Insulin stimulates movement of sorting nexin 9 between cellular compartments: a putative role mediating cell surface receptor expression and insulin action

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SNX9 (sorting nexin 9) is one member of a family of proteins implicated in protein trafficking. This family is characterized by a unique PX (Phox homology) domain that includes a proline-rich sequence and an upstream phospholipid binding domain. Many sorting nexins, including SNX9, also have a C-terminal coiled region. SNX9 additionally has an N-terminal SH3 (Src homology 3) domain. Here we have investigated the cellular localization of SNX9 and the potential role it plays in insulin action. SNX9 had a cytosolic and punctate distribution, consistent with endosomal and cytosolic localization, in 3T3L1 adipocytes. It was excluded from the nucleus. The SH3 domain was responsible, at least in part, for the membrane localization of SNX9, since expression of an SH3-domain-deleted GFP (green fluorescent protein)–SNX9 fusion protein in HEK293T cells rendered the protein cytosolic. Membrane localization may also be attributed in part to the PX domain, since *in vitro* phospholipid binding studies demonstrated SNX9 binding to polyphosphoinositides. Insulin induced movement of SNX9 to membrane fractions from the cytosol. A GST (glutathione S-transferase)–SNX9 fusion protein was associated with IGF1 (insulin-like growth factor 1) and insulin receptors *in vitro*. A GFP–SNX9 fusion protein, overexpressed in 3T3L1 adipocytes, co-immunoprecipitated with insulin receptors. Furthermore, overexpression of this GFP–SNX9 fusion protein in CHOT cells decreased insulin binding, consistent with a role for SNX9 in the trafficking of insulin receptors. Microinjection of 3T3L1 cells with an antibody against SNX9 inhibited stimulation by insulin of GLUT4 translocation. These results support the involvement of SNX9 in insulin action, via an influence on the processing/trafficking of insulin receptors. A secondary role in regulation of the cellular processing, transport and/or subcellular localization of GLUT4 is also suggested.

Key words: glucose transport, GLUT4, insulin action, insulin receptor, SH3PX1, sorting nexin 9 (SNX9).

INTRODUCTION

The mechanisms mediating protein trafficking between cellular compartments in response to insulin remain unclear, although this represents a key aspect of cellular regulation by the hormone. Trafficking of the insulin receptor to and from the plasma membrane has the potential to modify the magnitude and nature of the cellular response to insulin. In one of its more major actions, the insulin-responsive glucose transporter, GLUT4, is moved to the cell surface in response to hormone stimulation to mediate the effects of the hormone on glucose transport. Recent years have seen the description of much of the machinery for exocytic trafficking, including that for GLUT4, and it has become apparent that many of the proteins involved are conserved throughout evolution and regulate multiple cell functions, such as cargo selection, vesicle fusion and targeting to different compartments. However, the proteins involved in endocytic trafficking, including those mediating the response to insulin, are less well characterized.

The sorting nexins are a family of recently described proteins implicated in protein trafficking (e.g. see [1,2]). SNX1 (sorting nexin 1) was originally identified in a yeast two-hybrid screen as an interactor with the EGF (epidermal growth factor) receptor kinase domain, and binds to a region containing a lysosomal targeting code [3]. Overexpression studies showed that SNX1, and the related sorting nexins SNX2 and SNX4, co-immunoprecipitate with receptors for EGF, insulin, platelet-derived growth factor and the long form of the leptin receptor [2], while SNX6 was shown to associate with the transforming growth factor β receptor [4]. Thus it is possible that these proteins are involved in a generalized way in the trafficking of receptors. Consistent with this postulated function, homologues of these proteins in yeast (i.e. Vps5p, Mvp1p and Grd19p) were shown to have roles in protein trafficking. For example, Vps5p, the yeast homologue of SNX1, was identified as a component of the 'retromer complex' involved in the retrograde transport of proteins from prevacuolar endosomes to the *trans*-Golgi network.

To date, 17 mammalian sorting nexins have been deposited with GenBank™, having been identified largely by homology searching of EST (expressed sequence tag) databases [1]. Each of these proteins is hydrophilic, and shares with other family members a conserved 70–110-residue PX (Phox homology) domain referred to as the SNX-PX domain [1]. PX domains contain a proline motif, PXXP, that has been proposed to interact with proteins containing SH3 (Src homology 3) domains. Upstream of this proline motif is a phospholipid interaction motif. The SNX3-PX domain was shown to have specificity for PtdIns3*P* [5], similar to the $p40^{phox}$ and $p47^{phox}$ PX domains [6,7]. Many of the SNX family also have a coiled domain downstream of the PX domain that may participate in membrane or protein binding. SNX9 additionally has an SH3 domain upstream of its SNX-PX domain [8]. Interaction of this domain with the PXXP motif within its own PX domain, or within the PX domain of other SNX9 molecules

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; FCS, foetal calf serum; GFP, green fluorescent protein; GST, glutathione S-transferase; IGF, insulin-like growth factor; IRS, insulin receptor substrate; MEM, minimal essential medium; PH, pleckstrin homology; PKB, protein kinase B; PX domain, Phox homology domain; SH3 domain, Src homology 3 domain; SNX9, sorting nexin 9; WASP, Wiskott–Aldrich syndrome protein.

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or other sorting nexins to form multimers, offer potential modes for regulation of activity. Additionally, a recent study [9] demonstrated that DSH3PX1 (*Drosophila* SNX9) interacts with itself most strongly through a mechanism involving the C-terminal coiled portion of the protein, suggesting that a dimerization motif exists in the C-terminal coiled tail.

Recent studies with DSH3PX1 suggest a mechanism by which its mammalian homologue, SNX9, might participate as a trafficking protein in insulin action. DSH3PX1 was shown to interact with WASP (Wiskott–Aldrich syndrome protein), a known regulator of the actin cytoskeleton that is also involved in endocytosis [10] and implicated in trafficking of GLUT4 [11]. It was also found to interact with AP-50 (also known as $\mu 2$) [9], one of the clathrin coat adaptor proteins that have been implicated in the endocytic trafficking of insulin receptors and GLUT4. Lastly, it has been shown to interact with Dock, the fly orthologue of the mammalian adaptor protein Nck [that interacts strongly with IRS-1 (insulin receptor substrate-1)] [9,12]. These interactions were dependent upon the tyrosine phosphorylation state, consistent with tyrosine kinase mediating the association. Interestingly, recent studies have shown that Nck and PtdIns(4,5)P_2 synergistically activate actin polymerization through N-WASP *in vitro* [13].

Insulin-stimulated glucose transport in muscle and fat is mediated by tyrosine phosphorylation of its receptor and a signalling cascade that results in the translocation of GLUT4 from intracellular vesicles located within the tubovesicular network to the plasma membrane. Several studies have suggested that this process involves actin rearrangement [14], and WASP has also been implicated in the process [15,16]. The insulin receptor and GLUT4 are endocytosed separately through clathrin-coated pits. Thus SNX9 is a candidate protein for involvement in insulin receptor recycling and/or the trafficking of GLUT4. The present paper explores this possibility and demonstrates (1) that the subcellular localization pattern of SNX9 changes in response to insulin moving out of the cytosol, (2) that SNX9 associates with the insulin receptor and that its overexpression reduces cell surface insulin receptor expression, and (3) that sequestration of SNX9 by antibody microinjection inhibits insulin-stimulated GLUT4 translocation. These studies are thus consistent with a role for SNX9 in insulin signalling.

EXPERIMENTAL

Antibodies

Rabbit polyclonal antibody against SNX9 (SH3PX1) was a gift from Dr Carl Blobel (Sloan Kettering Cancer Center, New York, NY, U.S.A.) [8]. For microinjection studies, the antibody was dialysed with five changes of 5 mM sodium phosphate, pH 7.2, 100 mM KCl prior to use. Prebleed control serum was processed in parallel. Polyclonal antibody against GST (glutathione S-transferase) was from Amrad Biotech. Anti-GFP (green fluorescent protein) A.v. Living Colours® monoclonal antibody (for immunoblotting) and polyclonal antibody (for immunoprecipitation) were from Clontech. CT-1 and 83-7 anti-(insulin receptor) monoclonal antibodies, and 23-60 and 1-2 anti-[IGF1 (insulin-like growth factor 1) receptor] monoclonal antibodies, were gifts from Dr Ken Siddle (University of Cambridge, Cambridge, U.K.) [17–19].

Constructs and plasmid preparation

Full-length SNX9 (SH3PX1) in pcDNA3 was kindly donated by Dr Linda Howard and Dr Carl Blobel (Sloan Kettering Cancer Center) [8] (GenBank accession number AF131214). SNX9 was cloned as a *Bam*HI-excised fragment to blunted *Xba*I fragment into the *Bgl*II/*Sma*I sites of pEGFP-C1 (EGFP is enhanced GFP). SNX9-(1–364) and SNX9-(63–595) peptides were generated by PCR from pcDNA3/SNX9 using primers that had engineered an *Eco*RI site in the forward primer, and a stop and *Kpn*I sites into the reverse primer. These were cloned as inserts into the *Eco*RI/*Kpn*I sites of pEGFP-C1. pGEX-4T-1/SNX9 was ligated and cloned as *Eco*RI/*Xho*I fragments out of pcDNA3/SNX9 and pGEX-4T-1. For retroviral infection of 3T3L1 cells, GFP–SNX9 was ligated as a blunted *Nhe*I/*Bam*HI fragment into the *Hpa*I/*Bam*HI sites of the retroviral expression vector pLXIN. GFP–SNX9-(63–595) was inserted into pLXIN the same way. GFP–SNX9-(1–364) was inserted into pLXIN as a blunted *Kpn*I/*Bam*HI fragment. A fusion protein of GST with PKB1^{PH} (protein kinase B1^{PH}, where PH denotes pleckstrin homology) was prepared from viral PKB1, obtained from Dr Philip Tsichlis (Fox Chase Cancer Center, Philadelphia, PA, U.S.A.), by insertion of PKB1^{PH} into pGEX4T-1. GST–Grp1^{PH} was prepared from pEGFPC1-Grp1^{PH} (a gift from Mark Lemmon, University of Pennsylvania, Philadelphia, PA, U.S.A.) by insertion of Grp1^{PH} into pGEX4T-3 (GST–Grp1^{PH}). Correct insertion of all constructs was checked by restriction enzyme analysis and sequencing across the cloning sites for the full-length constructs. Products produced by PCR were fully sequenced.

Cell culture

3T3L1 fibroblasts obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) were maintained and passaged as pre-confluent cultures in DMEM (Dulbecco's modified Eagle's medium; Sigma) containing 5 % (v/v) FCS (foetal calf serum; CSL Ltd.). Cells for differentiation were maintained at confluence for 48 h, then induced to differentiate by the addition of DMEM containing 5 % FCS, 4 μg/ml insulin, 0.25 mM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine. After 72 h, the induction medium was replaced with fresh FCS/DMEM containing 4 μg/ml insulin; the cells were maintained in DMEM with 5 % FCS for 3 days thereafter. Cells were used 7–14 days after initiation of differentiation, after which time > 90 % of fibroblasts had differentiated into mature adipocytes.

HEK293T cells were maintained in DMEM containing 5 % FCS. CHOT cells engineered to express approx. 1×10^6 human insulin receptors/cell were a gift from Dr W. Rutter (UCSF, San Francisco, CA, U.S.A.), and were maintained in α-MEM (α-minimal essential medium) containing 5 % FCS [20].

Retroviral infection and transfection

3T3L1 cells were infected with retrovirus generated from BOSC 23 cells transfected with SNX9 constructs in the replication-incompetent retroviral vector, pLXIN (Clontech). The pLXIN: SNX9 constructs (6–8 μg of DNA/175 cm^2 dish) were transfected into BOSC 23 packaging cells [21] (Clontech) using FuGENE™ (Roche). Medium was supplemented to 20 ml, and virus was harvested 48 h later and frozen in 5 ml aliquots. A 5 ml aliquot of virus supernatant was used to infect a 55 cm^2 dish of 3T3L1 fibroblasts. G418 (0.8 mg/ml; Gibco, Life Technologies) for selection was placed on cells 72 h after infection. Selection was further enhanced by flow cytometric selection of fluorescent cells on three successive passages. Cells were used as mixed populations. Parallel infections using virus from BOSC 23 cells transfected with pLEIN (Clontech; pLXIN vector encoding EGFP) were performed as a control. For experiments, 3T3L1 cells were

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used in the differentiated state. In one series of experiments, 293T cells were transfected using FuGENE™ as described above with the pEGFP-C1 constructs. In another series, CHOT cells were transfected with the pEGFP-C1 constructs, and selected for expression by flow cytometry on three successive passages, by which stage all cells were fluorescent; cells were used for experiments as mixed populations.

Purification of GST fusion proteins

GST–SNX9, GST–Grp1^{PH}, GST–PKB1^{PH} and other GST fusion proteins were produced in *Escherichia coli* and purified on glutathione resin according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Phospholipid binding assay

The binding of GST–SNX9, GST–Grp1^{PH}, GST–PKB1^{PH} and GST to phospholipids was assessed as described previously [22]. GST fusion proteins at 1 μg/ml (GST–SNX9 and GST) or 0.25 μg/ml (GST–Grp1^{PH} and GST–PKB1^{PH}), in 5 ml of Tris-buffered saline/0.05 % Tween 20 containing 3 % (w/v) fatty acid-free BSA, were incubated with phospholipids bound to nitrocellulose at the concentrations indicated overnight at 4 °C. Samples were washed in the same buffer three times and incubated with anti-GST antibody (1:1000) for 60 min prior to washing and incubation with anti-rabbit IgG conjugated to horseradish peroxidase (1:3000), followed by enhanced chemiluminescence detection with Supersignal® (Pierce).

Cell lysis, immunoprecipitation and *in vitro* binding studies

Cells in 55 cm^2 dishes were washed twice with ice-cold PBS, pH 7.4, and then lysed by the addition of 800 μl/dish of 50 mM Hepes buffer, pH 7.2, containing 150 mM NaCl, 1 mM EGTA, 10 % (v/v) glycerol, 1 % Triton X-100, 200 units/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 2 mM PMSF, essentially as detailed previously [23]. For receptor extraction following stimulation of cells with insulin or IGF1, 20 mM NaF and 100 μM sodium orthovanadate were also included in the lysis buffer. Dishes were incubated for 10 min on ice, and the lysate was collected, incubated for a further 5 min on ice and then clarified by centrifugation at 12 500 ***g*** for 2 min. Full-length SNX9 or domain mutants in the supernatants were immunoprecipitated by incubation overnight at 4 °C with 5 μl/ml anti-SH3PX1 antibody or anti-GFP antibody and 10 μl/ml Protein A–Sepharose (Zymed) slurry as appropriate. The Sepharose beads were pelleted by centrifugation, washed three times in 0.5 ml of 20 mM Hepes, pH 7.2, 150 mM NaCl, 0.1 % Triton X-100 and 10 % glycerol (HNTG buffer), and used for SDS/PAGE and Western transfer. Blots were probed with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:3000) with enhanced chemiluminescence (Supersignal®; Pierce) detection.

Interaction of SNX9 with insulin and IGF1 receptors

Insulin and IGF1 receptors were purified from two 10 cm dishes of CHOT cells (a gift from W. Rutter) and P6 cells (gift from R. Baserga, Thomas Jefferson University Philadelphia, PA, U.S.A.) respectively [24,25] following stimulation with or without 70 nM insulin or IGF1 respectively for 10 min at 37 °C. Cells expressing Y960F mutated insulin receptors (a gift from Dr Y. Kaburagi, Institute for Diabetes Care and Research, Asahi Life Foundation, Tokyo, Japan) [26] were also examined. The receptors were extracted from cell lysates prepared as described above by immunoprecipitation with 10 μg of antibody 83-7 [anti-(insulin receptor)] or antibody 1-2 [anti-(IGF1 receptor)] and 25 μl of Protein A–Sepharose for 6 h. Immunoprecipitated receptor was washed with four changes of HNTG buffer containing 100 μM sodium orthovanadate, divided into two equal portions and incubated with 10 μg of GST–SNX9 or GST alone overnight at 4 °C in 200 μl of HNTG buffer. The receptor preparations were then washed four times as described above, and binding of GST–SNX9 to the receptors was assessed after SDS/PAGE and Western blotting with anti-GST antibody as described for the phospholipid binding studies.

Insulin binding studies

Insulin binding to CHOT cells overexpressing GFP–SNX9 or GFP was assessed by time-resolved fluorescence with Eu-labelled insulin (P. E. Hoyne, T. E. Adams and C. W. Ward, unpublished work). Cells were seeded at 1×10^5 cells/well in a 48-well plate, allowed to adhere overnight, then serum-starved the following night in α-MEM/0.5 % (v/v) FCS. They were then incubated for 1 h in α-MEM supplemented with 1 % (w/v) BSA, washed in the same medium and incubated with tracer Eu-conjugated insulin at a dilution of 1:20 000 in 250 μl of α-MEM/1 % (w/v) BSA with increasing concentrations of unlabelled insulin from 1 pM to 1 μM. Cells were incubated for 2.5–3 h at room temperature, then washed four times with PBS. A 200 μl aliquot of Delfia® Enhancement Solution (Perkin Elmer Life Sciences) was added to each well, followed by incubation for 20 min, then 150 μl aliquots were read on a Wallac VICTOR^2 1420 Multilabel Counter (Perkin Elmer). Cell counts and total protein were measured in duplicate wells.

Subcellular fractionation of 3T3L1 adipocytes

The subcellular distribution of SNX9 was determined after preparation of cell fractions by differential centrifugation as previously described [27]. Three separate membrane fractions were collected, designated as plasma membranes, low-density microsomal membranes and high-density microsomal membranes. In addition, a cytosol fraction was collected.

Microinjection

Our procedure for microinjection of 3T3L1 adipocytes and quantification of insulin-stimulated GLUT4 translocation by confocal microscopy has been described elsewhere [28,29]. Briefly, cells grown to confluence and differentiated on coverslips were transferred for 45 min to Krebs–Ringer bicarbonate Hepes buffer, pH 7.4, containing 2 mM pyruvate, 0.5 % BSA and 2.5 mM glucose. Antibody in a buffer containing 5 mM sodium phosphate (pH 7.2)/100 mM KCl or buffer alone was microinjected over a 45 min period using a Zeiss automated injection system (Carl Zeiss) coupled to an Eppendorf microinjector. Micropipettes were prepared using a Sutter P-97 micropipette puller. Cells were transferred into fresh medium and allowed to recover for 60–90 min following injection, prior to stimulation with insulin (100 nM) for 15 min and analysis of GLUT4 translocation using the plasma membrane lawn assay. GLUT4 translocation in microinjected cells was compared with that in non-injected cells in the immediate vicinity on the same coverslip.

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Glucose transport assay

Glucose transport was measured as 2-deoxy-[U-^{14}C]glucose uptake, as described previously [30]. Cells at least 48 h post-differentiation in 24-well plates were washed twice in Krebs–Ringer bicarbonate Hepes buffer containing 1 % BSA, 2 mM pyruvate and half the normal calcium concentration (1.15 mM $CaCl_2$), pH 7.4, and then allowed to equilibrate in the incubator for 90 min. Insulin was added over the concentration range 0.07–7 nM for 30 min, and uptake of 5 mM 2-deoxy-[U-^{14}C]glucose was measured over the final 10 min.

GLUT4 and SNX9 plasma membrane lawn assay

Translocation of GLUT4 and SNX9 to the plasma membrane was determined using the plasma membrane lawn assay, as described previously [29,31–33]. Briefly, after cell treatment, 3T3L1 cells grown on coverslips were washed in poly-L-lysine, shocked hypotonically with three washes in 1:3 (v/v) membrane buffer (70 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 1 mM dithiothreitol, 30 mM Hepes, pH 7.2) and sonicated using a probe sonicator (Microson) at setting 0, in membrane buffer, to generate a lawn of plasma membrane fragments that remained attached to the coverslip. The fragments were then immunolabelled with an in-house rabbit anti-GLUT4 polyclonal antibody (R10; 1:100; generated against a peptide encompassing the C-terminal 19 amino acids of GLUT4) or anti-SNX9 (SH3PX1) antibody and Alexa 488 goat anti-rabbit secondary antibody (Molecular Probes; 1:300). Coverslips were visualized and imaged using an Optiscan confocal laser scanning immunofluorescence microscope. Data were analysed using ImageJ (NIH) imaging software. At least six fields were examined within any one experiment for each condition. Efforts were made to maintain confocal microscope gain settings over the period of experiments, although alterations in these as well as differences in 3T3L1 populations contribute to between-experiment variability.

RESULTS

SNX9 is expressed in 3T3L1 cells

The presence of SNX9 in 3T3L1 cells was investigated following immunoprecipitation of SNX9 from cell lysates with an anti-SNX9 antibody, SDS/PAGE, Western transfer and immunoblotting with the same antibody (Figure 1). Lysates from both fibroblasts and adipocytes were used to address potential effects of differentiation on expression levels of SNX9. In addition to the immunoprecipitating antibody bands seen in the prebleed serum control lane, a major band of molecular mass 78 kDa was detected, as well as a minor band of approx. 75 kDa. The 78 kDa band was of similar molecular mass to that reported by Howard et al. [8] for the protein in 293, COS-7 and HeLa cells. Insulin stimulation of 3T3L1 adipocytes for 30 min did not regulate the abundance of either form (results not shown). The 75 kDa band was not detected consistently in all experiments, indicating that it may represent a proteolytically cleaved form of the protein or a protein interacting non-specifically with the antibody. The band was unlikely to arise from cross-reaction of the antibody with other sorting nexins, because in preliminary studies we found that it failed to blot GST–SNX6 (results not shown).

Immunofluorescence was used to confirm the presence of SNX9 in 3T3L1 cells (Figure 2). Cells were fixed, permeabilized, and probed with the same polyclonal antibody used for immunoprecipitation (Figure 1), followed by Alexa 488 anti-rabbit secondary antibody, prior to analysis by fluorescence microscopy.

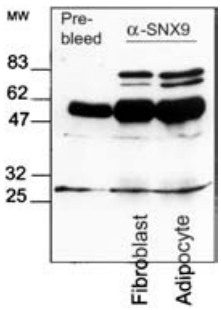

Figure 1 Western blot analysis of endogenous SNX9 in 3T3L1 fibroblasts and adipocytes

SNX9 was immunoprecipitated from lysates of 3T3L1 fibroblasts or adipocytes prepared from two 55 cm^2 dishes with 5 μl of anti-SH3PX1 polyclonal antibody (α-SNX9) and 20 μl of Protein A–Sepharose slurry. Prebleed serum was used for immunoprecipitation with adipocyte lysate to detect non-specific reactivity. Samples were analysed by Western blotting with the same antibody (1:1000) after SDS/PAGE on 10 % (w/v) acrylamide gels. The experiment was repeated three times. MW, molecular mass markers (kDa).

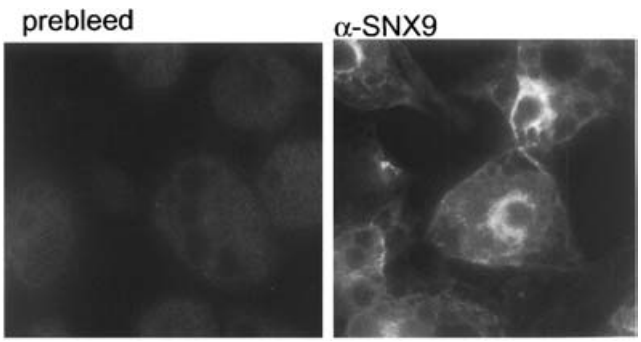

Figure 2 Immunolocalization of SNX9 in 3T3L1 adipocytes

3T3L1 adipocytes were fixed in 2 % paraformaldehyde, permeabilized and labelled with anti-SH3PX1 antibody (α-SNX9) (1:100), followed by Alexa 488 goat anti-rabbit antibody at a 1:1000 dilution. Representative fields are shown of cells analysed by fluorescence microscopy and digitally imaged.

Very little fluorescence was detected in cells probed with prebleed serum. Cells probed with anti-SNX9 (SH3PX1) serum showed some perinuclear staining, as well as punctate staining throughout the cytoplasm. The protein was excluded from the nucleus (Figure 2).

Insulin stimulates movement of SNX9 out of the cytosol and into membrane fractions

The effects of insulin on the cellular localization of SNX9 were investigated following subcellular fractionation of cells and analysis of SNX9 in fractions by immunoblotting (Figure 3). Endogenous SNX9 was expressed predominantly in the cytosol, but also to some extent in each membrane fraction collected (low-density microsomal, high-density microsomal and plasma membrane fractions) basally (Figure 3A), consistent with the immunofluorescence data in Figure 2. The 78 kDa band was the predominant band in the cytosol fraction, although bands isolated from the membranes were of both 78 and 75 kDa. Whether the difference in molecular mass was due to post-translational modification of the protein was unclear; however, variable extraction of the two bands in membrane fractions suggested that it was possibly due to proteolytic cleavage of the higher-molecular-mass form, even though high concentrations of protease inhibitors were used.

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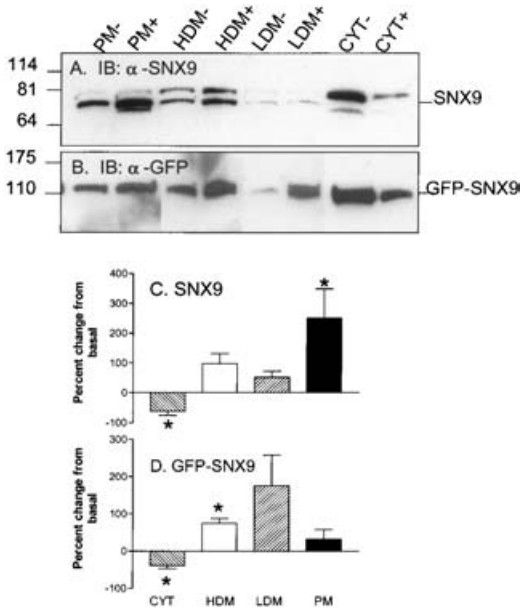

Figure 3 Subcellular localization of SNX9

3T3L1 adipocytes, uninfected (**A** and **C**) or expressing GFP–SNX9 (**B** and **D**), were serum starved overnight in DMEM/0.5 % (v/v) FCS and transferred to Krebs–Ringer bicarbonate Hepes buffer, pH 7.4, containing 1 % (w/v) BSA and 5 mM glucose for 90 min prior to treatment with (+) or without (−) 70 nM insulin for 20 min. Cells from ten 55 cm^2 dishes were fractionated by differential centrifugation as described in the Experimental section to yield plasma membrane (PM), cytosol (CYT), low-density microsomal (LDM) and high-density microsomal (HDM) fractions. Protein samples of 20 μg were analysed after SDS/PAGE and transferred to nitrocellulose by Western blotting (IB) with anti-SH3PX1 antibody (α-SNX9) (1:1000) (**A**) [8], or Living colours™ A.v. monoclonal anti-GFP antibody (**B**) with enhanced chemiluminescence detection. Similar loadings of samples treated or not with insulin were confirmed in parallel Coomassie Blue-stained gels (results not shown). Data from blots were scanned and analysed by densitometry using Image J software (NIH) (**C** and **D**) and are expressed as means ± S.E.M. of three or more experiments. Significance at $P < 0.05$ compared to basal is indicated (*). Left-hand side (**A** and **B**), molecular mass markers (kDa).

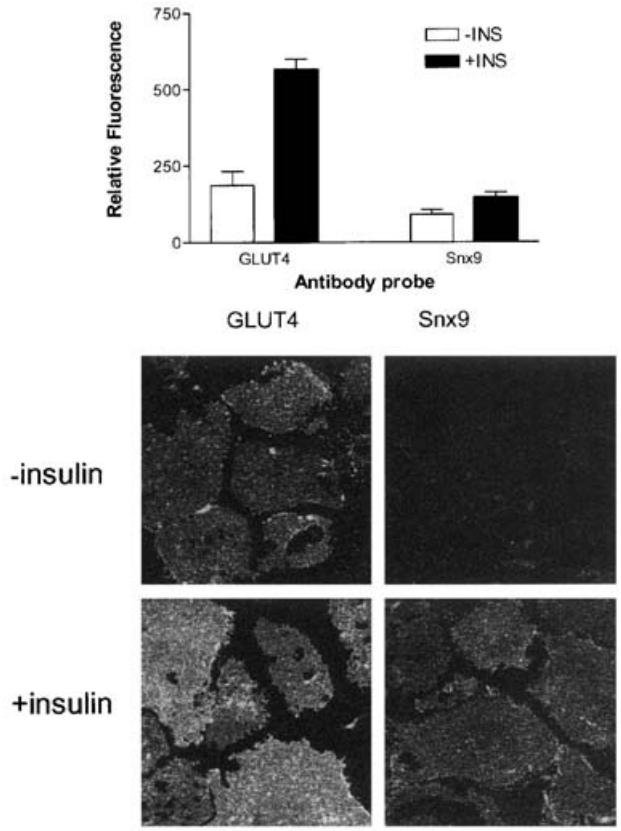

Figure 4 Insulin stimulates plasma membrane targeting of SNX9

Differentiated 3T3L1 adipocytes on coverslips were preincubated in Krebs–Ringer bicarbonate Hepes buffer, pH 7.4, containing 1 % BSA, and stimulated or not with 70 nM insulin (INS), as for the subcellular fractionation studies in Figure 3. The cells on coverslips were then washed briefly, and plasma membrane lawns were prepared on the coverslips on ice, as described in the Experimental section. The lawns were probed for the presence of GLUT4 with an anti-GLUT4 antibody (1:50) or for SNX9 with the anti-SH3PX1 antibody (1:50) and Alexa 488 anti-rabbit antibody prior to analysis by confocal microscopy and densitometric analysis of images by Image J. Results are means ± S.E.M. from analysis of six images for each condition within the same experiment. Similar results were obtained in two separate experiments.

Insulin stimulated the movement of SNX9 out of the cytosol fraction (amount decreased to 37.4 ± 12 % of basal; $P < 0.002$, $n = 7$), and into each of the membrane fractions, particularly the plasma membrane and high-density microsomal membrane fractions, although only the plasma membrane association reached statistical significance ($P < 0.05$, $n = 6$). Movement into the other membrane fractions was of borderline significance ($P < 0.1$, $n = 6$) in each case. The likelihood that the 75 kDa band is derived from the 78 kDa band is supported by the fact that the decreased cytosolic abundance of protein following insulin treatment is reflected by appropriate increases in membrane-associated lower-molecular-mass bands. Similar results were obtained in generated 3T3L1 cell lines that overexpressed GFP–SNX9 (Figures 3B and 3D). The major difference between GFP–SNX9 data and those for the endogenous protein was that more protein was membrane-associated basally with the former. Insulin stimulated movement of GFP–SNX9 out of the cytosol (to 61 ± 8 % of basal; $P < 0.01$, $n = 3$) and into each of the membrane fractions, particularly the high-density microsomal fraction ($P < 0.02$, $n = 3$), consistent with the results obtained for the endogenous protein. Movement into the low-density microsomal fraction was of borderline significance ($P < 0.1$, $n = 3$).

The movement of SNX9 to membrane fractions following stimulation by insulin suggests that it could be of significance in insulin action. SNX9 could be involved in the trafficking of insulin receptors and/or the trafficking of GLUT4, since insulin-stimulated GLUT4 vesicle translocation to the plasma membrane is the major mechanism by which insulin mediates its effects on glucose transport. The appearance of SNX9 at the plasma membrane was therefore addressed additionally by a plasma membrane lawn assay (Figure 4). 3T3L1 adipocytes on coverslips that had been stimulated or not with insulin were sonicated, leaving sheets of plasma membranes that were then probed for the presence of GLUT4, or SNX9, using immunofluorescence coupled with confocal microscopy. As we and others have reported in the past, insulin stimulated translocation of GLUT4 to the plasma membrane. A 3-fold stimulation of translocation was seen in response to insulin in the experiment shown. Using this approach, we also detected a 60 % increase in the level of SNX9 at the plasma membrane following stimulation by insulin. This result was consistent with the increased level of SNX9 seen in the plasma membrane in the fractionation studies (Figure 3).

Association of SNX9 with GLUT4 vesicles?

Since SNX9 was found to be recruited to membranes in response to insulin, we addressed the potential co-localization of SNX9

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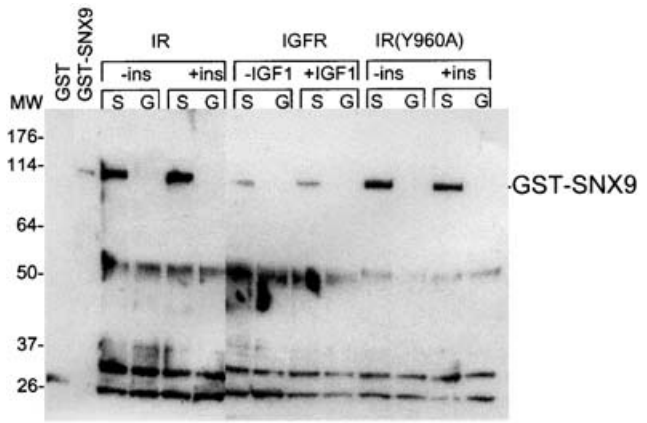

Figure 5 GST–SNX9 binds to insulin and IGF1 receptors

Insulin receptor (IR), Y960A mutated insulin receptor [IR(Y960A)] and IGF1 receptor (IGF1R) were immunoprecipitated with 10 μg of antibody 83-7 (against IR) or antibody 23-60 (against IGF1R) and Protein A–Sepharose, as described in the Experimental section. The immunopurified receptors were then incubated with 10 μg of GST–SNX9 (S) or GST (G) overnight at 4 °C. Protein bound to the immunoprecipitated receptors was analysed by SDS/PAGE and Western blotting with anti-GST antibody, as described in the Experimental section. Results are from a representative experiment that was repeated three times. MW, molecular mass markers (kDa).

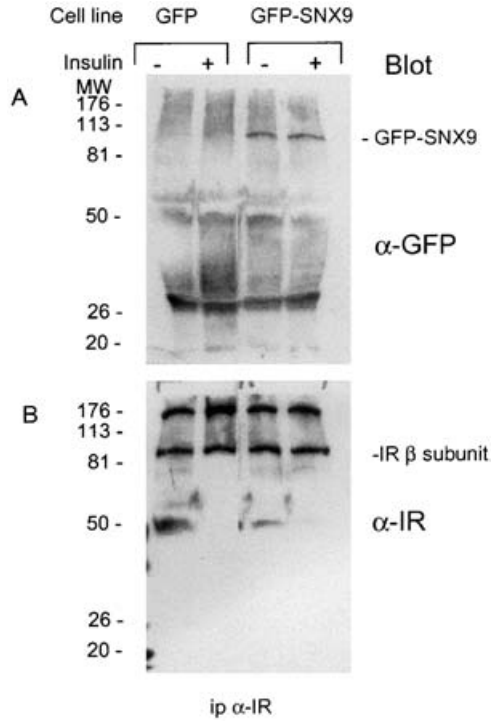

Figure 6 GFP–SNX9 associates with the insulin receptor in 3T3L1 adipocytes

Insulin receptor was immunoprecipitated with CT-1 antibody from 3T3L1 adipocytes expressing either GFP or GFP–SNX9, which had been treated with (+) or without (−) 70 nM insulin for 10 min. Association of GFP–SNX9 with immunoprecipitated receptors was analysed, following SDS/PAGE and Western transfer, by immunoblotting with anti-GFP antibody (**A**). Receptor abundance was assessed after stripping by reprobing blots with the CT-1 antibody (**B**). Results are from a representative experiment repeated twice. MW, molecular mass markers (kDa).

with GLUT4-containing vesicles that are translocated to the plasma membrane in response to insulin. GLUT4 vesicles in low-density microsomal fractions from 3T3L1 adipocytes were immunoprecipitated with 1F8 anti-GLUT4 antibody, and the potential association of SNX9 in these fractions was examined by probing Western blots with anti-SNX9 antibody after separation of proteins by SDS/PAGE. This methodology was used in the past by Kandror and Pilch [34] to identify GLUT4-interacting proteins. In our study, no SNX9 was detected (results not shown). Consistent with this finding, sucrose gradient centrifugation analysis of 3T3L1 extracts with anti-SNX9 and anti-GLUT4 antibodies showed SNX9 to separate at the top of the gradient in the free soluble protein fraction, away from membrane fractions including GLUT4 vesicles (K. V. Kandror and S. L. Macaulay, unpublished work). These results are perhaps not surprising, given that other peripheral membrane proteins, such as Rab4 and EEA1 (early endosome antigen 1), were not detected with this methodology in previous studies [35]. However, electron microscopic studies of 3T3L1 adipocytes with anti-SNX9 antibody detected with gold-labelled secondary antibody showed low-level association of SNX9 with endosomal compartments (R. Parton, personal communication).

Association of SNX9 with insulin and IGF receptors

Insulin and EGF receptors have been reported previously to bind SNX1, SNX2 and SNX4 [2]. We therefore assessed whether insulin or IGF receptors bound SNX9 (Figure 5). Recombinant GST–SNX9, or GST alone, was incubated overnight at 4 °C with immunopurified insulin or IGF receptors extracted from cells that had been stimulated or not with hormone for 10 min. SNX9 associated with receptors for both IGF1 and insulin. Association did not appear to be dependent on phosphorylation state of the receptors, since it was clearly demonstrated with receptors from both basal and hormone-stimulated cells. Interestingly, GST–SNX9 associated with receptors in which the Tyr-960 residue (the IRS1 binding residue and also part of an internalization motif) had been mutated to alanine (Figure 5). Binding to the Y960F mutant was also observed (results not shown). An important internalization motif, GPLY (Gly-Pro-Leu-Tyr), that lies just upstream of Tyr-960, represents an attractive candidate for further studies.

The potential association of SNX9 with insulin receptors was assessed further following immunoprecipitation of insulin receptors from 3T3L1 adipocytes with the CT-1 monoclonal antibody, and separation of receptor-associated proteins by SDS/PAGE, Western transfer and blotting with anti-SNX9 antibody. Only very low binding was detected (results not shown). To enhance detection, the experiment was repeated using 3T3L1 adipocytes engineered to overexpress GFP–SNX9 or GFP (as a control), as described in the Experimental section. GFP–SNX9 association was examined by blotting with anti-GFP antibody (Figure 6A). A band of approx. 110 kDa, consistent with the molecular mass of GFP–SNX9, was detected in insulin receptor immunoprecipitates of GFP–SNX9-expressing cells, but not GFP-expressing cells. There was no effect of insulin on association. Reprobing the blot for insulin receptor after stripping demonstrated similar receptor levels for each immunoprecipitation (Figure 6B). The reverse experiment, in which GFP–SNX9 was immunoprecipitated and analysed for insulin receptor binding, also demonstrated association (results not shown).

Since SNX9 associated with insulin receptors and previous studies had shown that overexpression of SNX1 decreased cell surface EGF receptor expression [3], we examined the effects on insulin binding to CHOT cells overexpressing GFP–SNX9 (Figure 7). CHOT cells were used, as these overexpress human insulin receptors [20]. CHOT cells expressing GFP–SNX9 showed

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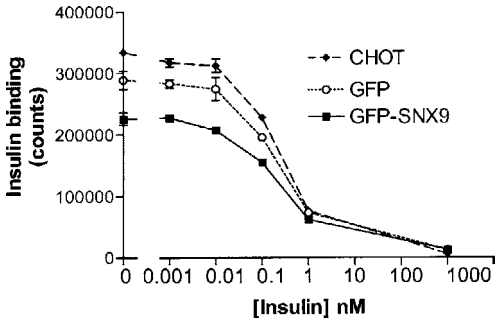

Figure 7 Overexpression of GFP–SNX9 decreases insulin binding to CHOT cells

CHOT cells expressing GFP–SNX9 or GFP alone were generated as mixed populations following transfection and selection of expressing cells by flow cytometry over three successive passages, as described in the Experimental section. Binding of Eu-labelled insulin to each cell population was analysed in displacement curves over the range of insulin concentrations indicated by time-resolved fluorimetry, as described in the Experimental section. Results are means ± S.E.M. of four determinations within the same experiment. Similar results were obtained in three separate experiments. Protein and cell counts confirmed similar levels in both GFP and GFP–SNX9 cells.

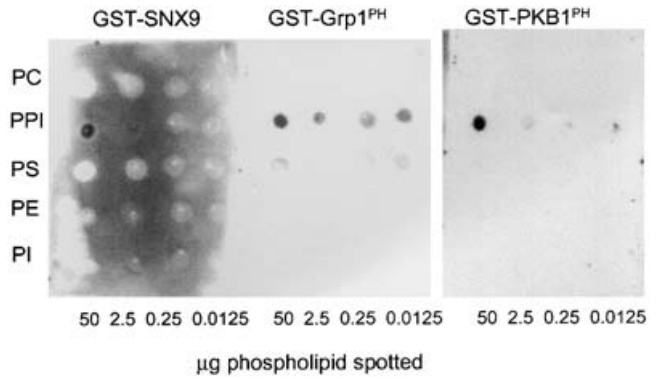

Figure 8 Phospholipid binding specificity of SNX9

The phospholipid binding specificity of GST–SNX9 fusion protein was assessed in a binding assay with phospholipids [phosphatidylcholine (PC), polyphosphoinositides (PPI), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI)] immobilized on nitrocellulose strips in the amounts indicated, as described in the Experimental section. Each strip was incubated in a 5 ml volume with GST–SNX9 or GST at 1 μg/ml, or GST–Grp1^{PH} or GST–PKB1^{PH} fusion proteins at 0.25 μg/ml. Binding was detected with anti-GST antibody (Amrad Biotech). No binding of GST to any phospholipid was detected (results not shown). Similar results were obtained in four separate experiments.

a small, but significant, reduction in binding of Eu-labelled insulin compared with cells expressing GFP or cells that had not been transfected with either construct. The experiment was repeated three times, with GFP–SNX9-expressing cells in each case displaying reduced binding compared with the other cell lines. Insulin binding to GFP-expressing cells was similar to or slightly higher than that to untransfected cells. Insulin binding to 3T3L1 cells expressing GFP–SNX9 could not be measured, as these cells had unacceptable inherent fluorescence associated with them that prevented analysis by the methodology used.

Targeting domains of SNX9

SNX9 shares a common SNX-PX domain with other members of the SNX family. Some PX domains have been shown to have specific phospholipid binding characteristics. Since insulin stimulates phosphoinositide 3-kinase activity, it was of interest to assess whether SNX9 membrane labelling was dependent upon binding to polyphosphoinositides. CHO cells or 3T3L1 adipocytes expressing full-length GFP–SNX9 were incubated in the presence or absence of 100 nM wortmannin for 30 min. The localization of SNX9 was then assessed. No differences in SNX9 localization were detected (results not shown). We therefore directly assessed the phospholipid binding specificity of SNX9 using GST–SNX9 produced in *E. coli*. Nitrocellulose blotted with various phospholipids was incubated with 0.25 μg/ml GST–Grp1^{PH} or GST–PKB1^{PH}, or 1 μg/ml GST or GST–SNX9. GST–SNX9 showed a high degree of specificity, and bound only to polyphosphoinositides (Figure 8). Very little binding was detected to phosphatidylinositol, phosphatidylserine or other phospholipids tested. GST–Grp1^{PH} domain and GST–PKB1^{PH} domain showed a similar specificity, binding predominantly to polyphosphoinositides. Grp1^{PH} has been reported previously to bind specifically to polyphosphoinositides, in particular PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, with lower affinity [36]. GST bound to none of the phospholipids tested (results not shown).

In addition to the SNX-PX domain that is conserved among SNX family members [1], SNX9 contains an N-terminal SH3 domain and a C-terminal helical region, either of which could mediate SNX9 localization. To test this possibility, GFP-fused deletion constructs were prepared in which either the SH3 domain or the C-terminal helical region was deleted. Transient expression of these constructs was then examined in HEK293T cells (Figure 9). Full-length GFP–SNX9 showed a punctate expression pattern, with some cytoplasmic expression, but was excluded from the nucleus, consistent with the fractionation data. The construct in which the C-terminal helical region was deleted showed the same expression pattern as the full-length construct. In contrast, the SH3 domain-deleted construct did not show punctate staining, and was found exclusively in the cytoplasm.

Effects of overexpression of SNX9 constructs on insulin stimulation of glucose transport

The effects of overexpressed truncated GFP–SNX9 proteins on insulin stimulation of glucose transport were examined in 3T3L1 adipocytes. These cells represented mixed populations that had been infected with retrovirus and sorted on the basis of GFP fluorescence. Insulin caused a 4–7-fold stimulation of glucose transport in each cell line tested that did not differ from that in untransfected cells. The lack of effect of these constructs on glucose transport indicates that any requirement for SNX9 in glucose transport is likely to be permissive. Moreover, expression of the SNX9 truncation constructs must not function in a dominant negative fashion.

Microinjection of anti-SNX9 antibody inhibits insulin-stimulated GLUT4 translocation

The effect of sequestering SNX9 on GLUT4 translocation in 3T3L1 adipocytes was examined following microinjection of anti-SNX9 polyclonal antibody in conjuction with the GLUT4 lawn assay, as described previously [29] (Figure 10). In this approach, 3T3L1 adipocytes were not injected, microinjected with anti-SNX9 antibody or microinjected with prebleed serum. Insulin stimulated GLUT4 translocation by approx. 3-fold in cells that had not been microinjected with antibody. Microinjection of control prebleed serum had no effect on this stimulation. Microinjection of anti-SNX9 antibody inhibited insulin-stimulated GLUT4

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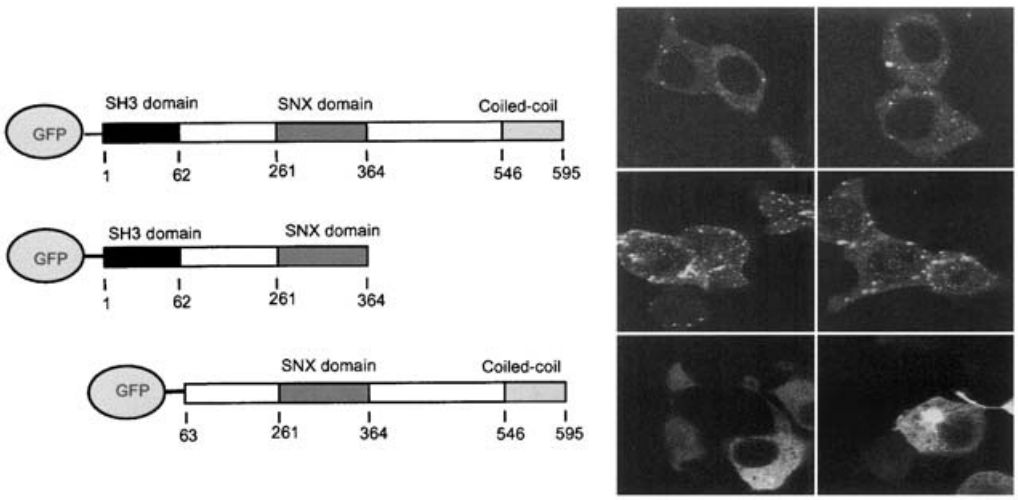

Figure 9 The SH3 domain directs membrane targeting of SNX9

293T cells were transiently transfected with each of the constructs shown on the left of the Figure in pEGFP-C1, as described in the Experimental section. GFP-expressing cells were examined after fixation in 2 % paraformaldehyde by confocal microscopy. Representative images of GFP–SNX9 (top panels), GFP–SNX9-(1–364) (middle panels) and GFP–SNX9-(63–595) (bottom panels) are shown.

translocation almost completely, supporting a functional role for SNX9 in GLUT4 trafficking or in insulin signalling to glucose transport.

DISCUSSION

The present study examined the cellular localization of SNX9 in relation to insulin action. Previous studies had demonstrated generalized, but varied, tissue distribution levels of SNX9 [8]. One of the highest expression levels was noted in skeletal muscle, a highly insulin-responsive tissue. In the present studies, SNX9 was isolated from 3T3L1 fibroblasts (poorly insulin responsive) and fat cells (highly insulin responsive) in approximately equivalent amounts, localized to both cytosolic and membrane compartments. The SH3 domain of SNX9 was found to be responsible in part for membrane targeting of the protein. SNX9 also showed avidity towards polyphosphoinositides, indicating that membrane targeting may also be manifested in part through the phospholipid binding motif within the SNX (SNX-PX) domain. We found that insulin caused redistribution of SNX9 out of the cytosol into membrane fractions. An overexpressed GFP-fused SNX9 protein showed a similar redistribution to membrane fractions in response to insulin. Overexpression of this full-length SNX9 protein, or the membrane targeting-defective SNX9 SH3 domain-deleted protein, failed to markedly affect insulin stimulation of glucose transport. Microinjection of an anti-SNX9 antibody into 3T3L1 cells, however, inhibited insulin-stimulated GLUT4 translocation, indicative of a role for SNX9 in insulin action, trafficking of GLUT4 and/or the insulin receptor. Consistent with the latter possibility, an SNX9 fusion protein, GST–SNX9, associated with purified insulin and IGF1 receptors *in vitro*, and GFP–SNX9 was immunoprecipitated with insulin receptor from 3T3L1 adipocytes engineered to express GFP–SNX9. Further, CHOT cells overexpressing GFP–SNX9 displayed reduced insulin binding compared with control, GFP-transfected or non-transfected cells.

The studies reported here are the first to investigate the cellular localization of SNX9. They show in 293T cells, consistent with studies of other sorting nexins, that SNX9 has a punctate membrane and cytosolic distribution and is excluded from the nucleus, consistent with an endosomal and cytosolic localization. In 3T3L1 adipocytes, SNX9 has a punctate and cytosolic distribution, as in 293T cells, and an additional perinuclear distribution that also is excluded from the nucleus. This cell distribution is perhaps not surprising, given that other members of the sorting nexin family have been shown to associate with themselves and with other sorting nexins [4,37]. The fly homologue of SNX9, DSH3PX1, was shown to self-associate via its C-terminal coiled domain [9]. For SNX1 and SNX5, membrane localization was also shown to be mediated by the C-terminal coiled domains [1]. The present studies show, surprisingly, that SNX9 targeting is mediated, at least in part, through its SH3 domain, since expression of SNX9 protein with a deletion of the SH3 domain rendered the protein cytosolic. A role for the PX domain in membrane targeting, however, cannot be excluded, since this domain was not deleted in any of the constructs tested. The SNX-PX domain itself is clearly not sufficient for membrane targeting, since the SH3-deleted protein that contained the SNX-PX domain was expressed only in the cytosol.

As indicated above, the SNX-PX domain may be involved, in part, in membrane targeting of the SNX family. For SNX9, it is unlikely to be the primary determinant. However, recent studies from several groups have demonstrated the phospholipid binding specificity of PX domains from a variety of proteins, including SNX3 and SNX4 [5,38]. Several of these proteins have high affinities for PtdIns3*P*, including SNX3 as well as the cytokine survival kinase CISK, Vam7P and $p40^{phox}$ [5–7,38–40]. Others have lower affinities for PtdIns3*P*, including SNX4 and Vps5p [38]. The C2-containing phosphoinositide 3-kinase reportedly binds specifically to PtdIns(4,5)P_2 [40], while $p47^{phox}$ binds preferably to PtdIns(3,4)P_2 [41]. Here we find that, like other PX domain-containing proteins, SNX9 has binding specificity for polyphosphoinositides, although the specificity of binding within this group of phospholipids could not be determined. The binding specificity was similar to that noted for the PH domains of Grp1 and PKB1. In whole cell expression studies, these PH domains are targeted to membranes when expressed as fusion proteins with GFP (D. F. Tucker and S. L.

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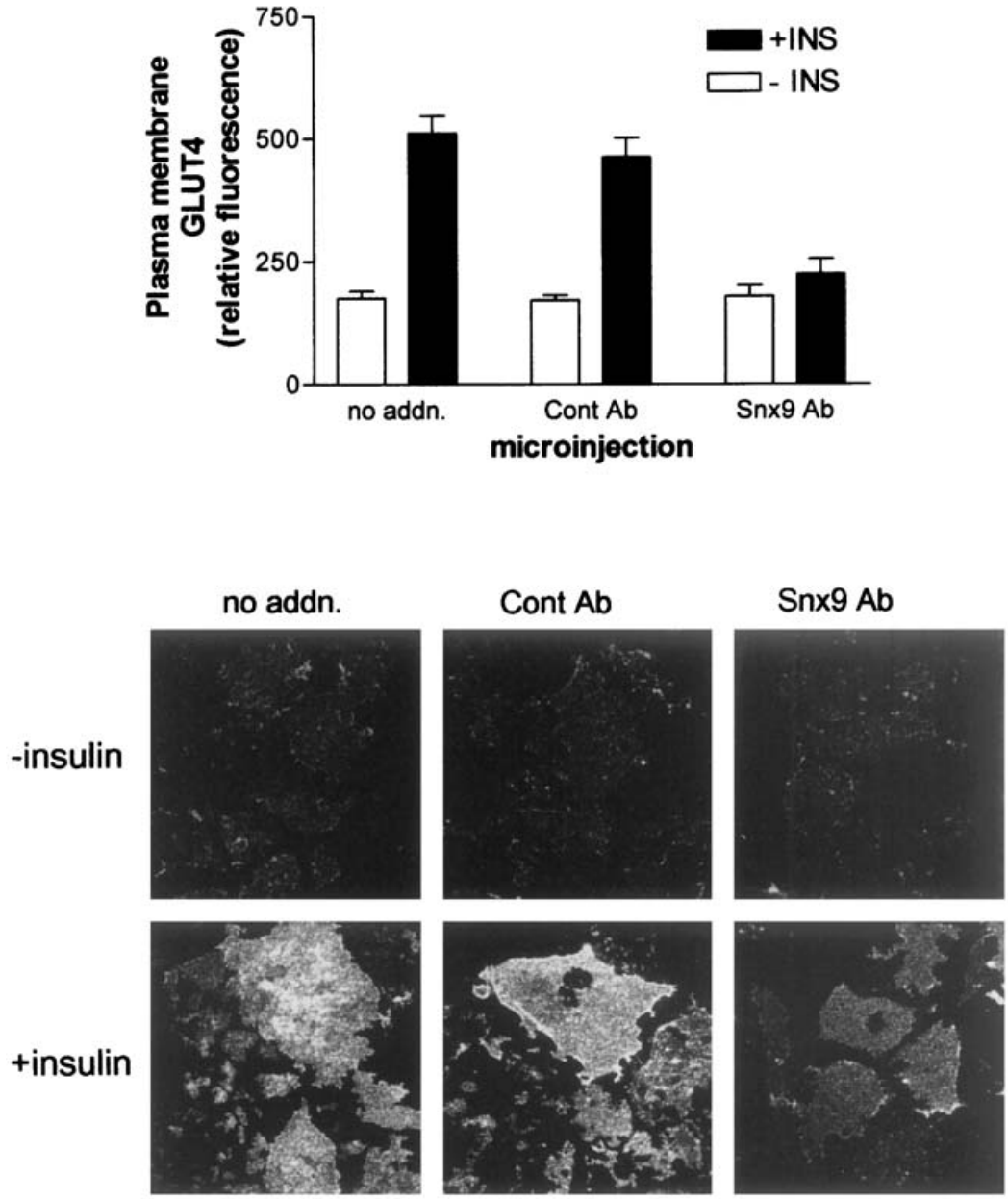

Figure 10 Microinjection of anti-SNX9 antibody into 3T3L1 adipocytes inhibits insulin-stimulated GLUT4 translocation

Differentiated 3T3L1 adipocytes were preincubated in Krebs–Ringer bicarbonate Hepes buffer, pH 7.4, containing 0.5 % BSA and 2.5 mM glucose, for 60 min. Cells were then microinjected with anti-SNX9 antibody (SNX9 Ab) or prebleed serum (Cont Ab), prepared for microinjection as described in the Experimental section. The buffer was then changed and the cells allowed to recover for 60 min prior to incubation in the presence or absence of 70 nM insulin for 15 min. GLUT4 translocation was then determined by a plasma membrane lawn assay, as described in the Experimental section. Data are means ± S.E.M. from a representative experiment in which fluorescence was measured in six separate fields for each condition. Representative lawns are shown for each condition; the experiment was repeated three times with similar results.

Macaulay, unpublished work). Our GFP-fused SNX9 protein that lacked the SH3 domain was located predominantly in the cytosol. Interestingly, the membrane targeting of two other SNX proteins, SNX1 and SNX5, was found to be mediated through the C-terminal coiled-coil domains [1,41] rather than the SNX-PX domain. These findings raise the question of the role of phospholipid binding in SNX function. For some sorting nexins, the PX domain is critically important for membrane targeting. Deletion of the SNX-PX domain from SNX15 resulted in it being mistargeted to the cytosol [37]. Endosomal association of SNX3 is dependent on its phospholipid binding, since a mutant lacking phospholipid binding activity, SNX3(Y71A), mainly localized to cytosol [5]. Interestingly, in preliminary studies examining the phospholipid binding specificity of the equivalent mutant of SNX9, SNX9(Y297A), we found no difference in its binding to phospholipid (V. Stoichevska and S. L. Macaulay, unpublished work). SNX3 has specificity for PtdIns3*P*, and this may be directed in part through the Tyr-297 residue. It seems likely that the phospholipid binding specificities of the various SNX proteins are involved in determining their membrane localization or in

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regulating their interaction with membranes. The 3′-phosphorylation specificity may imply a role for co-ordinated regulation by phosphoinositide 3-kinase that is mediated via hormone stimulation, whereas other specificities may dictate constitutive association and/or other forms of regulation.

Our finding that SNX9 has polyphosphoinositide lipid binding specificity has implications when considering how microinjection of anti-SNX9 antibody inhibits insulin-stimulated GLUT4 translocation. These effects could be manifested through effects of SNX9 on insulin receptor cycling, since the studies reported here demonstrated that SNX9 associates with insulin receptors. Alternatively, SNX9 may be involved in trafficking of GLUT4, even though we were unable to detect a direct association with GLUT4 vesicles. This inability to detect an association may not be surprising, given the previously reported difficulties in detecting association of peripheral membrane proteins with these vesicles in the past [35]. It is of interest in this regard that phosphoinositide 4-kinase has been reported to associate with GLUT4 vesicles [42,43]. It is thus possible, given the phospholipid binding specificity, that phosphoinositide 4-kinase may, in part, regulate association of SNX with GLUT4 vesicles.

The *Drosophila* homologue of SNX9, DSH3PX1, has recently been demonstrated to bind directly to the fly homologue of WASP [9], a multidomain protein that serves as a scaffold to bring signalling components together with machinery promoting actin polymerization and microfilament reorganization [44–46]. It also associates with Dock, the *Drosophila* homologue of the adaptor molecule Nck, which contains three tandem SH3 domains and an SH2 domain.

A potential interaction of SNX9 with mammalian N-WASP, although untested, raises the possibility that SNX9 may exert its effects through the actin cytoskeleton. N-WASP, when activated by the molecular switch cdc42 GTPase and PtdIns(4,5)P_2, induces long actin microspike formation and participates in vesicle trafficking by transmitting signals from tyrosine kinases to cause polarized rearrangement of cortical actin filaments [47]. *In vitro* studies have demonstrated that PtdIns(4,5)P_2 and cdc42 activate actin polymerization synergistically [13]. The translocation of GLUT4 has been linked with cortical actin remodelling [9,48,49]. Recent studies in cultured adipocytes show that expression of N-WASP-delta WA [N-WASP with a deletion of the WA domain, that includes the Arp (actin-related protein) and actin binding regions] attenuates cortical actin rearrangement by insulin and inhibits the action of insulin on GLUT4 translocation and glucose transport [11]. Interestingly, the small GTP binding protein TC10, which shows identity with cdc42, also binds N-WASP. Jiang et al. [11] showed, moreover, that an inhibitory TC10 mutant (T31N) that abrogates insulin-stimulated GLUT4 translocation [50] inhibits cortical localization of N-WASP. These studies support the possibility that WASP lies downstream of TC10 in the phosphoinositide 3-kinase-independent pathway to promote GLUT4 responsiveness. The demonstrated association between WASP and the *Drosophila* homologue of SNX9, DSH3PX1, places SNX9 as a potential linking molecule between GLUT4 vesicles, WASP and cortical actin remodelling induced by the association of TC10 with WASP. However, these issues remain to be addressed. They are consistent with the antibody microinjection data.

Although the above studies propose a potential mechanism for the involvement of SNX9 in GLUT4 translocation, we were unable to demonstrate a direct association of SNX9 with GLUT4 vesicles. An additional or alternative potential mechanism for the involvement of SNX9 in insulin-stimulated glucose transport is via its demonstrated association with the insulin receptor. The studies presented here demonstrated an *in vitro* association of a GST–SNX9 fusion protein with receptors for insulin and IGF1. This association was not dependent on the phosphorylation state of the insulin receptor, nor on insulin receptor residue Tyr-960, a key residue involved in IRS association and in insulin signalling. Association of SNX1, SNX2 and SNX4 with receptors for insulin and EGF has been demonstrated previously [2], and SNX1 has been implicated in the endocytosis of EGF receptors [3,51,52]. Our studies implicate SNX9 in the endocytosis of insulin receptors, since overexpression of GFP–SNX9 reduced insulin binding to CHOT cells significantly compared with that to untransfected CHOT cells or CHOT cells overexpressing GFP. Whether specific sorting nexins are involved in endocytic trafficking, or whether they form part of a signalling complex that includes multiple isoforms, remains to be seen; in support of the latter possibility, as discussed already, they do form oligomers with themselves and with other isoforms. It is unlikely that SNX9-mediated cortical actin changes postulated for GLUT4 could mediate the effects of SNX9 on insulin receptor binding, since proximal receptor events, including insulin receptor kinase, IRS phosphorylation and PKB activation, are unaffected by cortical actin remodelling [49]. However, the *Drosophila* homologue of SNX9, DSH3PX1, has been shown to co-immunoprecipitate with AP-50 [9], also known as $\mu 2$, part of the clathrin lattice complex involved in the endocytosis of a number of receptors (including the insulin receptor) and GLUT4 [33]. It thus may have a role in endocytosis, as proposed previously for SNX1.

If SNX9 is important in the mechanism of insulin-stimulated GLUT4 translocation, as suggested by the microinjection studies, the question arises as to why overexpressed SNX9 domain deletion constructs do not behave in a dominant negative manner, since deletion of the SH3 domain renders the protein cytosolic. It is possible that the co-localization of the protein with other components of the signalling complex, such as WASP, may be important for its interaction with other components. Mistargeting of overexpressed SNX9 to other cellular compartments may then not affect targeting of the endogenous protein to the signalling complex. The finding that overexpression of SNX9 inhibits insulin binding supports a role for SNX9 in receptor endocytosis, or alternatively in slowing trafficking of the insulin receptors to the plasma membrane, similar to that reported previously for SNX1 and the EGF receptor [3]. Our studies showed that this effect was not sufficient to affect insulin stimulation of glucose transport. However, microinjection of anti-SNX9 antibody into 3T3L1 cells, presumably resulting in the sequestration of SNX9, markedly inhibited insulin-stimulated GLUT4 translocation. These data suggest that SNX9 is involved additionally in trafficking of GLUT4 to either an insulin-responsive compartment or the cell surface in response to insulin.

In conclusion, the studies reported here identify SNX proteins, and SNX9 in particular, as interesting candidate proteins linking the insulin receptor and/or GLUT4 vesicles to WASP, clathrin and trafficking. The development of dominant negative constructs for SNX9 will be important for addressing further the role of SNX9 in insulin receptor and possibly also GLUT4 trafficking.

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