result of a higher GLUT4 expression or insulin-like effects in Arfrp1 knockdown cells we analyzed lysates of transfected 3T3-L1 adipocytes for GLUT4 expression and levels of phosphorylated Akt and FoxO1, two prominent downstream signals of the insulin receptor. Expression of GLUT4, Akt and FoxO1 as well as the levels of phosphorylated Akt and FoxO1 were not affected in two sets of experiments (Fig. 3D).

In order to characterize the role of ARFRP1 for the Golgi structure we stained some Golgi proteins in 3T3-L1 adipocytes after transfection with scrambled and Arfrp1-specific siRNA. In control cells (si-scrambled), the trans-Golgi marker TGN38 was colocalized with GLUT4 in the perinuclear region. In contrast, TGN38 was nearby not detectable after downregulation of Arfrp1 when GLUT4 was located at the cell surface (Supplementary Fig. 2A). In addition, ARL1 (ARF-like 1) which has been described to associate with the trans-Golgi only in the presence of Arfrp1 [4] was detected in the perinuclear compartment in control cells, whereas it was rarely located at the Golgi apparatus after downregulation of Arfrp1 (Supplementary Fig. 2B, upper panel). In contrast, distribution of the cis-Golgi marker p115 was not affected in 3T3-L1 adipocytes transfected with Arfrp1-specific siRNA (Supplementary Fig. 2B, lower panel).

FIG. 2. Altered targeting of GLUT4 in Arfrp1<sup>1<sup>−/−</sup></sup> adipocytes. Immunohistochemical detection of GLUT4 in BAT of Arfrp1<sup>lox/lox</sup> and Arfrp1<sup>1<sup>−/−</sup></sup> embryos at day 18.5 (left panels). Paraffin sections were stained with a polyclonal anti-GLUT4 antiserum in combination with an Alexa488-conjugated secondary antibody. mRNA expression of aP2 and Slc2a4 (GLUT4) in BAT of embryos at day 18.5 detected by qRT-PCR analysis (upper right panel). Western blot of GLUT4 in lysates of BAT from two days old Arfrp1<sup>lox/lox</sup> and Arfrp1<sup>1<sup>−/−</sup></sup> mice (lower right panel).
cytosol [4–6]. Mice lacking Arfp1 died during early gastrulation [7,8] due to defective E-cadherin targeting to the plasma membrane which resulted in adhesion defects. Here we demonstrated that in adipocytes targeting of the insulin-sensitive glucose transporter GLUT4 was affected in the absence of ARFRP1. At early stages after deletion of Arfp1 in adipocytes (Arfp1^{ad/−} embryos...
GLUT4 protein specifically accumulated at the plasma membrane (Fig. 4). The cell surface localization of GLUT4 could be the result of (i) an insulin stimulation which induces the translocation of GLUT4 to the plasma membrane, or (ii) defects in GLUT4 trafficking. An increase in insulin levels as the trigger of GLUT4 translocation is unlikely, as embryonic BAT was exposed to the maternal environment of normoglycemic and normoinsulinemic mothers. Since inhibition of Arfrp1 expression in fully differentiated 3T3-L1 adipocytes also resulted in plasma membrane localization of GLUT4 in Arfrp1 knockdown 3T3-L1 adipocytes was also evident by measuring elevated basal glucose transport rates (Fig. 3C) and an increased immunoreactivity obtained in isolated plasma membranes (Fig. 4). We conclude that cell surface localization of GLUT4 is a consequence of defects in Golgi structure as detected in Arfrp1−/− adipocytes.

The Golgi and trans-Golgi network (TGN) are major sorting compartments for proteins and membrane traffic [21]. Important biosynthesis processing steps of the insulin-regulated glucose transporter GLUT4 are believed to be initiated in the sorting compartments of the TGN [22]. Peripheral Golgi proteins such as golgin-160 [23] and golgin-p115 [24] have been described to be required for correct intracellular tethering of GLUT4. Williams et al. [23] who downregulated golgin-160 in differentiated 3T3-L1 adipocytes observed a similar mistargeting of GLUT4 as detected in cells lacking ARFRP1. Thus, it was assumed that in the absence of golgin-160, GLUT4 was targeted to the plasma membrane by default, bypassing the sorting to the insulin-responsive compartment [23]. These data are consistent with a scenario in which ARFRP1 is a crucial component of golgin targeting. Mistargeting of GLUT4 has to be a consequence of specific Golgi alterations. Treatment of adipocytes with the fungal metabolite brefeldin A (BFA) for instance did not affect the intracellular sequestration of GLUT4 or its insulin-dependent translocation to the plasma membrane [25] indicating that not alterations of Golgi structures per se result in a cell surface location of GLUT4.

The finding that Golgi structures of Arfrp1−/− adipocytes exhibited pronounced alterations of the trans-Golgi (Fig. 1B) is consistent with previous conclusions derived from in vitro studies with heterologous expression systems. We and others have shown previously that downregulation of ARFRP1 in HeLa cells by shRNA disrupted the recruitment of the GTPase ARL1 and of its effector golgin-245 to Golgi membranes [4–6]. Golgin proteins are coiled-coil proteins which are required for the structure of the Golgi apparatus in eukaryotic cells. A number of recent studies reveal a common theme for the targeting of golgins containing the ARL-binding GRIP domain [26]. Similarly, other golgins such as the vesicle tethering factor p115 are targeted by the Rab GTases, Rab1 and Rab6, respectively. Together, golgins and their regulatory GTases form a complex network, commonly known as the Golgi matrix, which organizes Golgi membranes and regulates membrane trafficking [27]. The present data support this concept, and provide definitive proof for the essential role of ARFRP1 in Golgi function.

In conclusion, the present data demonstrate that deletion of the GTase Arfrp1 severely alters GLUT4 sorting as well as Golgi structures.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (Schu 750/5-2; Schu 750/5-3; GK1208). The skilful technical assistance of Brigitte Rischke, Anett Seelig, and Michaela Rath is gratefully acknowledged.

Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2010.03.059.

References


