Sac3 Is an Insulin-regulated Phosphatidylinositol 3,5-Bisphosphate Phosphatase

GAIN IN INSULIN RESPONSIVENESS THROUGH Sac3 DOWN-REGULATION IN ADIPOCYTES

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Insulin-regulated stimulation of glucose entry and mobilization of fat/muscle-specific glucose transporter GLUT4 onto the cell surface require the phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2) pathway for optimal performance. The reduced insulin responsiveness observed under ablation of the PtdIns(3,5)P2-synthesizing PIKfyve and its associated activator ArPIKfyve in 3T3L1 adipocytes suggests that dysfunction of the PtdIns(3,5)P2-specific phosphatase Sac3 may yield the opposite effect. Paradoxically, as uncovered recently, in addition to turnover Sac3 also supports PtdIns(3,5)P2 biosynthesis by allowing optimal PIKfyve-ArPIKfyve association. These opposing inputs raise the key question as to whether reduced Sac3 protein levels and/or hydrolyzing activity will produce gain in insulin responsiveness. Here we report that small interfering RNA-mediated knockdown of endogenous Sac3 by ~60%, which resulted in a slight but significant elevation of PtdIns(3,5)P2 in 3T3L1 adipocytes, increased GLUT4 translocation and glucose entry in response to insulin. In contrast, ectopic expression of Sac3WT, but not phosphatase-deficient Sac3D488A, reduced GLUT4 surface abundance in the presence of insulin. Endogenous Sac3 physically assembled with PIKfyve and ArPIKfyve in both membrane and soluble fractions of 3T3L1 adipocytes, but this remained insulin-insensitive. Importantly, acute insulin markedly reduced the in vitro C8-PtdIns(3,5)P2 hydrolyzing activity of Sac3. The insulin-sensitive Sac3 pool likely controls a discrete PtdIns(3,5)P2 subfraction as the high pressure liquid chromatography-measurable insulin-dependent elevation in total [3H]inositol-PtdIns(3,5)P2 was minor. Together, our data identify Sac3 as an insulin-sensitive phosphatase whose down-regulation increases insulin responsiveness, thus implicating Sac3 as a novel drug target in insulin resistance.

Insulin simulation of glucose uptake in fat and muscle, which is mediated by the facilitative fat/muscle-specific glucose transporter GLUT4, is essential for maintenance of whole-body glucose homeostasis (1–7). In basal states GLUT4 is localized in the cell interior, cycling slowly between the plasma membrane and one or more intracellular compartments. Insulin action profoundly activates movements of preformed postendosomal GLUT4 storage vesicles toward the cell surface and their subsequent plasma membrane fusion, thereby increasing the rate of glucose transport >10-fold. Defective signaling-execution of GLUT4 translocation is considered to be a common feature in insulin resistance and type 2 diabetes (8, 9). However, the cellular and regulatory mechanisms whereby insulin activates GLUT4 membrane dynamics and glucose transport are not fully understood. More than 60 protein and phospholipid intermediate players are currently implicated in orchestrating the overall process (1–7). A central role is attributed to the highest phosphorylated member of the phosphoinositide (PI)3 family, i.e. phosphatidylinositol (PtdIns) (3,4,5)P3 (3). PtdIns(3,4,5)P3 is generated at the cell surface by the action of wortmannin-sensitive class 1A PI3K which is activated via the insulin-stimulated IR/IR receptor substrate signaling pathway. Inositol polyphosphate 5-phosphatases SHIP and SKIP and 3-phosphatase PTEN rapidly convert PtdIns(3,4,5)P3 to PtdIns(3,4)P2 and PtdIns(4,5)P2, respectively, thereby terminating insulin signal through class 1A PI3K (10–13). The class 1A PI3K-opposing function of these lipid phosphatases has provided an appealing prospect that inhibition of their hydrolyzing activities could produce significant efficacy in the treatment of type 2 diabetes and obesity (14–16).

It has recently become apparent that signals by other PIs act in parallel with that of PtdIns(3,4,5)P3 in integrating the IR-issued signal with GLUT4 surface translocation (3, 4). One such signaling molecule is PtdIns(3,5)P2, whose functioning as a positive regulator in 3T3L1 adipocyte responsiveness to insulin has been supported by several lines of experimental evidence. Thus, 3 The abbreviations used are: PI, phosphoinositide; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PIKfyve, phosphoinositide kinase for position five containing a fyve domain; ArPIKfyve, associated regulator of PIKfyve; Sac3, Sac domain-containing phosphatase 3; PAS, PIKfyve-ArPIKfyve-Sac3; PM, plasma membrane; IM, intracellular membrane; GFP, green fluorescence protein; eGFP, enhanced green fluorescence protein; IR, insulin receptor; GroPts, glycophosphoinositol; HA, hemagglutinin; 2DG, 2-deoxyglucose; HPLC, high pressure liquid chromatography; siRNA, small interfering RNA; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PTEN, phosphatase and tensin homolog.
expression of dominant-negative kinase-deficient mutants of PIKfyve, the sole enzyme for PtdIns(3,5)P₂ synthesis (17, 18), inhibits insulin-induced gain of surface GLUT4 without noticeable aberrations of cell morphology (19). Likewise, reduction in the intracellular PtdIns(3,5)P₂ pool through siRNA-mediated PIKfyve depletion reduces GLUT4 cell-surface accumulation and glucose transport activation in response to insulin (20). Concordantly, loss of ArPIKfyve, a PIKfyve activator that physically associates with PIKfyve to facilitate PtdIns(3,5)P₂ intracellular production (21, 22), also decreases insulin-stimulated glucose uptake in 3T3L1 adipocytes (20). Combined ablation of PIKfyve and ArPIKfyve produces a greater decrease in this effect, correlating with a greater reduction in the intracellular PtdIns(3,5)P₂ pool (20). Finally, pharmacological inhibition of PIKfyve activity powerfully reduces the net insulin effect on glucose uptake (23). These observations indicate positive signaling through the PtdIns(3,5)P₂ pathway and suggest that arrested PtdIns(3,5)P₂ turnover might potentiate insulin-regulated activation of glucose uptake.

Sac3, a product of a single-copy gene in mammals, is a recently characterized phosphatase implicated in PtdIns(3,5)P₂ turnover (24). Our observations in several mammalian cell types have revealed that Sac3 plays an intracellular role in the PtdIns(3,5)P₂ homeostatic mechanism. It is a constituent of the PtdIns(3,5)P₂ biosynthetic PIKfyve-ArPIKfyve complex and facilitates the association of these two (24, 25). Intriguingly, only if the PIKfyve-ArPIKfyve-Sac3 triad (known as the “PAS complex”) is intact will the PIKfyve enzymatic activity be activated (25). Thus, Sac3 not only catalyzes PtdIns(3,5)P₂ turnover but also promotes PtdIns(3,5)P₂ synthesis by functioning as an adaptor for the efficient association of PIKfyve with, and activation by, ArPIKfyve (25). Given these two seemingly opposing inputs, a critical question is whether reduction in Sac3 protein levels or phosphatase activity would facilitate or mitigate insulin action on glucose uptake and GLUT4 translocation. We demonstrate here that reduced levels of Sac3 potentiate, whereas ectopic expression of active Sac3 phosphatase reduces insulin responsiveness of GLUT4 translocation and glucose transport in 3T3L1 adipocytes. Whereas insulin action does not affect the PIKfyve kinase-Sac3 phosphatase association, it markedly inhibits the Sac3 hydrolyzing activity. We suggest that increased PtdIns(3,5)P₂ local availability through Sac3 phosphatase inhibition links insulin signaling to its effect on GLUT4 vesicle dynamics and glucose transport.

**EXPERIMENTAL PROCEDURES**

**Antibodies, siRNAs, and cDNA Constructs**—Polyclonal anti-PIKfyve (R7069; East-Acres, MA), anti-Sac3, and anti-ArPIKfyve antisera (WS047; Covance, Denver, PA), as characterized previously (18, 21, 24), were used for immunoprecipitation. For immunoblotting, the anti-ArPIKfyve antiserum was affinity-purified on recombinant ArPIKfyve as detailed previously (21). Monoclonal anti-α-tubulin antibody and anti-Myc producing 9E10.2 hybridoma cells were purchased from Sigma and ATCC, respectively. Polyclonal antibodies against GLUT4 (R4289) and HA epitope (R4289) were gifts by Dr. Mike Czech. Smart Pool™ siRNA duplexes targeting the mouse sequences of Sac3 (M-052024-00) or control mouse cyclophilin B (D-001136-01) were from Dharmacon. pEF-BOS-Myc- or pEGFP-Sac3WT and pEGFP-Sac3D488A constructs were described previously (24). pEGFP-HA-GLUT4 and the pcDNA3.1-Myc7-GLUT4 constructs, harboring HA- and 7-Myc epitopes, respectively, in the first exofacial loop were gifts by Drs. Jeff Pessin and Kostya Kandror.

**3T3L1 Cell Differentiation and Transfection**—Mouse 3T3L1 fibroblasts were differentiated into adipocytes following a standard differentiation protocol (25). 3T3L1 adipocytes were transfected by electroporation with the indicated siRNA duplexes or cDNA constructs on day 5 of the differentiation program as specified previously (20, 22). Electroporated cells were re-seeded onto 24-well (for glucose transport) or 6-well plates (for GLUT4 translocation, 32P labeling, or immunoblotting) and used 24–64 h post-transfection as specified below.

**Glucose Transporter Translocation and Fluorescence Microscopy**—These were performed essentially as we described previously (20, 25). Briefly, 3T3L1 adipocytes coelectroporated with the HA-GLUT4-eGFP cDNA (45 μg) and Sac3 siRNAs (0.2–0.4 nmol/5 × 10⁶ cells) or with Myc7-GLUT4 and eGFP-Sac3 cDNAs (25 μg each) were seeded on glass coverslips placed into a 6-well plate. Fifty five and 24 h post-transfection, respectively, cells were serum-starved (3 h) in DMEM and then incubated with insulin (0–100 nM; 30 min at 37 °C), washed with PBS, fixed in freshly prepared methanol-free formaldehyde (2% in PBS; 15 min), and stained with anti-HA (for HA-GLUT4-eGFP) or anti-Myc antibodies (for Myc7-GLUT4) without cell permeabilization to detect levels of cell-surface GLUT4 reporters. Anti-HA- and anti-Myc antibodies were visualized with Cy3-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) and Alexa568-conjugated mouse anti-IgG (Invitrogen), respectively. To detect Myc7-GLUT4 total expression, cells were subsequently permeabilized (0.5% Triton X-100 in PBS, 1% FBS), then incubated with the C-terminal GLUT4 antibodies at a dilution detecting only overexpressed but not endogenous GLUT4, and stained with Alexa350-conjugated goat anti-rabbit IgG (Invitrogen). Transporter surface abundance was quantified by two independent procedures. First, randomly selected GFP-positive (for HA-GLUT4-eGFP) or GLUT4-positive (for Myc7-GLUT4) adipocytes (at least 100 cells/condition), observed by a Nikon Eclipse TE 2000 microscope (×40 objective) by standard green and blue fluorescence channels, respectively, were evaluated for the presence of a complete HA- or Myc-plasma membrane rim on the red channel. Cells with an incomplete rim were considered negative. In the second procedure, we quantified the surface/total fluorescence intensity ratio of the HA-GLUT4-eGFP or Myc7-GLUT4 reporters. Randomly selected HA-GLUT4-eGFP-positive or Myc7-GLUT4/eGFP-Sac3-positive cells (15–20 cells/condition) were observed with an inverted confocal microscope (model 1X81, Olympus) with a cooled charge-coupled device 12-bit camera (Hamamatsu). Images were collected using a ×40 objective. The exposure times for each fluorescence channel were always set below the saturation threshold of the camera. The area of the selected cell was determined using a Nikon camera. The area of the selected cell was determined using a Nikon camera.
GLUT4) and Alexa350 (total Myc7-GLUT4) by IPLab software (Scanalytics, Inc., Fairfax, VA). After subtracting the background fluorescence of a nontransfected neighboring cell, quantified in a similar way, the Cy3/GFP and Alexa568/Alexa350 ratios were calculated. Slides from separate experiments were analyzed simultaneously.

**Glucose Transport**—Glucose transport was determined by measuring 2-deoxyglucose uptake as described previously (20). Briefly, transfected 3T3L1 adipocytes (24-well dish) were serum-deprived (3 h) in DMEM supplemented with 0.5% FBS. Cells were then incubated in KRH buffer (NaCl, 120 mM; KCl, 6 mM; MgSO_4_, 1.2 mM; CaCl_2_,2H_2O, 1 mM; Na_2HPO_4_, 0.6 mM; NaH_2PO_4_, 0.4 mM and Heps, pH 7.4, 30 mM), containing 0.5% FBS and 2 mM sodium pyruvate, in the presence or absence of insulin (1 or 100 nM; 30 min). 2-[1,2-3H]Deoxy-D-glucose (1 mM phenylmethylsulfonyl fluoride, 5 μM aprotinin, 1 μM benzamidine) was then added to a final concentration of 100 μM (0.5 μCi per well) for 5 min at 37 °C. Reactions were stopped with 2DG (20 mM). Cells were washed, then solubilized in 1% Triton X-100, and analyzed for radioactivity and protein concentration. Nonspecific glucose uptake was measured in the presence of 10 μM cytochalasin B and was subtracted for each determination. Values were normalized for protein content.

**3T3L1 Adipocyte Subcellular Fractionation**—Serum-starved 3T3L1 adipocytes (3 h in DMEM) seeded on 100-mm plates were stimulated or not with insulin (100 nM; 10 min) and then subjected to subcellular fractionation as described (20, 26). Briefly, cells were washed at 25 °C with PBS, then with the homogenization buffer (HES ++ buffer, 20 mM Hepes/NaOH, pH 7.5, 1 mM EDTA, 255 mM sucrose, containing 1× protease (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM benzamidine) and 1× phosphatase inhibitor mixtures (50 mM NaF, 10 mM sodium pyrophosphate, 25 mM sodium β-glycerophosphate, and 2 mM sodium metavanadate)), and scraped at 4 °C in HES ++ buffer and homogenized. Subcellular fractionation to obtain plasma membrane (PM), intracellular membrane (IM), and cytosol fractions was performed by sequential centrifugations with an SS-34 rotor (Sorvall Instrument Division) and a Beckman TLA 100.3 rotor (Beckman Instruments Inc.) as specified elsewhere (20). In this fractionation protocol, the classical high density and low density microsomal fractions are combined, representing the IM fraction (20). Pellets were resuspended in HES ++ buffer to a protein concentration of ~2 mg/ml and, after solubilization, analyzed by immunoprecipitation and immunoblotting.

**Cell Labeling and Detection of Radiolabeled PIs or Phosphoproteins**—PIs were labeled by [32P]orthophosphate in phosphate-free DMEM, containing 0.5% bovine serum albumin and 2 mM sodium pyruvate and stimulated or not, with insulin (100 nM; 10 min; 37 °C). Lysates, collected with RIPA buffer (50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 1% Nonidet P-40, and 0.5% sodium deoxycholate) supplemented with 1× protease inhibitor mixture, 1× phosphatase inhibitor mixture (RIPA ++), 1 μM pervanadate (29), and 50 mM okadaic acid, were immediately subjected to immunoprecipitation.

**Immunoprecipitation and Immunoblotting**—Whole-cell RIPA lysates, containing the specified inhibitors, were clarified by centrifugation (14,000 × g, 15 min, 4 °C) and then subjected to immunoprecipitation with the indicated immune or nonimmune sera (16 h, 4 °C), with protein A-Sepharose added during the last 1.5 h of incubation. The cytosol and HES ++-resuspended membrane fractions were supplemented with the RIPA buffer detergents (1% Nonidet P-40 and 0.5% sodium deoxycholate) and after clarification were subjected to immunoprecipitation under the same conditions. Immunoprecipitates were washed in RIPA buffer supplemented with protease inhibitors, resolved by SDS-PAGE, and after electrotransfer onto nitrocellulose membranes were analyzed by autoradiography and/or immunoblotting.

**In Vitro Phosphatase Assay**—Sac3 hydrolyzing activity was determined by a malachite green-based assay that measures the released inorganic phosphate from PI substrates as described previously (24). Briefly, RIPA ++ lysates derived from insulin-treated (100 nM; 10 min) serum-starved 3T3L1 adipocytes were subjected to immunoprecipitation with Sac3 antibodies as described above. Protein A-Sepharose beads were washed three times with the same buffer, then six times with the phosphatase “assay buffer” (50 mM Tris/HCl, pH 7.4, 1 mM MgCl_2_, and 1 mM dithiothreitol), and finally resuspended in the assay buffer (final volume 55 μl), containing soluble di-C8 PtdIns(3,5)P_2, di-C8 PtdIns(4,5)P_2, or di-C8 PtdIns(3,4,5)P_3 substrates (75 μM) (Echelon Generic Phosphatase assay kit). Reactions were incubated at 37 °C for 60 min and terminated by adding 35 μl of cold assay buffer at 4 °C. Supernatants were mixed with the mala-
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![Graph showing changes in Sac3 levels](image)

**RESULTS**

**Sac3 Depletion Enhances and Sac3 WT Overexpression Reduces GLUT4 Translocation by Insulin**—The Sac3 phosphatase is present in various degrees in all mammalian tissues and cells examined thus far, including 3T3L1 fibroblasts and adipocytes (24). To assess if endogenous Sac3 undergoes changes upon fibroblast differentiation into adipocytes, we analyzed equal protein amounts of lysates collected from the 3T3L1 cell line 2 days prior to or 8 days after the onset of the differentiation program by immunoblotting (Fig. 1). Quantification of four separate experiments revealed substantially lower levels of Sac3 (3-±0.2-fold) in 3T3L1 preadipocytes compared with fully differentiated 3T3L1 adipocytes. This result indicates that acquisition of the adipocyte phenotype and transition into insulin-responsive cells is associated with increases in Sac3 expression levels consistent with the role for the Sac3 phosphatase in 3T3L1 adipocyte functions.

To reveal whether Sac3 is part of the mechanisms underlying insulin-induced GLUT4 cell-surface translocation in 3T3L1 adipocytes, we examined GLUT4 vesicle dynamics under reduced levels of endogenous Sac3 protein. Cells were transfected with the HA-GLUT4-eGFP reporter construct that harbors HA and eGFP epitopes at the first exofacial loop and the C terminus, respectively, allowing immunofluorescence microscopy quantitation of both the cell-surface translocation and fusion in nonpermeabilized cells relative to reporter total expression. To knock down Sac3, cells were cotransfected with mouse Sac3-specific siRNA duplexes at low doses that yielded highly selective yet efficient ablation of the endogenous protein (58-64%) 60 h post-transfection (Fig. 2A). Importantly, under these conditions, expression levels of the two currently known Sac3-associated protein partners, i.e. the PtdIns(3,5)P2-synthesizing PIKfyve and the regulator ArPIKfyve (30), both reportedly enhancing GLUT4 cell-surface abundance in response to insulin (20), remained unaltered (Fig. 2A). As expected, in cells cotransfected with control siRNAs and HA-GLUT4-eGFP, insulin increased levels of plasma membrane-localized GLUT4 by ~8-fold, as compared with unstimulated cells (Fig. 2, B and C). Remarkably, reduced Sac3 protein levels resulted in an ~11-fold increase of insulin-stimulated cell-surface HA-GLUT4-eGFP signal versus unstimulated cells that displayed the basal state distribution of the transporter (Fig. 2C). Qualification of cells from four independent experiments revealed that Sac3 ablation resulted in 33±4% net insulin gain of cell-surface HA-GLUT4-eGFP (Fig. 2C). These data clearly indicate that attenuation of Sac3 protein levels increases insulin responsiveness of GLUT4 translocation without affecting the basal distribution of the transporter.

We next examined the contribution of the Sac3 enzymatic activity in the observed gain in insulin-regulated GLUT4 translocation. For this purpose, 3T3L1 adipocytes cotransfected with a Myc-GLUT4 reporter and the eGFP-based constructs of Sac3 WT or Sac3 D488A, a previously characterized mutation abrogating hydrolysis of PtdInsP2 substrates (24), were examined by immunofluorescence microscopy. The (Myc)-GLUT4 reporter used in this set of experiments harbors a 7-Myc epitope tag on the first exofacial loop allowing evaluation of both transporter surface translocation and insertion in nonpermeabilized cells (31). To quantify (Myc)-GLUT4 surface translocation/fusion relative to reporter total expression, cells were subsequently permeabilized and stained with polyclonal anti-GLUT4 antibodies at a dilution detecting only GLUT4 overexpressed but not endogenous levels (Fig. 3A). Quantitative analyses of the ratio of cell-surface Myc versus total Myc-GLUT4 fluorescence revealed that ectopic expression of eGFP-Sac3 WT resulted in a 35% decrease of the (Myc)-GLUT4 surface accumulation in the presence of insulin (Fig. 3B). Similar reduction in insulin responsiveness (~30%) due to eGFP-Sac3 WT expression was quantified by counting the number of (Myc)-GLUT4-expressing adipocytes that displayed a full Myc plasma membrane rim in response to insulin (Fig. 3C). In contrast, the (Myc)-GLUT4 surface accumulation in insulin-stimulated adipocytes expressing the phosphatase-deficient eGFP-Sac3 D488A remained unaltered, resembling that seen in insulin-stimulated control cells expressing only eGFP (Fig. 3C).

**Loss of Endogenous Sac3 Enhances Insulin-activated Glucose Uptake**—To reveal whether the gain in insulin responsiveness for GLUT4 translocation under Sac3 depletion is translated...
into enhanced glucose transport activation, we measured 2-deoxyglucose uptake in 3T3L1 adipocytes. Importantly, reduced Sac3 expression levels (by ~60%) significantly enhanced insulin-activated 2DG transport (Fig. 4). The potentiation effect was quantified to be 128 ± 6 and 132 ± 6% at submaximally (1 nM) and maximally effective insulin doses (100 nM), respectively. Consistently with our observation for unaltered basal GLUT4 cell-surface accumulation (see Fig. 2B), the basal 2DG uptake in the Sac3-depleted cells was not significantly affected (Fig. 4). This gain in cell-surface GLUT4 and glucose uptake by insulin under Sac3 protein ablation, combined with the data for inhibition of these effects by expression of the active, but not the phosphatase-dead Sac3, indicate that the Sac3 hydrolyzing activity negatively regulates insulin responsiveness of GLUT4-mediated glucose uptake.

Sac3 Association with the PtdIns(3,5)P2 Biosynthetic Complex Is Insulin-insensitive—As indicated above, Sac3 is involved in an intricate network of interactions to control PtdIns(3,5)P2 levels. It is essential for the formation of a higher order oligomeric complex, the PAS core complex, which in mammalian cells, including 3T3L1 adipocytes, incorporates PtdIns(3,5)P2-synthesizing PIKfyve and the ArPIKfyve activator (24, 25). Therefore, a plausible molecular mechanism modulating insulin responsiveness through the PtdIns(3,5)P2 pathway, observed previously under PIKfyve/ArPIKfyve depletion (20) and herein, with the Sac3 reagents (Figs. 2–4), may involve the phosphatase disassembly from the PAS complex, thereby reducing PtdIns(3,5)P2 turnover at the place of biosynthesis. Such a mechanism has been previously proposed based on findings for unaltered PIKfyve-ArPIKfyve association by insulin in 3T3L1 adipocytes yet insulin responsiveness attenuated upon knockdown of both proteins (20). To assess whether insulin regulates the lipid kinase-phosphatase interactions, anti-PIKfyve immunoprecipitates, derived from total lysates of 3T3L1 adipocytes stimulated with insulin for different time intervals, were quantitatively evaluated for coimmunoprecipitated Sac3. Intriguingly, the amounts of Sac3 that coimmunoprecipitated with PIKfyve remained largely unchanged over a 0–60-min time course of insulin challenge (Fig. 5A).

As PtdIns(3,5)P2 is membrane-bound, we hypothesized that insulin action might selectively disassemble Sac3 from the PAS complex on membranes, but changes became insignificant in analyses of whole-cell detergent-soluble extracts. Indeed, subcellular fractionation in basal 3T3L1 adipocytes revealed that, much like PIKfyve (26), a substantial subfraction (~50%) of immunoreactive Sac3 is recovered on membranes unevenly distributed between the IM (~36%) and the PM fractions (~14%). The remaining 50% was recovered exclusively in the cytosol; no immunoreactive Sac3 was detectable in the nuclear fraction (data not shown). Therefore, we next tested for plausi-

**FIGURE 2.** Sac3 depletion enhances insulin-induced GLUT4 cell-surface accumulation. A, 3T3L1 adipocytes transfected with indicated siRNA duplexes were lysed 60 h post-transfection. Duplicate samples immunoblotted (IB) with the indicated antibodies show ~60% reduction in Sac3 protein but not in PIKfyve or ArPIKfyve. B and C, 3T3L1 adipocytes were cotransfected with the indicated siRNAs and HA-GLUT4-eGFP cDNA. After 60 h, serum-starved cells were treated with or without insulin (1 nM; 20 min), stained with anti-HA and Cy3-conjugated anti-rabbit IgG without permeabilization, and analyzed by confocal microscopy. B, typical images depicting GFP fluorescence and exofacial HA (Cy3) staining in basal (panels a, b, e, and f) or insulin-stimulated cells (panels c, d, g, and h) under control (panels a, b, c, and d) or Sac3 depletion (panels e, f, g, and h). C, cell-surface HA (Cy3) signal-to-total GFP fluorescence ratio in HA-GLUT4-eGFP-expressing cells by quantitative fluorescence microscopy (means ± S.E.; 4 independent experiments; *, p < 0.01 versus insulin-stimulated control group).
ble insulin-dependent alterations in the relative amounts of Sac3 associated with PIKfyve in the membrane versus soluble fractions. Following insulin treatment of 3T3L1 adipocytes, IM, PM, and cytosolic fractions were obtained, and after lysing in the RIPA buffer detergents, cleared fractions were subjected to immunoprecipitation with anti-PIKfyve antibodies. As seen in Fig. 5B, Sac3 was readily coimmunoprecipitated with PIKfyve in all fractions, indicative of PIKfyve-Sac3 complexes both in cytosol and on membranes. The lowest degree of PIKfyve-Sac3 assembly was detected in PM, where both proteins were least abundant. Intriguingly, despite higher levels of both PIKfyve and Sac3 in cytosol versus IM (50% versus 35%), the Sac3 amounts coimmunoprecipitated with PIKfyve were almost equal in these two fractions (Fig. 5B). These data indicate a more prominent PIKfyve-Sac3 association on membranes versus cytosol. Nonetheless, similarly to our observations with total lysates (Fig. 5A), the relative amounts of Sac3 coimmunoprecipitated with PIKfyve in either fraction remained insignificantly affected by insulin (Fig. 5B). A slight increase of coimmunoprecipitated Sac3 in IM and a decrease in the cytosol were noted (Fig. 5B), but these changes were commensurate to the insulin-dependent variations in immunoprecipitated amounts of PIKfyve, found previously (26) and herein to be recruited to the 3T3L1 adipocyte IM fraction in response to insulin.

In addition to associating with the PIKfyve kinase in the PAS triad, Sac3 interacts with ArPIKfyve to form a distinct complex that does not contain PIKfyve (25). Therefore, we next tested for plausible insulin-regulated changes in the ArPIKfyve-Sac3 association by inspecting anti-ArPIKfyve immunoprecipitates of the individual subcellular fractions for the amounts of coimmunoprecipitated Sac3. Sac3 coimmunoprecipitated with ArPIKfyve in each fraction, indicative of ArPIKfyve-Sac3 complexes formed both on membranes and in the cytosol (Fig. 5C).
Sac3 and ArPIKfyve (25). Nonetheless, despite these changes, the existence of a distinct, PIKfyve-independent complex between cytosol. This observation lends further support to the view for Sac3 association with ArPIKfyve occurs predominantly in the cytosol. In Figure 5A, serum-starved 3T3L1 adipocytes were treated with or without insulin (100 nM) for the indicated time intervals (A) or for 10 min (B). Aliquots were immunoprecipitated (IP), resolved by SDS-PAGE, and immunoblotted (IB) as indicated. Shown are chemiluminescence detections of immunoblots from representative experiments out of 3–7 independent experiments with similar results. A, aliquots (800 μg of protein) of the RIPA buffer-lysed cells were immunoprecipitated with preimmune (Preim) or anti-PIKfyve. B, cells were fractionated to isolate IM, PM, and cytosol. Equal percentage (90%±1.9%) of the RIPA detergent solubilized fractions (protein content: IM, 106 μg; PM, 63 μg; cytosol, 772 μg) was immunoprecipitated with anti-PIKfyve antibodies. C, cells were fractionated to isolate IM, PM, and cytosol. Equal percentage (90%±1.7%) of the RIPA detergent solubilized fractions (protein content: IM, 106 μg; PM, 63 μg; cytosol, 772 μg) was immunoprecipitated with a preimmune serum (P) or anti-ArPIKfyve antibodies (I). Electrophoretic mobility of Sac3 and ArPIKfyve standards (St.) is denoted. A and B, membranes were cut horizontally and immunoblotted. C, membrane was stripped between the antibodies. The data in Figure 5A indicate that, unlike with PIKfyve-Sac3 association in the PAS complex being found more prominent on membranes (see Fig. 5B), the Sac3 association with ArPIKfyve occurs predominantly in the cytosol. This observation lends further support to the view for existence of a distinct, PIKfyve-independent complex between Sac3 and ArPIKfyve (25). Nonetheless, despite these changes, as with the PIKfyve-Sac3 interaction, insulin action failed to induce statistically significant alterations in the association of ArPIKfyve with Sac3 in either fraction (data not shown). Thus, whereas an insulin-triggered disassembly of the Sac3 phosphatase from the complex with PIKfyve and/or ArPIKfyve may mechanistically underlie the positive effect of the PIKfyve-ArPIKfyve-PtdIns(3,5)P2 pathway in insulin responsiveness (17), the data presented above are inconsistent with such a regulatory mechanism in 3T3L1 adipocytes, at least as determined by coimmunoprecipitation analyses under the specified conditions.

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Intriguingly, we observed more Sac3 coimmunoprecipitated with ArPIKfyve in the cytosol fraction versus each individual or combined membrane fractions (Fig. 5C). These data indicate that, unlike with PIKfyve-Sac3 association in the PAS complex being found more prominent on membranes (see Fig. 5B), the Sac3 association with ArPIKfyve occurs predominantly in the cytosol. This observation lends further support to the view for existence of a distinct, PIKfyve-independent complex between Sac3 and ArPIKfyve (25). Nonetheless, despite these changes, as with the PIKfyve-Sac3 interaction, insulin action failed to induce statistically significant alterations in the association of ArPIKfyve with Sac3 in either fraction (data not shown). Thus, whereas an insulin-triggered disassembly of the Sac3 phosphatase from the complex with PIKfyve and/or ArPIKfyve may mechanistically underlie the positive effect of the PIKfyve-ArPIKfyve-PtdIns(3,5)P2 pathway in insulin responsiveness (17), the data presented above are inconsistent with such a regulatory mechanism in 3T3L1 adipocytes, at least as determined by coimmunoprecipitation analyses under the specified conditions.

**PtdIns(3,5)P2 Hydrolyzing Activity of Sac3 Is Inhibited by Insulin**

Because insulin failed to disassemble Sac3 from the complexes with PIKfyve and/or ArPIKfyve, we next considered the Sac3 phosphatase activity as a plausible target of the hormonal effect. It has been demonstrated that ectopically expressed Sac3WT is an active phosphatase dephosphorylating in vitro higher 5-phosphorylated PI substrates, such as PtdIns(3,5)P2, PtdIns(4,5)P2, and PtdIns(3,4,5)P3 with activity decreasing in the indicated order, but is inefficient with PtdInsP (24). Reasoning that insulin action in 3T3L1 adipocytes might reduce the Sac3 hydrolyzing activity as one mechanism of increasing signaling through the PtdIns(3,5)P2 pathway, we tested the in vitro phosphatase activity of endogenous Sac3 utilizing the malachite green assay and di-C8 PI substrates. Similarly to our data with the recombinant Sac3 protein (24), endogenous Sac3 immunopurified from lysates of basal 3T3L1 adipocytes on anti-Sac3 IgG preferentially dephosphorylated PtdIns(3,5)P2 and, less so, PtdIns(3,4,5)P3 (Fig. 6). Control immunoprecipitates on nonimmune IgG were almost ineffective in hydrolyzing either substrate under the conditions of the experiment as was immunopurified Sac3 with di-C8 PtdIns(4,5)P2 substrate (Fig. 6 and data not shown). Notably, in response to insulin there was a marked inhibition (by 67±8%; n = 4) of the endogenous Sac3 hydrolyzing activity toward the PtdIns(3,5)P2 substrate (Fig. 6). To a lesser extent,
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PIKfyve displays intrinsic phosphatase activity in response to insulin. Therefore, we next tested the possibility that reversible protein phosphorylation might internally coordinate the opposing kinase-phosphatase activities within the PAS complex. Therefore, we next tested if the subtraction of Sac3 that is engaged in the association within the PAS complex is subjected to insulin-regulated phosphorylation. For this purpose, anti-PIKfyve immunoprecipitates from 32P-labeled 3T3L1 adipocyte lysates were quantitatively evaluated for the amounts of coprecipitated 32P-Sac3. As illustrated on the autoradiogram in Fig. 7, a Sac3 subtraction that coimmunoprecipitated with PIKfyve was substantially phosphorylated at basal conditions, as was PIKfyve itself, in agreement with previous observations (28). However, insulin action failed to significantly affect the phosphorylation of Sac3 nor did it alter that of PIKfyve, as revealed by normalizing the 32P-labeled bands on the autoradiogram for the PIKfyve amounts detected by subsequent immunoblotting (Fig. 7). Likewise, ArPIKfyve from the PAS complex also displayed some degree of basal phosphorylation, but this remained insulin-insensitive (Fig. 7). Thus, although a phosphorylation step to modulate the Sac3 phosphatase activity might mechanistically underlie insulin-dependent inhibition of PtdIns(3,5)P2 hydrolysis by Sac3, together the data presented above are inconsistent with such a regulatory mechanism in 3T3L1 adipocytes, as determined under the specified conditions.

PtdIns(3,5)P2 and PtdIns(3)P Accumulation in Radiolabeled 3T3L1 Adipocytes—The observed insulin-induced inhibition of Sac3 phosphatase activity (see Fig. 6) predicts hormone-dependent elevation in intracellular PtdIns(3,5)P2. However, HPLC inositol head group analysis in [32P]orthophosphate-labeled 3T3L1 adipocytes conducted previously (20, 22) and herein (see below) failed to document statistically significant insulin-dependent changes in [32P]PtdIns(3,5)P2 accumulation. Anticipating technical limitations of various nature, including low/uneven [32P]ATP-specific activity at discrete intracellular pools during the short term 32P-cell labeling (20), in this study we revisited this point by examining the insulin effect on PtdIns(3,5)P2 levels in cells labeled with myo-[3H]inositol under the course of 40 h. Importantly, we observed a slight but statistically significant insulin-regulated increase (21.4 ± 1.8%) in [3H]inositol-labeled PtdIns(3,5)P2 levels (Fig. 8A). Although the increment was substantially lower than that observed upon hyperosmotic stress in this cell type (see Ref. 22 and this study, data not shown), these data demonstrate for the first time that insulin action is associated with an increase in PtdIns(3,5)P2 that could be detected by an HPLC analysis of total cellular PIs.

We sought to relate the gain in insulin responsiveness under Sac3 depletion to changes in PtdIns(3,5)P2. Therefore, we assessed the PI levels in siRNAs-transfected 3T3L1 adipocytes by HPLC, following cell metabolic labeling with [32P]orthophosphate. It should be noted that the siRNA transfection protocol is incompatible with long term cell labeling by [3H]inositol. As illustrated in Fig. 8B and supplemental Fig. 1, depletion of Sac3 to about 58–64% (see Fig. 2A) induced a significant, although small, gain in [32P]PtdIns(3,5)P2-accumulated levels. Only modest elevation has also been registered under siRNA-mediated Sac3 knockdown in other mammalian cell types (24), which is likely associated with the dual function of Sac3 not only in PtdIns(3,5)P2 hydrolysis but also in PIKfyve-catalyzed conversion of PtdIns(3)P to PtdIns(3,5)P2 by promoting efficient PIKfyve-ArPIKfyve association (24, 25). Concordantly, Sac3 depletion in 3T3L1 adipocytes resulted in elevated [32P]PtdIns(3)P accumulation, in line with reduced PtdIns(3)P consumption by the PIKfyve-ArPIKfyve biosynthetic pathway (Fig. 8B and supplemental Fig. 1), an effect also seen in other cell types (24). However, unlike with the myo-[3H]inositol-labeled 3T3L1 adipocytes where [3H]inositol incorporation into PtdIns(3,5)P2 slightly increased (Fig. 8A), insulin action in the 32P-labeled siRNA-

FIGURE 7. Sac3 is phosphorylated in vivo in an insulin-independent manner. Serum-starved 3T3L1 adipocytes were labeled with [32P]orthophosphate, treated with or without insulin (100 nM; 10 min), and lysed in RIPA buffer, supplemented with 1 mM pervanadate and 50 nM okadaic acid. Aliquots were immunoprecipitated (IP) with preimmune (P), anti-PIKfyve, or anti-Sac3 sera as indicated. Immunoprecipitates were washed, resolved by SDS-PAGE, and electrotransferred onto nitrocellulose membranes that were subjected to autoradiography. The identity of the proteins is confirmed by subsequent immunoblotting with anti-PIKfyve, anti-Sac3, or anti-ArPIKfyve antibodies (mobility indicated by arrows). Insulin has no effect on Sac3, PIKfyve, and ArPIKfyve phosphorylation in two to three independent cell labelings. The slight increases in the phosphorylated bands under insulin (+) are due to slightly greater protein amounts (~20%) subjected to immunoprecipitation versus control (−).

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transfected cells failed to induce statistically significant changes in $[^{32}P]PtdIns(3,5)P_2$, as observed previously (20, 22) and herein. Collectively, the data in $[^{3}H]$inositol- and $^{32}$P-labeled adipocytes are consistent with the idea that insulin action might affect a subcellular PtdIns(3,5)P2 pool at a spatially discrete membrane microdomain, which results in only small or marginal changes of the HPLC-measurable total radiolabeled PtdIns(3,5)P$_2$.

**DISCUSSION**

Inhibition of the endogenous phosphatases PTEN, SHIP2, and SKIP, all antagonizing insulin signaling via the PtdIns(3,4,5)P$_3$ pathway, emerges as a potentially effective approach for alleviating insulin resistance associated with type 2 diabetes and obesity (13–16). Recent observations for a positive role of the PIKfyve-ArPIKfyve-PtdIns(3,5)P$_2$ pathway in insulin responsiveness (20, 23) suggest that the newly identified PtdIns(3,5)P$_2$-specific phosphatase Sac3 (24) might also represent a candidate drug discovery target in insulin resistance. However, unlike the first three PI phosphatases, Sac3 plays an elaborate role in the PtdIns(3,5)P2 biosynthetic complex. It facilitates the physical association of PIKfyve with its activator ArPIKfyve, thus promoting maximal PIKfyve/ArPIKfyve-dependent PtdIns(3,5)P$_2$ biosynthesis (20, 25). Consistent with these observations are recent findings in a Sac3 knock-out mouse model, where cellular PtdIns(3,5)P$_2$ levels, instead of being up-regulated, are reduced (34). This dual function of Sac3 in two seemingly opposing events poses the key question as to how modulations in the Sac3 protein levels and/or activity affect insulin responsiveness. This study was designed to assess the role of reduced Sac3 protein levels or up-regulated hydrolyzing activity in insulin-stimulated GLUT4 translocation and glucose uptake in 3T3L1 adipocytes. To this end, we used siRNA-based selective depletion of endogenous Sac3 or ectopic expression of Sac3$^{WT}$ in 3T3L1 adipocytes. The data presented herein unequivocally demonstrate that attenuation of the Sac3 protein levels by ~60% produces substantial gain in insulin responsiveness manifested by increased cell-surface HA-GLUT4-eGFP reporter and stimulation of deoxyglucose uptake (Figs. 2 and 4). Our observations for reduced cell-surface GLUT4 in response to insulin under ectopic expression of active Sac3$^{WT}$, but not the phosphatase-deficient mutant Sac3$^{D488A}$ (Fig. 3), implicate the Sac3 phosphatase activity in the negative regulation of insulin responsiveness in 3T3L1 adipocytes. These data determine that although a knock-out of Sac3 markedly reduces intracellular PtdIns(3,5)P$_2$ (34), a partial decrease in Sac3 protein expression (~60%) is associated with a net increase in PtdIns(3,5)P$_2$ and gain of insulin responsiveness in 3T3L1 adipocytes (Fig. 8B). To the best of our knowledge this is the first demonstration that a PI-polyphosphate phosphatase with specificity for PtdIns(3,5)P$_2$ suppresses insulin signaling to GLUT4 and glucose influx. These data are of significant importance as they could lead to new insights in our understanding of both basic and disease mechanisms of insulin action.

An observation with exceptional mechanistic significance in this study is the characterization of Sac3 as an insulin-regulated phosphatase, as judged by the marked inhibition of its PtdIns(3,5)P$_2$ hydrolyzing activity in response to insulin (Fig. 6). These data, taken together with our observations for unaltered Sac3 assembly with PIKfyve and/or ArPIKfyve (Fig. 5) and the
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PIKfyve relocation on intracellular membranes, reported previously and confirmed herein (24) (Fig. 5B), are consistent with the notion that insulin induces both membrane recruitment of preassembled PAS complexes and arrest of the Sac3 hydrolyzing activity but not disassembly of the PAS complex. Such a regulation may mechanistically explain how the opposing activities of the PIKfyve kinase and the Sac3 phosphatase are coordinated in a context of a common protein complex. The model is consistent with the requirement for physical integrity of the PAS core complex for optimal PIKfyve activity as shown recently in the case of GLUT4 surface translocation by insulin (25). Thus, a dual effect of insulin on both PtdIns(3,5)P$_2$ signal localization (through PAS complex membrane recruitment) and amplitude (through inhibition of Sac3 in the PAS core) may result in a robust net increase of localized PtdIns(3,5)P$_2$. Such an elevation has been demonstrated previously with isolated intracellular membranes of 3T3L1 adipocytes (20) and here in intact adipocytes that have been exposed to [3H]inositol for relatively longer periods (Fig. 8).

It is characterized by the presence of a C(\(X\))$_3$R(S/T) conserved sequence, a signature motif of the dual specificity protein-tyrosyl and lipid phosphatases. The invariant catalytic Cys residue in these enzymes functions as a nucleophile in the phosphor hydrolysis (35, 36). Cys is highly susceptible to oxidation due in these enzymes functions as a nucleophile in the phospholipid phosphatases. The invariant catalytic Cys residue in these enzymes functions as a nucleophile in the phosphor hydrolysis (35, 36). Cys is highly susceptible to oxidation, as observed in response to various extracellular stimuli that generate reactive oxygen species. Insulin action in target cells, including 3T3L1 adipocytes in culture, has long been known to induce endogenous production of H$_2$O$_2$ (37, 38). In fact, several enzymes with the C(\(X\))$_3$R(S/T) signature motif, including the PtdIns(3,4,5)P$_3$ phosphatase PTEN (39, 40) and the IR phosphatase PTP1B (41, 42), undergo transient oxidation and hence inhibition in response to insulin. Future studies should attempt to determine whether insulin-induced redox fluctuations regulate Sac3 hydrolyzing activity and signaling through the PtdIns(3,5)P$_2$ pathway in target cells. In any case, this present study demonstrates a striking potentiation of adipocyte insulin responsiveness under depletion of Sac3 and that insulin action arrests Sac3 phosphatase activity as one mechanism of promoting signaling through the PtdIns(3,5)P$_2$ pathway.

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REFERENCES

1. Huang, S., and Czech, M. P. (2007) Cell Metab. 5, 237–252
2. Watson, R. T., and Pessin, J. E. (2007) Cell. Signal. 19, 2209–2217
3. Shisheva, A. (2008) Am. J. Physiol. Endocrinol. Metab. 295, E536–E544
4. Zaid, H., Antonescu, C. N., Randhawa, V. K., and Klip, A. (2008) Biochem. J. 413, 201–215
5. Pilch, P. F. (2008) Acta Physiol. (Oxf) 192, 89–101
6. Larance, M., Ramm, G., and James, D. E. (2008) Mol. Endocrinol. 22, 226–233
7. Taniguchi, C. M., Emuennelli, B., and Kahn, C. R. (2006) Nat. Rev. Mol. Cell. Biol. 7, 85–96
8. Biddinger, S. B., and Kahn, C. R. (2006) Annu. Rev. Physiol. 68, 123–158
9. Pattaranit, R., van den Berg, H. A., and Spanwick, D. (2008) Sci. Prog. 91, 285–316
10. Ijuin, T., and Takenawa, T. (2003) Mol. Cell. Biol. 23, 1209–1220
11. Tang, X., Powelka, A. M., Soriano, N. A., Czech, M. P., and Guilhaume, A. (2005) J. Biol. Chem. 280, 22523–22529
12. Astle, M. V., Horan, K. A., Ooms, L. M., and Mitchell, C. A. (2007) Biochem. Soc. Symp. 74, 161–181
13. Vinciguerra, M., and Foti, M. (2006) Arch. Physiol. Biochem. 112, 89–104
14. Sasaoka, T., Wada, T., and Tsuneki, H. (2006) Pharmacol. Ther. 112, 799–809
15. Lazar, D. F., and Saltiel, A. R. (2006) Nat. Rev. Drug Discov. 5, 333–342
16. Ijuin, T., Yu, Y. E., Mizutani, K., Pao, A., Tateya, S., Tamori, Y., Bradley, A., and Takenawa, T. (2008) Mol. Cell. Biol. 28, 5184–5195
17. Shisheva, A., Sbrissa, D., and Ikonomov, O. (1999) Mol. Cell. Biol. 19, 623–634
18. Sbrissa, D., Ikonomov, O. C., and Shisheva, A. (1999) J. Biol. Chem. 274, 21589–21597
19. Ikonomov, O. C., Sbrissa, D., Mlak, K., and Shisheva, A. (2002) Endocrinology 143, 4742–4754
20. Ikonomov, O. C., Sbrissa, D., Dondapi, R., and Shisheva, A. (2007) Exp. Cell Res. 313, 2404–2416
21. Sbrissa, D., Ikonomov, O. C., Strakova, J., Dondapi, R., Mlak, K., Deeb, R., Silver, R., and Shisheva, A. (2004) Mol. Cell. Biol. 24, 10437–10447
22. Sbrissa, D., and Shisheva, A. (2005) J. Biol. Chem. 280, 7885–7889
23. Ikonomov, O. C., Sbrissa, D., and Shisheva, A. (2009) Biochem. Biophys. Res. Commun. 382, 566–570
24. Sbrissa, D., Ikonomov, O. C., Fu, Z., Ijuin, T., Gruenberg, J., Takenawa, T., and Shisheva, A. (2007) J. Biol. Chem. 282, 23878–23891
25. Sbrissa, D., Ikonomov, O. C., Fenner, H., and Shisheva, A. (2008) J. Mol. Biol. 384, 766–779
26. Shisheva, A., Rusin, B., Ikonomov, O. C., DeMarco, C., and Sbrissa, D. (2001) J. Biol. Chem. 276, 11859–11869
27. Ikonomov, O. C., Sbrissa, D., and Shisheva, A. (2001) J. Biol. Chem. 276, 26141–26147
28. Sbrissa, D., Ikonomov, O. C., and Shisheva, A. (2000) Biochemistry 39, 15980–15989
29. Shisheva, A., and Shechter, Y. (1993) Endocrinology 133, 1562–1568
30. Shisheva, A. (2008) Cell Biol. Int. 32, 591–604
31. Shi, J., and Kandror, K. V. (2005) Dev. Cell 9, 99–108
32. Botelho, R. J., Efe, J. A., Teis, D., and Emr, S. D. (2008) Mol. Biol. Cell 19, 4273–4286
33. Jin, N., Chow, C. Y., Liu, L., Zolov, S. N., Bronson, R., Davison, M., Petersen, J. L., Zhang, Y., Park, S., Duek, J. E., Goldowitz, D., Meisler, M. H.,
and Weisman, L. S. (2008) EMBO J. 27, 3221–3234
34. Chow, C. Y., Zhang, Y., Dowling, J. J., Jin, N., Adamska, M., Shiga, K., Szigeti, K., Shy, M. E., Li, J., Zhang, X., Lupski, J. R., Weisman, L. S., and Meisler, M. H. (2007) Nature 448, 68–72
35. Tonks, N. K. (2006) Nat. Rev. Mol. Cell Biol. 7, 833–846
36. Leslie, N. R., Batty, I. H., Maccario, H., Davidson, L., and Downes, C. P. (2008) Oncogene 27, 5464–5476
37. Goldstein, B. J., Mahadev, K., Kalyankar, M., and Wu, X. (2005) Diabetes 54, 311–321
38. Tonks, N. K. (2005) Cell 121, 667–670
39. Kwon, J., Lee, S. R., Yang, K. S., Ahn, Y., Kim, Y. J., Stadtman, E. R., and Rhee, S. G. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 16419–16424
40. Seo, J. H., Ahn, Y., Lee, S. R., Yeol Yeo, C., and Chung Hur, K. (2005) Mol. Biol. Cell 16, 348–357
41. Mahadev, K., Zilbering, A., Zhu, L., and Goldstein, B. J. (2001) J. Biol. Chem. 276, 21938–21942
42. Meng, T. C., Buckley, D. A., Galic, S., Tiganis, T., and Tonks, N. K. (2004) J. Biol. Chem. 279, 37716–37725