The first luminal loop confers insulin responsiveness to glucose transporter 4

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ABSTRACT **Glucose transporter isoform 4 (GLUT4), is the sole glucose transporter responsible for the effect of insulin on postprandial blood glucose clearance. However, the nature of the insulin sensitivity of GLUT4 remains unknown. In this study, we replaced the first luminal loop of cellugyrin, a 4-transmembrane protein that does not respond to insulin, with that of GLUT4. The chimera protein is targeted to the intracellular insulin-responsive vesicles and is translocated to the plasma membrane upon insulin stimulation. The faithful targeting of the chimera depends on the expression of the sorting receptor sortilin, which interacts with the unique amino acid residues in the first luminal loop of GLUT4. Thus the first luminal loop may confer insulin responsiveness to the GLUT4 molecule.**

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INTRODUCTION

Glucose represents a universally important, albeit membrane-impermeable, biological fuel for mammalian cells. Correspondingly, every cell in the body expresses at least one type of glucose transporter protein. Mammalian glucose transporters belong to the family of 14 closely related 12-transmembrane proteins that are expressed in a tissue-specific manner (Thorens and Mueckler, 2010). One member of this family, glucose transporter isoform 4 (GLUT4), attracted the attention of a wide range of scientists, from diabetologists to cell biologists, because of its unique biological properties and functions. GLUT4 is expressed in insulin-sensitive tissues (fat, skeletal muscle, and some neurons) and is the sole glucose transporter responsible for the effect of insulin on postprandial blood glucose clearance (Huang and Czech, 2007). Moreover, multiple lines of independent evidence that include a number of transgenic models (reviewed in Charron *et al.*, 1999; Minokoshi *et al.*, 2003) and in vivo NMR studies (reviewed in Shulman, 2000) prove that GLUT4-mediated glucose uptake represents the rate-limiting step of insulin-stimulated glucose disposal. Therefore mechanistic dissection of GLUT4 regulation is crucial for our understanding of the molecular nature of insulin resistance and diabetes mellitus.

Unlike most other glucose transporters, which are localized largely at the plasma membrane, GLUT4 is compartmentalized inside the cell primarily in small insulin-responsive vesicles (IRVs) that are translocated to the cell surface upon insulin stimulation (Kandror and Pilch, 2011). From the plasma membrane, GLUT4 is internalized into early endosomes and is rapidly delivered to a subdomain of the *trans*-Golgi network (TGN) and/or recycling endosomes in which the IRVs are formed. Each GLUT4 molecule undergoes multiple cycles of translocation to and from the cell surface before being rerouted to lysosomes for degradation.

Such a complicated pattern of the intracellular trafficking suggests the presence of unique signal sequences in the GLUT4 molecule, and many research groups have contributed to deciphering these signals. Earlier studies revealed that the N-terminal F^{5}QQI and the C-terminal L^{489}L motifs are required for the internalization and the intracellular retention of GLUT4 (reviewed in Holman and Sandoval, 2001). More recent experiments showed that the T^{498}ELEYL^{503} motif (or the partially overlapping L^{500}xxLx-PDExD^{509} motif (see Song *et al.*, 2008) in the C-terminus of GLUT4 is important for its insulin responsiveness (Shewan *et al.*, 2003; Blot and McGraw, 2008; Xiong *et al.*, 2010). Very recently, GLUT4 ubiquitination was proposed as playing a role in the acquisition of insulin sensitivity of the transporter (Lamb *et al.*, 2010).

Importantly, most targeting studies have been performed with the help of a loss-of-function approach, in which mutations introduced to the trafficking motifs attenuate the efficiency of intracellular

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Abbreviations used: BS, bovine serum; EGFP, enhanced green fluorescent protein; EV, empty vector; FBS, fetal bovine serum; GGA, Golgi-localized, γ-ear-containing, Arf-binding; GLUT4, glucose transporter isoform 4; IgG, immunoglobulin G; IRV, insulin-responsive vesicle; KRH, Krebs-Ringer-HEPES; S^{-}, sortilin-depleted; S2, high-speed supernatant; SDA, Sabouraud dextrose agar; shRNA, short hairpin RNA; TGN, *trans*-Golgi network.

retention and/or insulin-dependent translocation of GLUT4 to the plasma membrane. Thus it remains uncertain whether these signals are sufficient for the faithful targeting and insulin responsiveness of GLUT4. In addition, interfering with the targeting sequences of the transporter may alter its intracellular localization and translocation in various ways, some of which may be difficult to predict or interpret. For all these reasons, we decided to assess the role of the C-terminal trafficking motifs of GLUT4 in both loss-of-function and gain-of-function experiments. First, we exchanged the C-termini of GLUT4 and cellugyrin, a 4-transmembrane vesicular protein not localized in the IRVs and, correspondingly, not translocated to the plasma membrane in response to insulin (Kupriyanova and Kandror, 2000; Kupriyanova *et al.*, 2002; Jedrychowski *et al.*, 2010). We have found that the C-terminus of GLUT4 is required for its localization in the syntaxin 6–positive perinuclear compartment but does not confer insulin responsiveness to the molecule (Li *et al.*, 2009).

In this study, we explored the targeting role of the first luminal loop of GLUT4. We transferred it to cellugyrin and found that it is sufficient for the acquisition of insulin responsiveness by the chimera protein. We propose that the first luminal loop of GLUT4 targets proteins to the IRVs by interaction with the sorting receptor sortilin.

RESULTS

Previous results have shown that sortilin represents a component protein of the IRVs (Lin *et al.*, 1997; Morris *et al.*, 1998) and plays an important role in the biogenesis of this compartment (Shi and Kandror, 2005; Ariga *et al.*, 2008). In addition, we have previously found that the luminal Vps10p domain of sortilin directly interacts with the first luminal loop of GLUT4 (Shi and Kandror, 2005, 2007). On the basis of these results, we hypothesized that the first luminal loop of GLUT4 may play an important role in targeting the transporter to the IRVs. To test this hypothesis, we attempted to identify amino acid residues involved in the interaction with sortilin. For that, we carried out alanine-screening mutagenesis in a yeast two-hybrid system. As is shown in Supplemental Figure S1, A and B, replacement of amino acids 65–73 with alanines strongly reduced the interaction of the first luminal loop of GLUT4 with the Vps10p domain of sortilin. Significantly, most of these amino acids (shown in red) are unique for GLUT4 and are not conserved, even in the closest GLUT4 homologue, GLUT1, which has a significantly lower insulin responsiveness in adipocytes (reviewed in Kandror and Pilch, 1996).

A recent report showed that *N*-glycosylation of GLUT4 on Asn-57 is important for its stability and intracellular trafficking (Haga *et al.*, 2011). In our experiments, since the first luminal loop of GLUT4 is expressed as a cytosolic fusion protein with the GAL4 transcription activation domain, it is almost certainly not glycosylated. Additionally, Asn-57 lies outside the essential 65–73 sequence. Thus we believe that *N*-glycosylation of GLUT4 is not absolutely required for binding to sortilin; it is possible, however, that *N*-glycosylation facilitates the interaction between the two proteins.

We then prepared the deletion mutant of GLUT4, which lacked amino acids involved in the interaction with the Vps10p domain of sortilin. Deletion of this sequence produces an unstable mutant (Figure S1C), targeting of which cannot be analyzed. Thus sortilin-binding amino acids in the first luminal loop of GLUT4 are essential for the structure of the molecule. Hence, we changed our approach from loss of function to gain of function.

We exchanged the first luminal loop of GLUT4 with that of cellugyrin, as shown in Figure 1A. The chimera protein (myc$_7$-CgGLUT4), as well as control myc$_7$-cellugyrin, were stably expressed in 3T3-L1

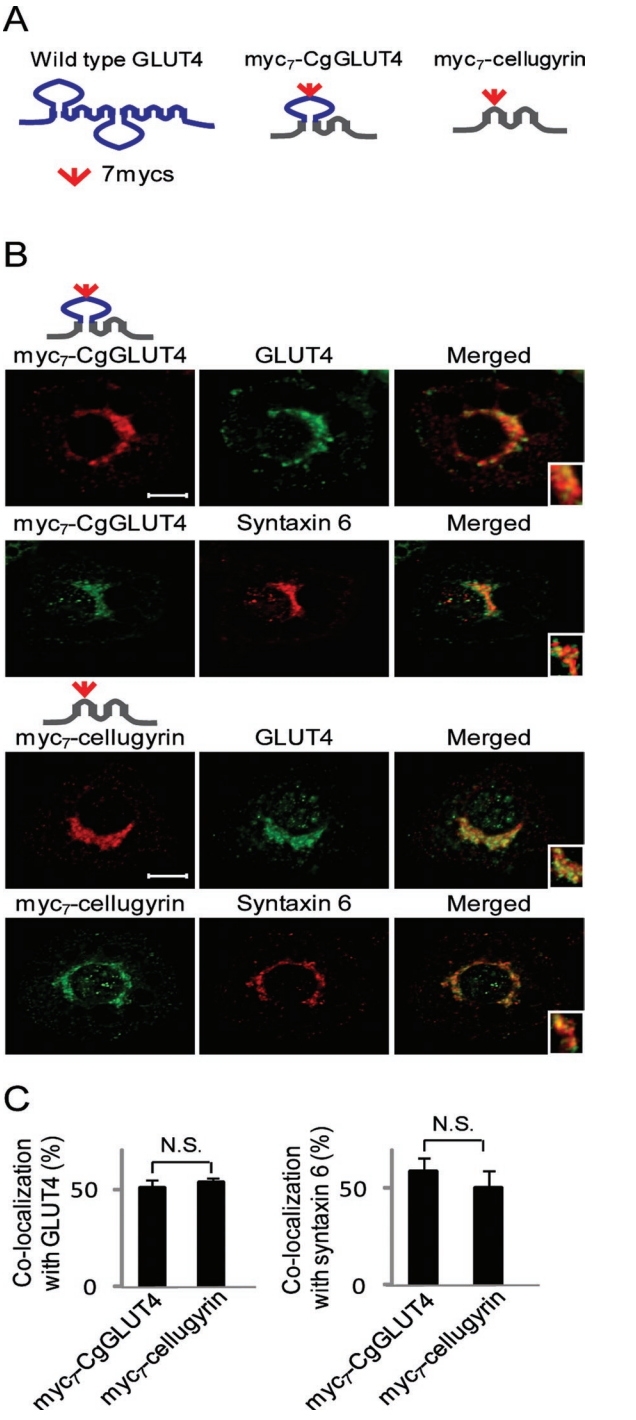

FIGURE 1: Intracellular localization of myc$_7$-CgGLUT4 and myc$_7$-cellugyrin in 3T3-L1 adipocytes. (A) Schematic representations of wild-type GLUT4, myc$_7$-CgGLUT4 and myc$_7$-cellugyrin. The cellugyrin backbone in is black, the first luminal loop of GLUT4 is in blue, and the myc$_7$ epitope is in red. (B) myc$_7$-CgGLUT4 and myc$_7$-cellugyrin were stably expressed in 3T3-L1 adipocytes. Serum-starved cells were permeabilized with Triton X-100 and stained with either monoclonal anti-myc antibody and polyclonal anti-GLUT4 antibody or with the polyclonal anti-myc antibody and monoclonal anti–syntaxin 6 antibody. Note that polyclonal antibody against GLUT4 was raised against the C-terminus of the transporter and thus does not recognize myc$_7$-CgGLUT4. Small boxes show enlarged images of the perinuclear area. Scale bar: 1 μm. (C) Quantitative analysis of colocalization of the reporter proteins with endogenous GLUT4 (left) and syntaxin 6 (right) obtained in three independent experiments.

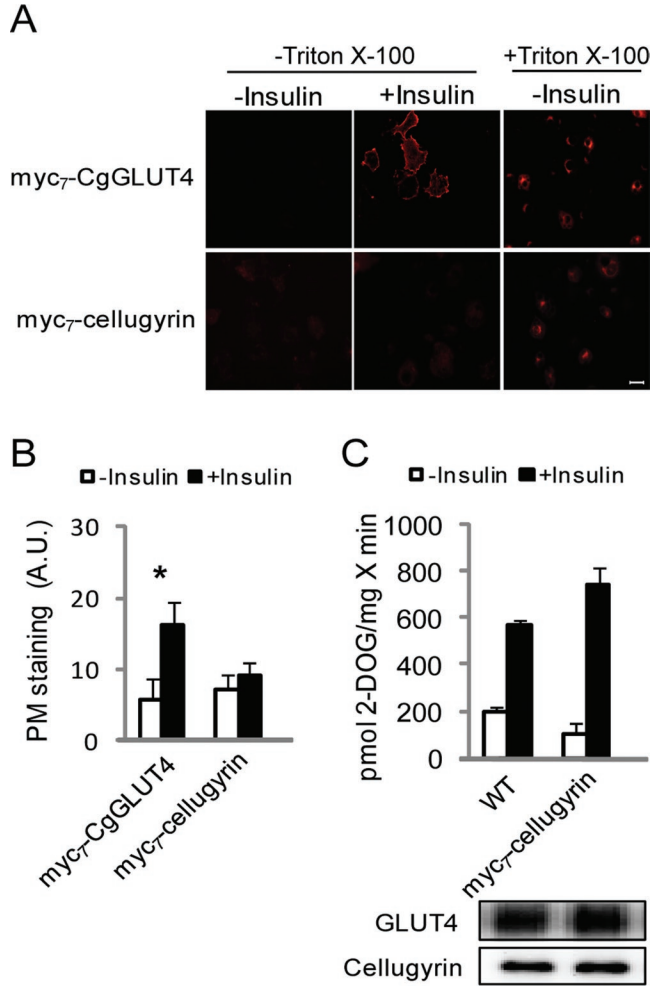

FIGURE 2: myc$_7$-CgGLUT4 but not myc$_7$-cellugyrin translocates to the plasma membrane in response to insulin stimulation. (A) myc$_7$-CgGLUT4– and myc$_7$-cellugyrin–expressing adipocytes were serum-starved for 3 h, treated with 100 nM insulin or carrier for 10 min, and stained with monoclonal anti-myc antibody. Right (staining in the presence of Triton X-100), total expression levels of the reporter proteins. Scale bar: 20 μm. (B) Quantitative analysis of translocation from three individual experiments. *, $p < 0.0001$. (C) [^{3}H]2-deoxyglucose (2-DOG) uptake in wild-type (WT) and myc$_7$-cellugyrin–expressing 3T3-L1 adipocytes. The figure shows normalized mean value ± SE of three experiments. Bottom, expression levels of endogenous GLUT4 and cellugyrin in analyzed cells.

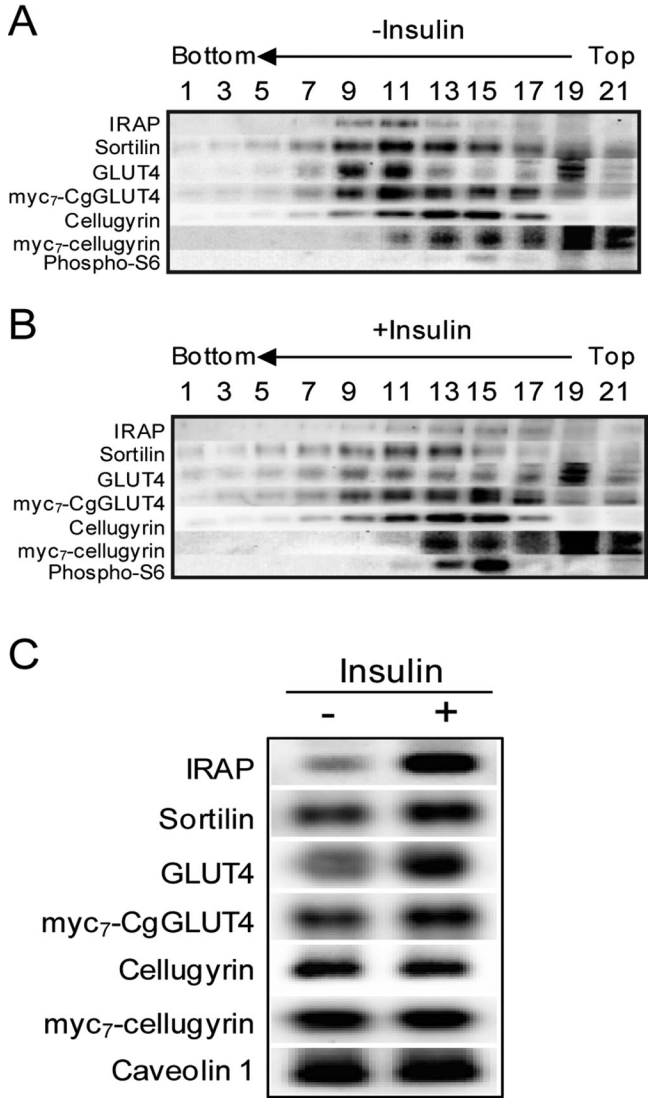

FIGURE 3: myc$_7$-CgGLUT4 is incorporated into the IRVs. myc$_7$-CgGLUT4– and myc$_7$-cellugyrin–expressing adipocytes were serum-starved for 3 h, treated with carrier (A) or 100 nM insulin (B) for 10 min, and fractionated in a 10–35% sucrose velocity gradient. Proteins were analyzed in odd gradient fractions by Western blotting. Arrows indicate the direction of sedimentation. The plasma membrane fraction isolated from cells treated or not treated with insulin was analyzed by Western blotting (C).

adipocytes with the help of lentiviral vectors. Both reporter proteins showed significant colocalization with the endogenous GLUT4 and endogenous syntaxin 6 (Figure 1, B and C), which represents a marker of the perinuclear GLUT4-containing compartment in adipocytes (Perera *et al.*, 2003; Shewan *et al.*, 2003). However, only the myc$_7$-CgGLUT4 demonstrated insulin-dependent translocation to the plasma membrane in response to insulin stimulation (Figure 2, A and B). Since expression of myc$_7$-cellugyrin may somehow interfere with insulin responsiveness of adipocytes, we measured insulin-stimulated glucose uptake in wild-type and in myc$_7$-cellugyrin–expressing cells. As there was no significant change in glucose uptake between these two cell lines (Figure 2C), we conclude that expression of myc$_7$-cellugyrin does not affect the endogenous GLUT4-transporting machinery. To exclude other possible artifacts of this experiment, we changed the method of the intracellular delivery of cDNA from lentiviral infection to electroporation. Also, since the myc$_7$ tag in the first luminal loop of cellugyrin can potentially interfere with cellugyrin's insulin responsiveness and diminish the value of this construct as a control, we reduced the number of myc epitopes on cellugyrin from seven to one and switched its position from the first intracellular loop to the C-terminus of the molecule. None of these changes, however, altered the results of the experiment (Figure S2). Thus the first luminal loop may confer insulin responsiveness to the GLUT4 molecule.

To explain this effect, we carried out biochemical fractionation of stably transfected cells treated and not treated with insulin in a continuous sucrose gradient. As is seen in Figure 3A, the major component proteins of the IRVs—GLUT4, IRAP, and sortilin—cosediment in fractions 9–13, whereas cellugyrin is localized in a lighter vesicular compartment and is recovered in fractions 11–17 (see also Li *et al.*,

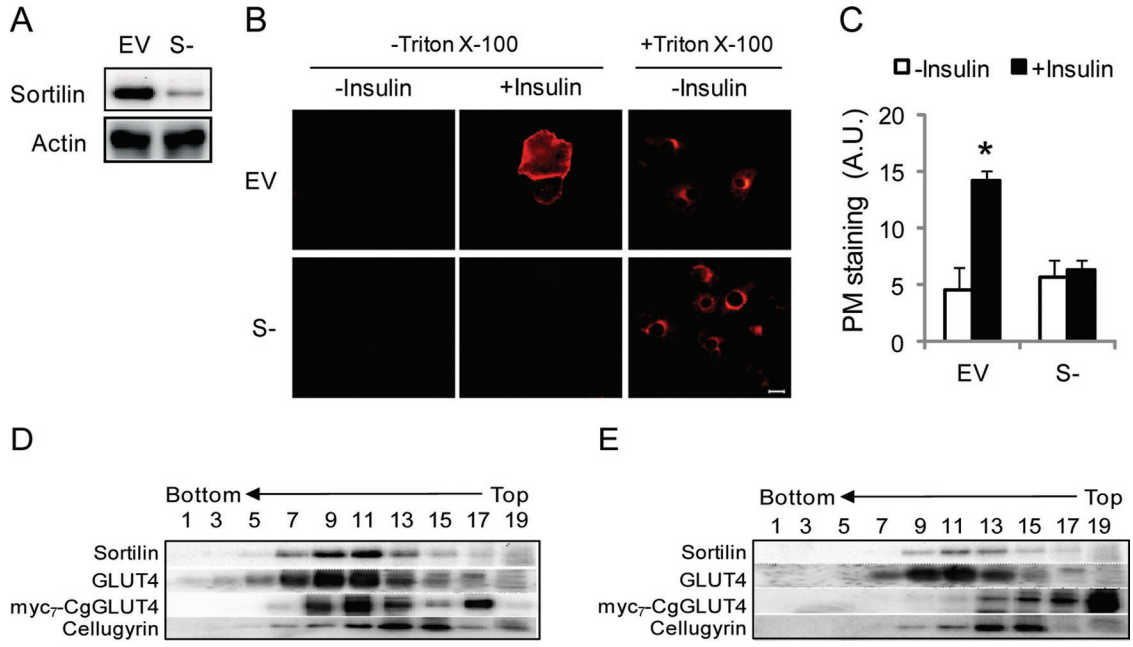

FIGURE 4: Sortilin is required for insulin-dependent translocation of myc$_7$-CgGLUT4 in stably transfected 3T3-L1 adipocytes. (A) Expression of sortilin was analyzed by Western blotting in EV and S$^-$ adipocytes. (B) myc$_7$-CgGLUT4 was stably expressed in sortilin-depleted (S$^-$) or control (EV) 3T3-L1 adipocytes. Serum-starved cells were treated with 100 nM insulin or carrier for 10 min and stained with monoclonal anti-myc antibody. Right (staining in the presence of Triton X-100), total expression levels of myc$_7$-CgGLUT4 in different cells. Scale bar: 20 μm. (C) Quantitative analysis of translocation from three individual experiments. *, $p < 0.0001$. (D and E) myc$_7$-CgGLUT4–expressing EV (D) and S$^-$ (E) adipocytes were serum-starved for 3 h and fractionated in a 10–35% sucrose velocity gradient. Proteins were analyzed in odd gradient fractions by Western blotting. Arrows indicate the direction of sedimentation.

2009; Jedrychowski *et al.*, 2010). The distribution of ectopically expressed myc$_7$-cellugyrin overlaps with endogenous cellugyrin (signal on top of the gradient in fractions 19–21 is likely to be nonspecific). Interestingly, the myc$_7$-CgGLUT4 chimera is distributed between the IRVs and cellugyrin-containing vesicles, suggesting that it may be localized in both compartments.

On insulin administration (note phosphorylation of the ribosomal protein S6), the IRVs fuse with the plasma membrane. Correspondingly, the amount of GLUT4, IRAP, and sortilin in the IRV zone (fractions 9–13) is decreased (Figure 3B). The amount of intracellular cellugyrin does not change, as cellugyrin-containing vesicles do not respond to insulin stimulation (Kupriyanova and Kandror, 2000; Kupriyanova *et al.*, 2002; Jedrychowski *et al.*, 2010). The amount of the myc$_7$-CgGLUT4 chimera is decreased in the IRV zone (fractions 9–13) but shows no change in the zone of cellugyrin-containing vesicles (fractions 15–17), suggesting that it is translocated to the plasma membrane by the IRVs. Biochemical isolation and analysis of the plasma membrane fraction from insulin-treated and untreated adipocytes (Figure 3C) have confirmed this conclusion. Note that the presence of endogenous cellugyrin and myc$_7$-cellugyrin in the plasma membrane fraction is most likely explained by inevitable endosomal contaminations.

To determine whether sortilin is essential for the targeting of the myc$_7$-CgGLUT4 chimera, we used 3T3-L1 adipocytes in which expression of sortilin was stably suppressed with the help of short hairpin RNA (shRNA; Shi and Kandror, 2005; Figure 4A). These S$^-$ cells and control empty vector (EV)-infected adipocytes were stably transfected with myc$_7$-CgGLUT4 with the help of the lentiviral expression vector (Figure 4, B and C). Alternatively, myc$_7$-CgGLUT4 was transiently expressed in S$^-$ and EV adipocytes by electroporation (Figure S3). In both stably and transiently transfected adipocytes, translocation of the myc$_7$-CgGLUT4 chimera took place only in sortilin-expressing cells, but not in the absence of sortilin (Figures 4, B and C, and S3). Sucrose gradient centrifugation demonstrated that targeting of myc$_7$-CgGLUT4 to the IRVs in S$^-$ cells is severely impaired (Figure 4, D and E), which likely explains the lack of insulin responsiveness of the chimera in sortilin-depleted cells.

Thus expression of sortilin is necessary for the faithful targeting and insulin responsiveness of myc$_7$-CgGLUT4. To determine whether it is sufficient for these effects, we carried out an experiment with undifferentiated myc$_7$-CgGLUT4-preadipocytes that do not express sortilin (Lin *et al.*, 1997; Morris *et al.*, 1998). These cells were electroporated with either cDNA for enhanced green fluorescent protein (EGFP) alone, or with the mixture of cDNAs for EGFP and sortilin. As expected, myc$_7$-CgGLUT4 shows no insulin responsiveness in EGFP-transfected cells; however, ectopic expression of sortilin confers strong insulin sensitivity to myc$_7$-CgGLUT4 in undifferentiated preadipocytes (Figure 5). We have determined previously that sortilin has an analogous effect on GLUT4 upon coexpression in undifferentiated 3T3-L1 preadipocytes and in several other cell types (Shi and Kandror, 2005). On the basis of these results, we conclude that targeting of GLUT4 to the IRVs is primarily mediated by the interaction between its first luminal loop and sortilin.

DISCUSSION

The search for the targeting sequences in GLUT4 has always been focused on the cytoplasmic regions of the molecule. Recent work, however, has revealed that formation of the IRVs requires expression of the putative sorting receptor, sortilin (Shi and Kandror, 2005; Ariga *et al.*, 2008). Since the protein-binding Vps10p domain of

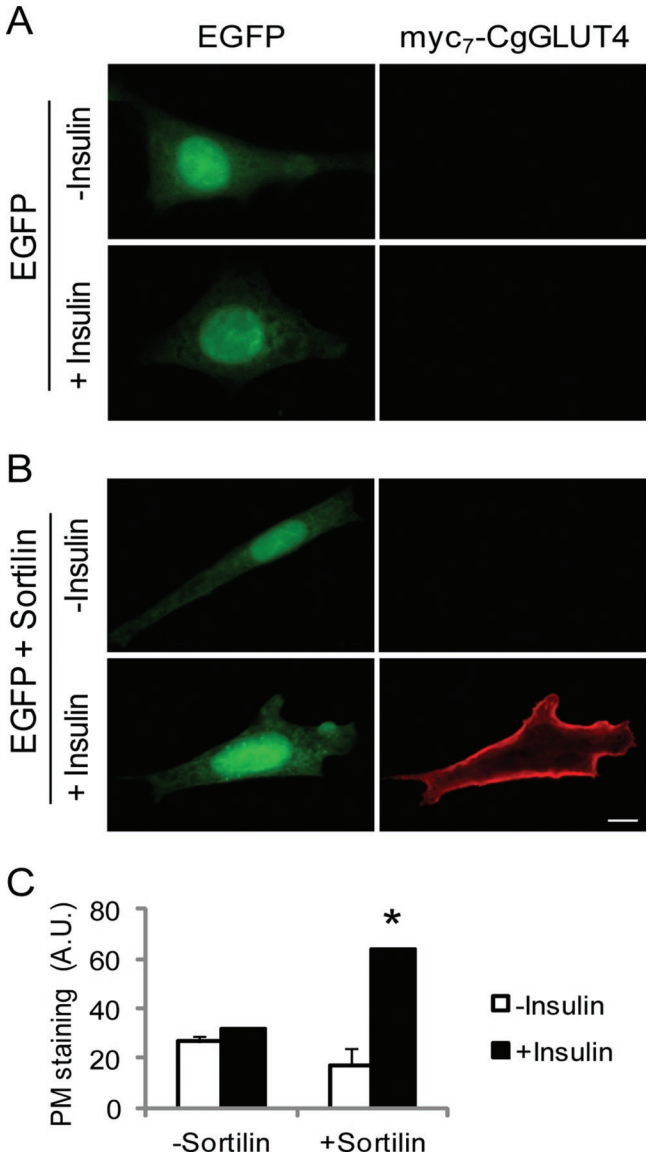

FIGURE 5: Sortilin is sufficient for insulin-dependent translocation of myc$_7$-CgGLUT4 in undifferentiated 3T3-L1 preadipocytes. Undifferentiated myc$_7$-CgGLUT4 preadipocytes were electroporated with either EGFP (A) or with EGFP and sortilin (B). After 2 d, cells were serum-starved for 3 h and stimulated with either insulin (100 nM) or carrier (5 mM HCl) for 10 min. Cells were stained with monoclonal anti-myc antibody, which was followed by Cy3-conjugated donkey anti-mouse secondary antibody in the absence of Triton X-100. Scale bar: 20 μm. (C) Quantitative analysis of translocation. *, $p < 0.0001$.

sortilin is localized in the vesicular lumen, we have suggested that sortilin recruits GLUT4 into the IRVs by interacting with its luminal domain(s). This hypothesis has been further supported by the observation that sortilin physically interacts with the first luminal loop of GLUT4 (Shi and Kandror, 2005; Figure S1). Thus, to determine the role of the first luminal loop of GLUT4 in its targeting to the IRVs, we exchanged it with the first luminal loop of cellugyrin. For the following reasons, we believe that cellugyrin represents an appropriate reporter molecule for such studies. GLUT4 and cellugyrin are naturally coexpressed in adipocytes and share the same general molecular design, having several transmembrane domains. However, GLUT4 and cellugyrin are localized in different populations of intracellular vesicles that can be separated by gradient centrifugation (Figure 3; see also Kupriyanova *et al.*, 2002; Li *et al.*, 2009; Jedrychowski *et al.*, 2010). Most importantly, cellugyrin-containing vesicles are resistant to insulin stimulation, and cellugyrin is not translocated to the plasma membrane after insulin stimulation (Figure 2; see also Kupriyanova and Kandror, 2000; Kupriyanova *et al.*, 2002; Li *et al.*, 2009; Jedrychowski *et al.*, 2010).

Our results demonstrate that cellugyrin with the first luminal loop of GLUT4 (myc$_7$-CgGLUT4) is partially targeted to the IRVs (Figures 3 and 4D) and is translocated to the plasma membrane in response to insulin. Importantly, ablation of sortilin expression blocks insulin responsiveness of myc$_7$-CgGLUT4 (Figure 4), while ectopic coexpression of sortilin and myc$_7$-CgGLUT4 in undifferentiated fibroblasts is sufficient to confer insulin responsiveness to the latter construct.

How is it possible that myc$_7$-CgGLUT4 and myc$_7$-cellugyrin have different insulin responsiveness in spite of virtually identical intracellular localization (Figure 1)? The GLUT4/syntaxin 6–positive perinuclear region of the adipocyte in which both reporter proteins reside does not represent a homogeneous compartment. From the biochemical standpoint, perinuclear membranes represent a mixture of the IRVs and large "donor" membranes from which these vesicles originate (Shi *et al.*, 2008). The vesicular and donor compartments exist in a dynamic equilibrium (Karylowski *et al.*, 2004). Because of the compact nature of perinuclear membranes and the limited resolution of light microscopy, immunofluorescence-based techniques do not discriminate between small IRVs and the precursor donor membranes. Thus, if a protein is present in the same donor membranes as GLUT4 but is not sorted into the IRVs, it is likely to show a significant colocalization with GLUT4 by immunofluorescence microscopy, albeit much lower insulin responsiveness. Examples of such proteins may include syntaxin 6 (Perera *et al.*, 2003; Shewan *et al.*, 2003), vti1a (Bose *et al.*, 2005), and myc$_7$-cellugyrin (Figure 1).

According to our model, targeting of GLUT4 into the IRVs may require at least two distinct steps: 1) targeting into the perinuclear donor membranes, which may represent a subdomain of the TGN (Shewan *et al.*, 2003), and/or recycling endosomes (Karylowski *et al.*, 2004; Xiong *et al.*, 2010), and 2) targeting from the donor membranes into the IRVs per se. In other words, targeting of GLUT4 into the IRVs may be described in terms of "sorting for entry" and "sorting by retention" that have been previously proposed to explain biogenesis of secretory granules (Arvan and Castle, 1998).

Sorting for entry into the perinuclear IRV donor membranes primarily depends on the C-terminus of GLUT4 (Shewan *et al.*, 2003; Blot and McGraw, 2008; Song *et al.*, 2008; Li *et al.*, 2009; Xiong *et al.*, 2010). Replacement of the entire C-terminus or mutations in its targeting sequences decreases insulin responsiveness of GLUT4, as the mutant molecule is not accumulated in the IRV donor compartment and naturally has less chance of being packed into the IRVs (Shewan *et al.*, 2003; Blot and McGraw, 2008; Song *et al.*, 2008; Li *et al.*, 2009; Xiong *et al.*, 2010). By the same token, addition of the GLUT4 C-terminus to an irrelevant molecule, such as cellugyrin, increases its localization in the perinuclear donor membranes but is not sufficient for the efficient targeting into the IRVs (Li *et al.*, 2009). The latter step, or sorting by retention, may require luminal interactions between the major IRV components, such as GLUT4, sortilin, and IRAP (Shi *et al.*, 2008; Jordens *et al.*, 2010). Such a network of luminal interactions among the IRV component proteins may allow for their retention in the donor membranes in the form of clusters or aggregates. Following this step, the IRV proteins may distribute to the vesicular phase as a single entity with the help of Golgi-localized, γ-ear-containing, Arf-binding (GGA) and ArfGAP with coiled-coil,

ankyrin repeat and PH domain 1 adaptors that bind to the cytoplasmic regions of sortilin and GLUT4 correspondingly (reviewed in Kandror and Pilch, 2011). According to this model, sortilin plays the role of a transmembrane scaffold that interacts with the first loop of GLUT4 in the membrane lumen and recruits GGA-dependent vesicle-budding machinery to the cytoplasmic side of the membrane.

MATERIALS AND METHODS

Reagents and antibodies

Insulin and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum (BS) and fetal bovine serum (FBS) were from Atlanta Biologicals (Lawrenceville, GA). DMEM, Opti-MEM, D-PBS, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). SlowFade antifade solution and Alexa Fluor 488–conjugated donkey anti-rabbit immunoglobulin G (IgG) were from Molecular Probes (Carlsbad, CA). Monoclonal anti–mouse myc antibody, polyclonal anti–rabbit myc antibody, and polyclonal anti–phospho-S6 antibody were from Cell Signaling Technology (Danvers, MA). Anti–syntaxin 6 monoclonal antibody and anti-sortilin mouse antibody were from PharMingen, BD Biosciences (San Diego, CA). Rabbit polyclonal antibody against cellugyrin (Ac-CQNVETTEGYQPPPVY-OH) was raised and affinity-purified by BioSource International (Camarillo, CA; Xu and Kandror, 2002). Polyclonal antibodies against GLUT4 and IRAP were kind gifts of Samuel Cushman (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) and Paul Pilch (Boston University, School of Medicine, Boston, MA), respectively. Cy3-conjugated anti–mouse IgG was obtained from Jackson ImmunoResearch (West Grove, PA).

DNA manipulations

To obtain a myc$_7$-CgGLUT4 chimera containing myc$_7$-tagged first luminal loop of GLUT4, amino acids Glu-56–Cys-79 of cellugyrin were substituted with the GLUT4 sequence Gln-46–Gly-82 containing seven consecutive myc epitopes between Pro-66 and Gly-68 (a kind gift of Jonathan Bogan, Yale Medical School). For that, *Cla*1 and *Pac*1 restriction sites were engineered into GLUT4 and cellugyrin cDNAs. Briefly, Gln-46 and Ala-47 of GLUT4 and Glu-56 and Gly-57 of cellugyrin were replaced with the *Cla*1 restriction site by using nested PCR with the following primers: GLUT4 *Cla*1 forward primer (5′-GGAGTCATCGACGATCCACAGAAA-3′), GLUT4 *Cla*1 reverse primer (5′-CACTTTCTGTGGATCGATGATGACTCC-3′); cellugyrin *Cla*1 forward primer (5′-TGCATCTTCGGCATCGATTACATCAAC-3′), cellugyrin *Cla*1 reverse primer (5′-GTTGATGTAATCGATGCCGAAGATGCA-3′). Similarly, Leu-81 and Gly-82 of GLUT4 and Ala-78 and Cys-79 of cellugyrin were replaced with the *Pac*1 restriction site using the *Cla*1-bearing GLUT4 and cellugyrin cDNAs. The following primers were used: GLUT4 *Pac*1 forward primer (5′-CTCACCACCTTAATTAACCTCTCCGTG-3′), GLUT4 *Pac*1 reverse primer (5′-CACGGAGAGGTTAATTAAGGTGGTGAG-3′); cellugyrin *Pac*1 forward primer (5′-CGGAACGAAGTTAATTAACGCTACGGC-3′), cellugyrin *Pac*1 reverse primer (5′-GCCGTAGCGTTAATTAACTTCGTTCCG-3′). The myc$_7$-CgGLUT4 chimera was obtained by ligation of the GLUT4 insert into the cellugyrin cDNA. After that, the *Cla*1 and *Pac*1 restriction sites were erased also with the help of nested PCR. To erase the *Cla*1 site in the myc$_7$-CgGLUT4 cDNA, the forward primer was 5′-TGCATCTTCGGCAACGCCCCACAGAAA-3′ and the reverse primer was 5′-TTTCTGTGGGGCGTTGCCGAAGATGCA-3′. To erase the *Pac*1 site in the myc$_7$-CgGLUT4 cDNA, the forward primer was 5′-CTCACCACCTTATGGTGCCGCTACGGCAGC-3′, and the reverse primer was 5′-ACTGCCGTAGCGGCACCATAAGGTGGTGAG-3′. For the preparation of myc$_7$-cellugyrin, the cDNA for the myc$_7$-tag flanked by DraIII restriction sites was synthesized by Blue Heron Biotechnology (Bothell, WA) and ligated into the cellugyrin cDNA between Cys-69 and Val-70. pLenti-myc$_7$-CgGLUT4 and pLenti-myc$_7$-cellugyrin were obtained by subcloning myc$_7$-CgGLUT4 and myc$_7$-cellugyrin into the modified pLenti-m1 vectors (Shi and Kandror, 2005) using EcoR1 and Apa1 restriction sites.

Cell culture

Murine 3T3-L1 preadipocytes were cultured and differentiated as described previously (Stephens *et al.*, 1997). Briefly, cells were grown in DMEM containing 10% calf bovine serum. Two days after confluence, cells were transferred to the differentiation medium (DMEM with 10% FBS, 1.67 µM insulin, 1 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine). After 48 h, differentiation medium was replaced with DMEM containing 10% FBS. Differentiated adipocytes were used for experiments at day 5. The 3T3-L1 cells in which expression of sortilin was suppressed by constitutive production of corresponding shRNA (S$^-$ cells; Shi and Kandror, 2005) were maintained in DMEM with 10% BS and 3.75 µg/ml of puromycin as a selection marker.

Generation of stable cell lines

For the constitutive expression of myc$_7$-CgGLUT4 and myc$_7$-cellugyrin, wild-type 3T3-L1 preadipocytes or S$^-$ preadipocytes (Shi and Kandror, 2005) were infected with pLenti-myc$_7$-CgGLUT4 or pLenti-myc$_7$-cellugyrin according to the manufacturer's protocol. At least seven individual clones along with two pooled clones of infected cells were selected by treatment with 8 µg/ml of blasticidin and analyzed for expression of chimera proteins.

Subcellular fractionation

After 3-h starvation in serum-free DMEM, adipocytes were stimulated with either insulin (100 nM) or carrier (5 mM HCl) for 10 min, washed three times with warm HES buffer (250 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 µM aprotinin, 2 µM leupeptin, 1 µM pepstatin, 5 mM benzamidine, pH 7.4) and harvested in 500 µl HES buffer. Adipocytes were homogenized by 11 strokes in a ball-bearing homogenizer (European Molecular Biology Laboratory, Heidelberg, Germany) with 12-µm clearance. Homogenates were centrifuged at 1000 × *g* for 5 min to generate a postnuclear supernatant that was further centrifuged at 27,000 × *g* for 35 min in a Ti42.2 rotor (Beckman Coulter, Brea, CA) to produce high-speed supernatant (S2). For sucrose gradient centrifugation, 1 mg of S2 was layered on a 4.6-ml linear 10–35% (wt/vol) sucrose gradient in HES buffer. Gradients were centrifuged at 278,000 × *g* for 1 h in an AH-650 rotor using a Sorvall WX ultracentrifuge (Fisher Scientific, Asheville, NC). Fractions were collected from the bottom of the tube using a peristaltic pump and analyzed by SDS–PAGE and Western blotting. For isolation of the plasma membrane, adipocyte homogenates were centrifuged at 16,000 × *g* at 4°C for 20 min in an SA-600 rotor. The resulting pellet was thoroughly resuspended in 1 ml of HES buffer, laid on the sucrose cushion (4 ml of 1.12 M sucrose), and centrifuged in an AH-650 rotor at 11,600 × *g* at 4°C for 1 h using a Sorvall WX ultracentrifuge. The interphase layer was collected, resuspended in and brought to 15 ml with HES buffer, and centrifuged in an SA-600 rotor at 33,000 × *g* at 4°C for 20 min. The plasma membrane fraction was recovered in the pellet of the last centrifugation.

Immunofluorescence

Differentiated adipocytes were grown on coverslips coated with collagen IV (Sigma-Aldrich). Serum-starved adipocytes were treated with either insulin (100 nM) or carrier (5 mM HCl) for 10 min and fixed with

4% paraformaldehyde in PBS (pH 7.4) for 20 min. Fixed cells were permeabilized or not with 0.2% Triton X-100 in PBS for 3 min and stained overnight at 4°C with primary antibodies, which was followed by incubation with either Cy3-conjugated anti–mouse IgG or Alexa Fluor 488–conjugated anti–rabbit IgG for 1 h at room temperature. SlowFade antifade solution (Molecular Probes) was used for mounting cells on slides. Slides were examined with the help of the Axio Observer Z1 fluorescence microscope with the 40× Plan-Neofluar objective lens, numerical aperture 0.6, and 60× Plan-Apochromat objective lens, NA 1.4, equipped with the Hamamatsu digital camera C10600/ORCA-R2 and AxioVision 4.8.1 program with AxioVision 4 Module 3D Deconvolution (Carl Zeiss, Thornwood, NY).

Image analysis and statistics

In translocation experiments, cells were analyzed in three different experiments (at least nine coverslips and over 4000 cells per each experiment). The number of cells with clear plasma membrane staining was normalized by total number of myc-positive cells. Colocalization analysis was performed with the help of ImageJ software (National Institutes of Health [NIH]), and Student's *t* test was used to evaluate the statistical significance of results.

Glucose uptake assay

This assay was performed as described previously (Shi and Kandror, 2008). Briefly, adipocytes grown in six-well plates were washed three times with serum-free DMEM, which was followed by serum starvation for 3 h. Cells were then washed with Krebs-Ringer-HEPES (KRH) buffer without glucose (121 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 0.33 mM $CaCl_2$, 12 mM HEPES, pH 7.4) at 37°C and treated with 100 nM insulin or carrier (5 mM HCl) at 37°C for 15 min. Immediately after stimulation with insulin, [^{3}H]2-deoxyglucose was added to cells for 4 min. Reaction was stopped by aspiration of the radioactive media, and cells were rapidly washed with 2 ml of ice-cold KRH with 25 mM D-glucose. Cells were harvested in KRH buffer with 0.1% SDS (400 µl/well). Radioactivity of total-cell lysates (300 µl) was counted in a liquid scintillation counter (LKB-Wallac; Bromma, Sweden) and normalized by protein content.

cDNA constructs and alanine-scanning mutagenesis

Preparation of cellugyrin-myc cDNA and subcloning it into pcDNA3.1 was described previously (Li *et al.*, 2009). For alanine-scanning mutagenesis, groups of nine amino acids in the first luminal loop (EX1) of GLUT4 were replaced with alanines using the pGADT7-GLUT4-EX1 cDNA without the myc_7 tag (Shi and Kandror, 2007). Substitution of amino acids 46–54 with alanines was carried out by three consecutive PCR steps. The first one was carried out with the T7 forward primer and the reverse primer 5′-CTCATTGTAGCTCGCTGCAGCCGCCGCCGCAGCGGCAGCGAATTCACTGG-3′. For the second PCR step, the forward primer was 5′-GCCAGTGAATTCGCTGCCGCTGCGGCGGCGGCTGCAGCGAGCTACAATGA-3′ and the reverse primer was 5′-AGATGGTGCACGATGCACAG-3′ (also known as the 3′ AD-sequencing primer from the matchmaker Gal4 two-hybrid system 3; Clontech, Mountain View, CA). Products from the first two PCR steps were used as a template for the third round of PCR with the following primers: T7 sequencing primer and 3′ AD sequencing primer. The product of this third step was called Ala46-54. Ala55-63, Ala65-73, and Ala73-81 were generated using the same approach with the following pairs of primers: Ala55-63: forward primer 5′-GTGATTGAACAGGCCGCCGCTGCGGCGGCGGCGGCGCAGGGGCCTGA-3′, reverse primer 5′-TGGCTGGGGAGGGCGGCGGCTGCGGCAGCCGCCGCCGCCCCTCCAGGCAC-3′. Ala65-73: forward primer 5′-CCCAGCTCCATCGCTGCAGCCGCCGCCGCCGCCGCGGGGATCCTGAC-3′, reverse primer 5′-GGTGCCTGGAGGGGCGGCGGCGGCTGCCGCAGCCGCCGCCCTCCCCAGCC-3′. Ala73-81: forward primer 5′-CTCAGGCCCCTGCGCCGCCGCCGCCGCAGCAGCGGCGGCCTGTTCAATCA-3′, reverse primer 5′-CGTCAGGATCCCCGCGGCGGCGGCGGCGGCTGCAGCGATGGAGCTGG-3′. The preparation of the deletion mutant lacking amino acids 68–81 in the first luminal loop (Δ68–81) was carried out in three steps. First, DNA fragment encoding Met-1– Gln-67 was synthesized using pcDNA3.1-myc_7-GLUT4 with the T7 commercial forward primer and the reverse primer 5′-CGGAAAAGATGGCCACGGAGAGGGCCTTAAGCAAGTCCTCTTCAGAAATGAGCTT-3′. In parallel, DNA fragment encoding Ala-82–stop-510 was synthesized on the same pcDNA3.1-myc_7-GLUT4 using the forward primer 5′-CATTTCTGAAGAGGACTTGCTTAAGGCCCTCTCCGTGGCCATCTTTTCCGTGGGC-3′ and the BGH reverse primer. These two DNA fragments were used as a template for the third round of PCR to generate myc_7-GLUT4 cDNA Δ68–81 lacking 14 amino acids in the first luminal loop.

Yeast two-hybrid assay

Interaction between the first extracellular loop of Glut4, Ala46-54, Ala55-63, Ala65-73, Ala73-81, and the Vps10p domain of sortilin was examined using the Matchmaker GAL4 two-hybrid system 3 (Clontech). For that, the above-mentioned cDNAs were subcloned into the pGADT7 vector that encodes GAL4 transcription activation domain. Subcloning of the Vps10p domain of sortilin in the pGBKT7 vector that encodes GAL4 DNA-binding domain was described previously (Shi and Kandror, 2007). Transformation was carried out according to the manufacturer's instructions. Briefly, a mixture of 0.3 µg of each DNA and 5 µg of herring testes carrier DNA was added to 50 µl of competent cells. After that, 0.5 ml of polyethylene glycol/LiAc was added and the tubes were placed in a 42°C water bath for 15 min. Tubes were centrifuged for 15 s and supernatants were resuspended in yeast extract–peptone–dextrose plus liquid medium, which was followed by incubation at 30°C with shaking for 90 min. Competent cells were resuspended in 0.9% (wt/vol) NaCl and plated on Sabouraud dextrose agar (SDA)-Leu, SDA-Trp, SDA–Leu/-Trp, SDA–Leu/-Trp/X-α-gal, and SDA–Leu/-Trp/-His plates (MP Biomedicals, Solon, OH). After 4 d, colonies grown in the selection medium were counted, and results were expressed as normalized mean ± SE from three independent experiments.

Electroporation of 3T3-L1 cells

Equipment and reagents for this technique were purchased from Bio-Rad (Hercules, CA). Undifferentiated and differentiated 3T3-L1 cells were trypsinized, washed with D-PBS twice, and resuspended in 500 µl of electroporation buffer with 100 µg cDNA in a gene pulser cuvette with the 0.4-cm electrode gap. Electroporation was performed with the help of a Gene Pulser MXcell Electroporation System at 950 µF, 0.16 kV. After electroporation, 1 ml of DMEM containing 10% FBS was added to the cuvette, cells were left to recover for 10 min at room temperature, and were replated on collagen IV–coated cover slips (Fisher Scientific, Pittsburgh, PA).

Transient transfection of HEK293 cells

Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, ~80% confluent HEK293 cells were transfected with 1 µg of cDNA in the pcDNA 3.1 vector. After 2 d, cells were lysed in lysis buffer (20 mM Tris, pH 7.4, 120 mM NaCl, 1% Triton, 1 mM EGTA, 1 µM aprotinin, 2 µM leupeptin, 1 µM pepstatin, 5 mM benzamidine).

Gel electrophoresis and Western blotting

Proteins were separated in SDS–PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine. Following transfer, the membrane was blocked with 10% BSA in PBS with 0.5% Tween 20 for 1 h. Blots were probed overnight with specific primary antibodies at 4°C, which was followed by 1-h incubation at room temperature with horseradish peroxidase–conjugated secondary antibodies (Sigma-Aldrich). Protein bands were detected with the enhanced chemiluminescence substrate kit (Perkin Elmer-Cetus, Waltham, MA) using a Kodak Image Station 440CF (Eastman Kodak, Rochester, NY).

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