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Lipid phosphatase SHIP2 downregulates insulin signalling in podocytes

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Abstract

Podocyte injury plays an important role in the development of diabetic nephropathy. Podocytes are insulin responsive and can develop insulin resistance, but the mechanisms are unknown. To study the role of CD2-associated protein (CD2AP) in podocyte injury, we performed a yeast two-hybrid screening on a glomerular library, and found that CD2AP bound to SH2-domain-containing inositol polyphosphate 5-phosphatase 2 (SHIP2), a negative regulator of insulin signalling. SHIP2 interacts with CD2AP in glomeruli and is expressed in podocytes, where it translocates to plasma membrane after insulin stimulation. Overexpression of SHIP2 in cultured podocytes reduces Akt activation in response to insulin, and promotes apoptosis. SHIP2 is upregulated in glomeruli of insulin resistant obese Zucker rats. These results indicate that SHIP2 downregulates insulin signalling in podocytes. The upregulation of SHIP2 in Zucker rat glomeruli prior to the age of onset of proteinuria suggests a possible role for SHIP2 in the development of podocyte injury.

Keywords: diabetes, diabetic nephropathy, insulin resistance, podocyte, proteinuria

1. Introduction

Diabetic nephropathy is one of the leading causes of end stage renal disease and kidney transplantations, and is also associated with an increased risk for cardiovascular morbidity and mortality (Borch-Johnsen et al 1987). Diabetic nephropathy is characterized by early development of proteinuria indicating an injury in the glomerular filtration barrier, which consists of three layers: fenestrated endothelial cells, basement membrane, and glomerular epithelial cells or podocytes. Podocytes are highly specialized cells forming foot processes that surround the glomerular capillary walls and are interconnected with special cell-adhesion structures called slit diaphragms. One of the key components of the slit diaphragm is nephrin, a transmembrane protein of the immunoglobulin superfamily, mutated in the congenital nephrotic syndrome of the Finnish type (Kestila et al 1998). Interestingly, nephrin has been shown to be downregulated in diabetic nephropathy (Langham et al 2002).

It has become evident that podocyte injury plays an important role in the development of diabetic nephropathy like in many other proteinuric diseases (Wolf et al 2005). Podocyte number is reduced already after short duration of diabetes in renal biopsies from patients with type 1 diabetes (Steffes et al 2001) and podocyte loss is also described in type 2 diabetes where it correlates with disease progression (Meyer et al 1999). Podocyte foot process effacement (Steffes et al 1980, Ellis et al 1987) and detachment from the basement membrane (Toyoda et al 2007) are seen in both human patients and animal models. In addition to detachment, podocyte loss can result from increased apoptosis (Verzola et al 2007) which precedes the increase in urinary albumin excretion in mouse models of diabetes (Susztak et al 2006).

Insulin resistance is known to be associated with microalbuminuria in diabetic patients (Yip et al 1993, Parvanova et al 2006). Further, in type 1 diabetic patients insulin resistance has shown to predict (Orchard et al 2002) and the metabolic syndrome to associate with (Thorn et al 2005) overt nephropathy, but the mechanisms have remained unsolved. Podocytes have shown to be insulin-responsive and to increase their glucose

uptake after insulin stimulation (Coward et al 2005). This suggests that insulin resistance can have a direct effect on podocyte function. Indeed, in a recent article Tejada et al. showed that podocytes from diabetic db/db mice were unable to phosphorylate Akt in response to insulin (Tejada et al 2008).

CD2-associated protein (CD2AP) is a cytosolic adaptor protein with multiple protein-protein interaction domains (Dustin et al 1998). It interacts with nephrin (Palmen et al 2002), and as CD2AP associates with actin directly (Lehtonen et al 2002), it has been suggested to link nephrin to the actin cytoskeleton. Mice lacking CD2AP develop nephrotic syndrome, indicating that CD2AP is required to maintain the functional integrity of the glomerular slit diaphragm (Shih et al 1999). Heterozygous mice lacking one allele of CD2AP develop glomerular abnormalities at an advanced age and are more susceptible to glomerular damage than wild-type mice (Kim et al 2003). CD2AP mutations have also been reported in human patients with focal segmental glomerulosclerosis (Kim et al 2003).

In order to study the role of CD2AP in podocyte injury, we performed a yeast-two-hybrid screening of a glomerular library. Interestingly, we found that CD2AP bound to SH2-domain-containing inositol polyphosphate 5-phosphatase 2 (SHIP2), a negative regulator of insulin signalling and a molecule associated with the metabolic syndrome (Dyson et al 2005). Lipid phosphatase SHIP2 downregulates PI3K mediated signalling, induced by insulin and other growth factors, by hydrolyzing PI(3,4,5)₃P to PI(3,4)P₂ (Blero et al 2001, Pesesse et al 2001). SHIP2 overexpression in mice has a negative effect on glucose tolerance (Kagawa et al 2008, Fukui et al 2005) and SHIP2 knockout mice appear resistant to high fat diet- induced obesity and insulin resistance (Sleeman et al 2005), and in addition, inhibition of SHIP2 in the liver has been shown to improve glucose tolerance in diabetic mice (Grempler et al 2007). Taken together, SHIP2 is a downregulator of PI3K mediated insulin signalling, that is known to function in liver, muscle and adipose cells, but its role in the kidney has not been studied previously. We hypothesized that SHIP2 can mediate insulin resistance in podocytes, and investigated its expression and

function in cultured podocytes and in diabetic rat and mouse kidneys.

2. Materials and methods

2.1. Yeast two-hybrid screening

Construction of the rat glomerular yeast two-hybrid library has been described previously (Takeda et al 2001). The cDNA encoding the N-terminal part of mouse CD2AP (aa 1-330) was amplified by PCR and subcloned into NcoI and XhoI sites in the LexA DNA-binding domain- containing displayBAIT vector (Display System Biotech, Vista, CA). The displayBAIT-CD2AP and the displayREPORTER vector, containing green fluorescent protein gene as a reporter, were transformed into yeast strain EGY48 (Invitrogen, Carlsbad, CA). 50 µg of the rat glomerular yeast two-hybrid library in the displayTARGET vector was transformed into yeast pretransformed with displayBAIT-CD2AP and displayREPORTER vectors and plated on selective medium (-histidine, -uracil, -leucine, -tryptophane). Colonies growing in the selective medium were exposed to UV light (360 nm) to select the ones expressing the green fluorescent protein reporter. Plasmid DNA of the positive clones was electroporated into *Escherichia coli* KC8 (Clontech, Mountain View, CA) and the identity of the inserts was analyzed by sequencing and Blastn (nucleotide-nucleotide BLAST) search.

2.2. Animals

Male Sprague-Dawley rats and male FVB mice were used to study SHIP2 expression in normal rat and mouse. Male db/db mice and heterozygous db/+ controls in C57BLKS background (BKS.Cg-*m*^{+/+}*Lepr*^{db}/BomTac) were purchased from Taconic Europe (Ry, Denmark), and obese (fa/fa) and lean (fa/+) Zucker rats (CrI:ZUC-*Lepr*fa) from Charles River Laboratories (Sulzfeld, Germany). Animal care followed the guidelines of NIH and Finnish laws. Blood glucose values were measured from tail vein samples using OneTouch Ultra glucometer (Lifescan, Milpitas, CA). The urinary albumin to creatinine ratio was determined from spot urine samples. Albumin was measured with mouse or rat albumin ELISA kit (CellTrend, Luckenwalde, Germany) and creatinine using CREA plus

enzymatic assay (Roche, Basel, Switzerland) and Roche clinical chemistry analyzer. For histological analysis kidneys were fixed in 10% formalin, embedded in paraffin and analyzed by hematoxylin-eosin staining.

2.3. Cell culture

Immortalized human podocytes (Saleem et al 2002) were maintained as previously described in RPMI medium, supplemented with 10% FBS, and insulin, transferrin and selenite (ITS), at + 33 °C for proliferation and thermo-switched to + 37 °C for differentiation for 14 days. HIRc cells (rat fibroblasts overexpressing human insulin receptor) (McClain et al 1987) were maintained in DMEM medium, supplemented with 10% FBS, ultraglutamine, penicillin and streptomycin. All cell culture reagents were from Sigma (St. Louis, MO) except ultraglutamine from Lonza (Basel, Switzerland).

2.4. Immunoblotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped from the dish into ice-cold 1% Nonidet P-40 (NP-40), 20 mM Hepes, pH 7.5, 150 mM NaCl with 1x Complete™, EDTA-free proteinase inhibitor cocktail (Roche), 50 mM NaF and 1 mM Na₃VO₄ (lysis buffer) and rotated at 4°C for 15 min. Glomeruli and tubules were isolated from rat kidney cortices by graded sieving and lysed by rotation in lysis buffer at 4°C for 30 min. Detergent insoluble material was removed by centrifugation (10,000 x g for 10 min). For immunoblotting proteins were separated by SDS-PAGE and transferred to PVDF-FL membranes (Millipore, Billerica, MA), blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) diluted 1:1 with PBS, and incubated with goat anti-SHIP2 I-20 (Santa-Cruz Biotechnology, Santa-Cruz, CA), rabbit anti-CD2AP 1774 or 1764 (raised against mouse CD2AP as described (Lehtonen et al 2000)), rabbit anti-phospho-Akt (Ser473) (Cell Signalling Technology, Danvers, MA), mouse anti-Pan Akt (R&D Systems, Minneapolis, MN), mouse anti-phosphotyrosine PT-66 (Sigma) or mouse anti-actin (Sigma) and Alexa Fluor 680 (Invitrogen) or IRDye 800 (LI-COR) donkey anti-rabbit, anti-goat or anti-mouse IgG followed by detection and quantitation with an Odyssey Infrared Imager (LI-COR).

2.5. Immunoperoxidase staining

Kidney samples were fixed with 10% formalin, dehydrated and embedded in paraffin. Immunoperoxidase staining was performed with a VectaStain Elite kit (Vector Laboratories, Burlingame, CA). Sections were deparaffinized, antigen retrieval was done by boiling for 15 minutes in a microwave oven in 10 mM citric acid (pH 6,0), and endogenous peroxidase was inactivated by incubation in 0,5% H₂O₂ in methanol for 30 min. Sections were blocked with CAS-block (Zymed, South San Francisco, CA) and incubated with anti-SHIP2 I-20 or anti-CD2AP 1774 diluted in ChemMate (Dako, Glostrup, Denmark) and with biotinylated rabbit anti-goat or goat anti-rabbit secondary antibody followed by incubation with ABC-reagent and AEC (Dako) colour development. Sections were counterstained with hematoxylin and mounted with Shandon Immu-Mount (Thermo Scientific, Waltham, MA). In db/db mice SHIP2 expression was rated (scale 1-5) by two independent researchers blinded from the genotypes. From each mouse (6 diabetic and 6 control), 8 glomeruli were photographed with a Nikon Eclipse 800 microscope with the same microscope settings for analysis.

2.6. Pull-down assay

Generation of glutathione-S-transferase (GST)-tag fusion protein constructs of mouse CD2AP 1.SH3, 2.SH3 and 3.SH3 domains has been previously described (Palmen et al 2002). The NH₂-terminus of mouse CD2AP (aa 1-330) was subcloned into pGEX-4T-1 vector (Amersham Biosciences, Buckinghamshire, England) to produce GST-CD2AP-N. GST and GST-CD2AP fusion proteins were produced in *E. coli* BL21(DE3) (Stratagene; La Jolla, CA) and purified on glutathione-Sepharose beads (Amersham Biosciences). Cell and glomerular lysates were precleared with glutathione-Sepharose beads at 4°C for 1 h, followed by incubation at 4°C for 4 h with GST-CD2AP fusion proteins or GST alone (20 µg each) immobilized on beads. Beads were washed extensively with lysis buffer, boiled in Laemmli sample buffer and proteins were separated by 10% SDS-PAGE for immunoblotting with anti-SHIP2 I-20.

2.7. Immunoprecipitation

HIRc cells in basal state, starved 16 hours, or starved 16 hours and stimulated with 17 nM insulin for 7 minutes were lysed as described in 2.4.. Lysates were precleared with protein A-Sepharose beads (Invitrogen) or TrueBlot anti-goat Ig IP Beads (eBioscience, San Diego, CA) at 4°C for 1 h and incubated with anti-CD2AP 1764 (Lehtonen et al 2000) or normal rabbit serum for 16-20 h at 4°C, or alternatively, with anti SHIP2 I-20 or goat IgG (5 µg). Immune complexes were bound to protein A-Sepharose or goat TrueBlot beads at 4°C for 1 h, washed 3 times with lysis buffer and boiled in Laemmli sample buffer for immunoblotting with anti-SHIP2 I-20, anti-CD2AP 1764, or anti-phosphotyrosine.

2.8. Transient transfections and infections

Proliferating human podocytes were transfected with human SHIP2 cDNA with HA-tag in pCGN vector (SHIP2-pCGN) (Dyson et al 2001) or empty pCGN using Lipofectamine 2000 (Invitrogen) and used for experiments 48 h after transfection. In differentiated podocytes SHIP2 was overexpressed by lentiviral infection. SHIP2 with HA-tag was subcloned from SHIP2-pCGN into pSIN18.cppt.hEF1αp.WPRE vector (Gropp et al 2003). Empty pSIN18.cppt.hEF1αp.WPRE vector was used as control. For virus production, KS pCMVΔ8.9 and pHCMV packaging plasmids, together with SHIP2 or control lentiviral vector, were transfected into HEK 293FT cells with Lipofectamine 2000. Medias were collected after 3 days, filtrated through 0.45 µm filter, and ultracentrifuged 85000 x g at + 4 °C for 90 min, to concentrate the viruses. The viruses were resuspended in PBS and added to differentiated podocytes on day 11 or 12 of differentiation. Podocytes were first incubated at +37 °C for 10 min, followed by centrifugation 1360 x g at +4 °C for 30 min. Virus-containing medium was replaced with regular medium 10-12 hours after the infection.

2.9. Immunofluorescence

Differentiated podocytes were starved for 14 hours, and stimulated with 200 nM insulin (Actrapid, Novo Nordisk, Denmark) for 5 or 15 min, and fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with CAS-block

(Zymed). Cells were incubated with primary antibodies, rabbit anti-SHIP2 C76A7 (Cell Signalling Technology) or rabbit anti-CD2AP H-290 (Santa-Cruz) in ChemMate (Dako) for 1 h followed by secondary antibody Alexa Fluor 555 donkey anti-rabbit, in ChemMate for 1 h. Cells were mounted in Moviol and examined with a Zeiss Axiophot 2 microscope.

2.10. Akt activity measurements

Podocytes expressing SHIP2-pCGN or pCGN were serum and ITS starved overnight (18-20 h) and stimulated with 200 nM insulin for 15 min. Akt phosphorylation was measured by immunoblotting with Phospho-Akt (Ser473) antibody recognizing Akt1 phosphorylated at Ser473 and Akt2 and Akt3 phosphorylated at corresponding residues, and compared to total Akt level detected with Pan Akt antibody. Akt kinase activity was measured with ELISA-based Akt/PKB Kinase Activity Assay Kit (Assay Designs, Enzo Biochem, New York, NY).

2.11. Fluorescence-activated cell sorting (FACS)

Apoptosis in SHIP2 overexpressing differentiated podocytes was measured on day 13 or 14 of differentiation, 48 hours after the lentiviral infection, by fluorescence-activated cell sorting (FACS) using Annexin V-FITC Apoptosis Kit (Pharmingen, BD, Franklin Lakes, NJ) and double staining with 7-Aminoactinomycin D (7-AAD) (BD). Cells were trypsinized, combined with detached cells in media, and centrifuged. Cell pellet was washed twice with cold PBS and resuspended in Annexin V Binding Buffer (BD) containing Annexin V-FITC and 7-AAD. Cells were incubated for 15 min at room temperature in dark, washed twice with cold PBS, centrifuged and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). After fixation, cells were washed once with PBS and centrifuged, and then analyzed for apoptosis using FACS Aria (BD). Cells positive for Annexin V-FITC and negative for 7-AAD were regarded as apoptotic, positive for both as late apoptotic, positive for 7-AAD and negative for Annexin V-FITC as necrotic, and negative for both as viable.

2.12. High-glucose treatment

Human podocytes were cultured at + 37 °C for 14 days (the time of their differentiation) in RPMI medium containing 30 mM glucose, or mannitol resulting in the same osmolality as a control. Insulin stimulations were performed as described in 2.9. and cells lysed and immunoblotted as described in 2.4.

3. Results

3.1. CD2AP interacts with SHIP2 in glomeruli.

The NH₂-terminus of the adaptor protein CD2AP contains three SH3 domains, which can bind to proline-rich sequence motifs and thus link CD2AP to various signal transduction pathways. To identify proteins that specifically bind to the SH3-domains of CD2AP in glomeruli we performed yeast two-hybrid screening of a rat glomerular library using the NH₂-terminus of CD2AP (aa 1-330) as a bait. Screening of 1×10^6 yeast transformants led to the identification of two SHIP2 clones covering amino acids 726-1257. SHIP2 is a lipid phosphatase that hydrolyzes the 5-position phosphate of phosphoinositol(3,4,5)trisphosphate (PI(3,4,5),P₃) to generate phosphoinositol(3,4)bisphosphate (PI(3,4)P₂), and thus negatively regulates the phosphoinositol 3-kinase (PI3K) pathway mediating various metabolic effects of insulin (Dyson et al 2005).

SHIP2 has previously been shown to be widely expressed in different tissues (Schurmans et al 1999) but its function in the kidney has not been studied. To confirm that SHIP2 is expressed in glomeruli, we isolated rat glomeruli and tubuli by the sieving technique, and immunoblotted the fractions with SHIP2 antibodies. SHIP2 was found to be expressed in both glomeruli and tubuli in the kidney whereas CD2AP was found predominantly in the glomerular fraction (Fig. 1A). Immunoperoxidase staining of mouse kidney sections indicated that SHIP2 localizes in glomeruli (Fig. 1B), and staining of adjacent sections

for SHIP2 and podocyte marker CD2AP confirmed SHIP2 expression in podocytes (Fig. 1C-D).

3.2. Confirmation of the interaction between CD2AP and SHIP2.

The COOH-terminal region of SHIP2 that was identified in the yeast two-hybrid screening contains a proline-rich domain that, based on the screening, mediates the interaction of SHIP2 with the NH₂-terminus of CD2AP harboring the three SH3 domains. To map which of the SH3-domains of CD2AP mediates the interaction, we performed pull-down assays on human podocyte cell lysates with GST-CD2AP 1., 2. and 3. SH3 domains and the GST-CD2AP-NH₂-terminus. The 3. SH3-domain of CD2AP pulled down endogenous SHIP2 from podocyte lysate (Fig. 2A), and similar result was obtained when the pull-down assay was performed on rat glomerular lysates (Fig. 2B), further confirming the interaction between SHIP2 and CD2AP in glomeruli and suggesting that the binding is primarily mediated by the 3. SH3-domain of CD2AP.

To confirm the physiological interaction between CD2AP and SHIP2, we performed a co-immunoprecipitation assay from HIRc cells using CD2AP antibodies. Immunoblotting of the precipitates with SHIP2 antibodies showed that CD2AP co-immunoprecipitates SHIP2, confirming their interaction (Fig. 2C). The interaction was seen similarly in basal state, starved and insulin stimulated cells, indicating that the interaction is not dependent on insulin stimulation. Interestingly, two different forms of SHIP2 were detected in the cell lysate, but only the lower one was precipitated by CD2AP. Immunoprecipitation with SHIP2 antibodies from insulin stimulated HIRc cells and probing the same blot with anti-SHIP2 and anti-phosphotyrosine antibodies showed that only the upper band of SHIP2 is phosphorylated (Fig. 2D), proving that CD2AP binds to SHIP2 in its non-tyrosine phosphorylated form.

3.3. Insulin stimulation induces SHIP2 translocation to plasma membrane.

In differentiated podocytes, endogenous SHIP2 was located predominantly in the cytoplasm in basal and starved state (Fig. 3A-B), but after insulin stimulation, it was

partly translocated to plasma membrane (Fig. 3C-D). CD2AP was localized in cytoplasm and the localization did not change after insulin stimulation (Fig. 3E-F).

3.4. SHIP2 overexpression downregulates insulin signalling in podocytes.

Activation of the PI3K pathway leads to phosphorylation of Akt protein kinase. To study the regulative function of SHIP2 on this pathway in podocytes, we overexpressed SHIP2 in cultured podocytes, stimulated them with insulin and measured Akt phosphorylation level by immunoblotting. In unstimulated cells no difference in Akt phosphorylation was detected when SHIP2 overexpressing cells were compared with control cells transfected with an empty vector (Fig. 4A). But when podocytes were stimulated with insulin, Akt phosphorylation level remained 15.5% lower in SHIP2 overexpressing cells (Fig. 4B). The downregulation of Akt activation was confirmed with an ELISA-based Akt activity assay, showing that in SHIP2 overexpressing podocytes Akt activity was not increased after insulin stimulation (Fig. 5A), in contrast to control cells (Fig. 5B), where it was increased by 32%. The difference in Akt activity between SHIP2 overexpressing and control podocytes after insulin stimulation was 35% ($p=0.02$, Student's t-test). Together, these results indicate that SHIP2 downregulates the insulin signalling pathway in podocytes.

3.5. SHIP2 overexpression promotes apoptosis in cultured podocytes.

Apoptosis is normally detected in low rates in differentiated podocytes. To study if SHIP2 affects apoptosis, SHIP2 was overexpressed in differentiated podocytes by lentiviral infection and apoptosis was measured by FACS. In control podocytes, infected with empty vector, the apoptosis rate was 4.5%, whereas SHIP2 overexpression increased the proportion of apoptotic cells to 8.6% (Fig. 6A-C). The proportion of necrotic cells was not increased by SHIP2 overexpression.

3.6. SHIP2 is upregulated in glomeruli of diabetic rats and mice.

SHIP2 expression in vivo in glomeruli of insulin resistant animals was studied using obese Zucker rats, that are insulin resistant and slightly diabetic due to a mutation in the leptin receptor gene (Chua et al 1996). Kidneys were analyzed at two time points, 12 and

40 weeks of age, 40 weeks being the age when proteinuria has been reported to start (Coimbra et al 2000). Kidneys from 12 weeks old rats appeared histologically normal with hematoxylin-eosin staining (data not shown), but immunoblot analysis of isolated glomeruli showed marked upregulation of SHIP2 expression in obese (fa/fa) rats when compared to lean controls (fa/+) (Fig. 7A, D). At 40 weeks of age the obese rats had slightly higher weights (data not shown) and blood glucose levels (table 1) than the controls, but these differences were not statistically significant. At this time point the obese rats also had proteinuria, as the urine albumin/creatinine ratio was higher in obese rats than in controls (table 1). At 40 weeks of age the elevation of SHIP2 levels in isolated glomeruli of obese rats continued (Fig. 7B, E), whereas in tubuli SHIP2 was expressed at equal levels (Fig. 7C, F).

As another animal model of insulin resistant diabetes we used db/db mice that also have a mutation in the leptin receptor gene (Chua et al 1996). These mice were highly hyperglycaemic at the age of 8 weeks (table 1) and clearly obese compared to heterozygous db/+ controls. They also had notable albuminuria (table 1). SHIP2 expression was studied by immunohistochemistry and the level of SHIP2 was graded in eight glomeruli from each sample by two independent researchers blinded from the genotypes. SHIP2 expression level was rated higher in podocytes in the diabetic group (Fig. 8), showing a similar trend to the obese rats, but there was some variation between individual mice and the difference did not reach statistical significance ($p=0.059$).

3.7. SHIP2 expression in cultured podocytes is not regulated by high glucose.

Cultured human podocytes were used to test whether SHIP2 is upregulated in a high-glucose environment. Podocytes were cultured in cell culture medium containing high glucose concentration, 30 mmol/l, for the time of their differentiation (two weeks) or mannitol as an osmotic control. SHIP2 was expressed at equal levels in both conditions, and also the Akt phosphorylation response to insulin was similar (Fig. 9).

4. Discussion

Podocytes have been shown to belong to insulin target cells (Coward et al 2005) and to develop insulin resistance (Tejada et al 2008), but the mechanisms have not been elucidated. Considering the findings from clinical studies linking insulin resistance to diabetic nephropathy (Parvanova et al 2006, Orchard et al 2002), it can be hypothesized that disturbances in insulin signalling affect podocyte function. This study identifies SHIP2 as a new interaction partner for CD2AP in glomeruli and focuses on its role in the regulation of insulin signalling in podocytes.

The interaction of CD2AP and SHIP2 was mapped by pull down assay to be mediated by the SH3 domains of CD2AP, most strongly by the third one, that are known to bind to proline rich domains. SHIP2 has a proline rich domain with the consensus sequence for SH3 domain ligand (Marion et al 2002), and interestingly, this domain was covered by both SHIP2 clones identified in the yeast-two-hybrid screening. Immunoprecipitation experiments confirmed that endogenous CD2AP and SHIP2 proteins are in the same complex, and that the interaction is not regulated by insulin stimulation. However, CD2AP was found to bind SHIP2 in its non-tyrosine phosphorylated form. SHIP2 tyrosine phosphorylation has previously shown to be induced by insulin and other growth factors (Habib et al 1998) and suggested to alter its subcellular localization and enzymatic activity (Prasad et al 2009). Our results show that insulin stimulation induces partial SHIP2 translocation to plasma membrane in differentiated podocytes, a similar phenomenon as seen previously in COS-7 cells after EGF stimulation (Pesesse et al 2001, Dyson et al 2001). In that model, SHIP2 was also shown to regulate actin (Dyson et al 2001). Thus, alterations in SHIP2 expression in diabetes could affect the dynamic regulation of the actin cytoskeleton needed to maintain the functional structure of the podocyte foot processes. Interestingly, also CD2AP has been shown to participate in the regulation of actin organization (Kirsch et al 1999), and to function as a scaffolding protein that binds to membrane proteins (Dustin et al 1998, Palmen et al 2002), actin cytoskeleton (Lehtonen et al 2002), and signalling proteins (Huber et al 2003). Our results suggest that CD2AP could have a role in the formation of signalling complexes

including SHIP2 in podocytes.

In order to investigate the function of SHIP2 in podocytes we overexpressed it in cultured human podocytes and measured insulin sensitivity of the cells by measuring Akt phosphorylation and activation in response to insulin. SHIP2 overexpression downregulated the response like it has been previously shown to do in other insulin responsive cells (Sasaoka et al 2001, Wada et al 2001). The mechanisms of insulin resistance seem to be partly shared and partly separate in different cell types, and have been mainly studied in the traditional insulin target cells, myocytes, adipocytes and hepatocytes (Tomas et al 2002, Savage et al 2007, Houstis et al 2006). Podocyte cell line derived from db/db mice has earlier been shown to be insulin resistant compared to podocyte line from control mice (Tejada et al 2008). The results of this study identify the upregulation of SHIP2 as a mechanism inducing impaired insulin signalling in podocytes, leading to reduced Akt phosphorylation and activation.

Apoptosis rate was markedly increased in SHIP2 overexpressing differentiated podocytes, in which apoptosis is normally detected in low levels. Akt phosphorylation, also induced by CD2AP, has previously shown to act as a survival signal in podocytes protecting them from apoptosis (Huber et al 2003), suggesting that impaired insulin response might contribute to podocyte loss in diabetic nephropathy. In addition to the regulation of PI3K pathway, SHIP2 also modulates, via its docking properties, JNK activation involved in apoptotic pathways (Xie et al 2008), this being another possible mechanism by which SHIP2 could increase apoptosis.

In addition to the in vitro results, we found that SHIP2 is upregulated in vivo in glomeruli of obese Zucker rats and db/db mice, two well characterized models of insulin resistance and type II diabetes. In both of these models the development of proteinuria and renal injury has been analyzed, and they have been shown to share multiple similarities to early human diabetic nephropathy (Coimbra et al 2000, Sharma et al 2003). Our results show upregulation of SHIP2 in obese rat glomeruli already before histological changes and the reported age of onset of proteinuria. Thus SHIP2 upregulation is not a secondary effect of

podocyte damage but rather might be involved in the development of diabetic nephropathy. Interestingly, SHIP2 was not upregulated in tubuli of obese Zucker rats, which further underlines the nature of podocytes as targets of insulin action and resistance.

Hyperglycaemia is known to induce insulin resistance in muscle cells by reducing Akt phosphorylation in response to insulin (Tomas et al 2002). This prompted us to study if high glucose affects insulin signalling in podocytes by regulating SHIP2 expression. Our findings did not support this hypothesis as high glucose concentration did not induce SHIP2 upregulation in cultured podocytes, nor did it affect Akt phosphorylation. We cannot exclude the possibility that SHIP2 upregulation in glomeruli in vivo could result from glucotoxicity, when the podocytes can be exposed to hyperglycaemia for a longer time. It seems more likely though, that upregulation of SHIP2 in vivo could be due to long term programming of cells either genetically or by environmental factors, possibly including obesity. Also our results from animal models suggest that upregulation of SHIP2 does not depend on hyperglycaemia, as SHIP2 was upregulated in obese Zucker rats, which did not have significantly elevated blood glucose levels. Both animal models have a mutation in the leptin receptor gene, leading to hyperphagia and obesity (Chua et al 1996). Many of the factors promoting insulin resistance, namely adipocytokines, free fatty acids, inflammation and oxidative stress, are linked to obesity (Savage et al 2007, Houstis et al 2006). Obesity is a central feature of the metabolic syndrome, and interestingly, SHIP2 gene *INPPL1* polymorphisms have been associated to the components of the metabolic syndrome and type II diabetes in humans (Marion et al 2002, Kaisaki et al 2004).

In clinical studies, insulin resistance has been associated with proteinuria in both type 1 and type 2 diabetic patients (Parvanova et al 2006, Orchard et al 2002), and the insulin sensitizing agents thiazolidinediones have been shown to have a renoprotective effect in diabetic patients (Miyazaki et al 2007) and animal models (Zhang et al 2008), but the mechanism is not known. Previously SHIP2 levels have been found to be elevated in db/db mouse muscle and adipose tissues (Hori et al 2002), and could be lowered by

treatment with a thiazolidinedione drug, rosiglitazone. Considering the previous data together with our finding of SHIP2 expression in glomerular podocytes in db/db mice, it could be speculated that SHIP2 might have a role in the effects of thiazolidinediones in the kidney.

In conclusion, this study shows that glomerular podocytes express lipid phosphatase SHIP2 and that SHIP2 downregulates insulin response and promotes apoptosis in podocytes. Further, SHIP2 expression was found to be elevated in glomeruli of diabetic rats prior to the development of proteinuria. Collectively, these results demonstrate a possible mechanism for insulin resistance in podocytes and suggest the involvement of SHIP2 in altered podocyte function in diabetes.

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FIGURE LEGENDS

Fig. 1. SHIP2 is expressed in glomerular podocytes. (A) SHIP2 is expressed in both glomeruli (lane 1) and tubuli (lane 2) whereas CD2AP is expressed predominantly in glomeruli (lane 1). 100 µg of rat glomerular and tubular lysates were separated by SDS-PAGE and immunoblotted with anti-SHIP2, anti-CD2AP and anti-actin. (B) SHIP2 localizes along the capillary loops (arrows) of the glomerulus. (C-D) Higher magnification (inserts) of adjacent sections stained for SHIP2 (C) and CD2AP (D) confirms that SHIP2 is expressed by podocytes. Adult mouse kidney paraffin sections were processed for immunoperoxidase staining, labelled with anti-SHIP2 and anti-CD2AP, and examined by light microscopy. Bar (B), 20 µm.

Fig. 2. SHIP2 associates with CD2AP. (A) SHIP2 is pulled down from human podocyte cell lysate with GST- 3. SH3 domain of CD2AP (CD2AP-3.SH3, lane 4) but not with GST alone (lane 5). Lane 6, podocyte cell lysate. (B) Also from rat glomerular lysate SHIP2 is pulled down with GST- 3. SH3 domain of CD2AP (CD2AP-3.SH3, lane 4), and in addition, weaker bands are observed with GST-CD2AP NH₂-terminus and GST- 2. SH3 domain of CD2AP (CD2AP-N and CD2AP-2.SH3, lanes 1 and 3) but not with GST alone (lane 5). Lane 6, rat glomerular lysate. Human podocyte and rat glomerular lysates were incubated with GST-CD2AP fusion proteins or GST alone immobilized on beads. Beads were washed, proteins separated by SDS-PAGE and immunoblotted with anti-SHIP2. (C) SHIP2 is co-immunoprecipitated with CD2AP antibodies (lanes 1, 4 and 7), but not with control serum (lanes 2, 5 and 8), from HIRc cells in basal, starved or insulin stimulated state, indicating that the interaction is not dependent on insulin stimulation. SHIP2 is seen as two bands in lysate (lanes 3, 6 and 9) but only the lower one is co-precipitated with CD2AP. (D) Immunoprecipitation with SHIP2 antibodies from HIRc cells stimulated with 17 nM insulin for 7 min, and probing the same blot with anti-SHIP2 and anti-phosphotyrosine antibodies shows that the upper band of SHIP2 is

phosphorylated. Cells were lysed and immunoprecipitations performed with anti-CD2AP and control serum, or anti-SHIP2 and control IgG, followed by immunoblotting with anti-SHIP2, anti-CD2AP and anti-phosphotyrosine.

Fig. 3. Insulin stimulation induces SHIP2 translocation to plasma membrane.

(A-D) In differentiated podocytes, endogenous SHIP2 is localized mainly in the cytoplasm in basal state (A) and starved cells (B). After 5 minutes of insulin stimulation, SHIP2 is occasionally seen at the cell membrane (C, arrow), and after 15 minutes of stimulation the membrane localization becomes more frequent (D, arrows). (E-F) CD2AP is localized in cytoplasm in basal state (E), and its localization does not change after insulin stimulation (F). Bar, 40 μ m.

Fig. 4. SHIP2 downregulates insulin signalling in podocytes. (A) In unstimulated SHIP2 overexpressing human podocytes the phosphorylation level of Akt does not differ from control cells transfected with an empty vector. (B) After 15 min insulin stimulation the phosphorylation level of Akt remained 15.5% lower in SHIP2 overexpressing podocytes than in control cells. SHIP2 was transiently transfected into proliferating podocytes, cells were starved overnight and stimulated with 200 nM insulin for 15 min, lysed and immunoblotted with anti-P-Akt (Ser473) and anti-Pan Akt. Quantitation was done with Odyssey Infrared Imager, and the amount of phosphorylated Akt was adjusted to total Akt in each lane. Bars (above) show results from either 8 experiments (4 independent experiments in duplicate) (A) or 12 experiments (4 independent experiments in triplicate) (B). Representative immunoblots are shown below. Akt phosphorylation level in SHIP2 overexpressing podocytes is presented as percentage of control. Bars show mean and error bars STDEV. ** $p < 0.01$, Wilcoxon signed rank test.

Fig. 5. SHIP2 downregulates insulin induced Akt activation in podocytes. (A) In SHIP2 overexpressing podocytes Akt activity is not increased after insulin stimulation, in

contrast to control cells (B), where it is increased by 32%, (n=6, error bars SEM). Akt activity from SHIP2 transfected and control podocytes was measured with ELISA-based Akt kinase activity assay kit.

Fig. 6. SHIP2 overexpression increases apoptosis in cultured human podocytes. (A) SHIP2 overexpression increased the proportion of apoptotic cells to 8.6%, whereas in control podocytes infected with empty vector, the apoptosis rate was 4.5%. (B-C) Representative pictures from 3 independent experiments, each with 5-10 parallel samples. n=20, error bars STDEV, ** p<0.01, Student's t-test. SHIP2 was overexpressed in differentiated podocytes by lentiviral infection. Apoptosis was measured by fluorescence-activated cell sorting (FACS) using annexin V-FITC and 7-Aminoactinomycin D (7-AAD) double staining.

Fig. 7. SHIP2 is upregulated in obese Zucker rat glomeruli. (A-B) SHIP2 is upregulated in the glomeruli of 12 weeks (A) and 40 weeks (B) old obese Zucker rats when compared to lean controls. (C) In tubuli of 40 weeks old rats SHIP2 is expressed at equal levels in both groups. Glomeruli and tubuli were isolated, lysed and immunoblotted with anti-SHIP2 and anti-actin. SHIP2 expression was quantitated with Odyssey Infrared Imager and adjusted to actin in each lane. The bars (A-C) show the mean expression in arbitrary units (error bars STDEV). A, n=3. B and C, n =6. * p<0.05, student's t-test. (D) Immunoblot of SHIP2 in glomeruli of 12 weeks old Zucker rats. (E-F) Representative immunoblots of SHIP2 in glomeruli (E) and tubuli (F) of 40 weeks old rats.

Fig. 8. SHIP2 in diabetic mouse glomeruli. (A-B) SHIP2 staining in diabetic db/db mouse glomerular podocytes (B) compared to db/+ control (A). 8-week old db/+ and db/db mouse kidney paraffin sections were processed for immunoperoxidase staining and labelled with anti-SHIP2. Bar, 20 μ m. (C) SHIP2 expression was rated visually (scale 1-5) by two independent researchers blinded from the genotypes. Analysis of 6 db/+ and 6

db/db mice (eight randomly chosen glomeruli analyzed from each mouse) showed a trend of higher glomerular expression of SHIP2 in diabetic db/db mice, but the difference did not reach statistical significance. $p=0.059$, Students's t-test, error bars STDEV.

Fig. 9. SHIP2 expression in cultured podocytes is not regulated by high glucose. (A) High-glucose treatment does not induce SHIP2 upregulation in cultured human podocytes compared to mannitol treated cells. (B-C) The level of Akt phosphorylation was the same in high-glucose and mannitol treated cells both without (B) and with (C) insulin stimulation. Podocytes were cultured for two weeks in RPMI medium containing 30 mM glucose, or mannitol resulting in the same osmolality as a control. Quantitation of the immunoblots was performed with Odyssey Infrared Imager, and SHIP2 expression was adjusted to actin, and P-Akt to total Akt in each lane. The bars (A-C) show the mean results of 6 experiments (3 independent experiments done in duplicate) in arbitrary units (error bars STDEV). (D) A representative immunoblot of SHIP2 in high-glucose and mannitol treated podocytes. (E-F) Representative immunoblots of P-Akt in high-glucose and mannitol treated podocytes without (E) and with (F) insulin stimulation.

Table 1. Blood glucose and urinary albumin values of Zucker rats and db/db mice.

	Blood glucose, mmol/l (n=6)	Urine albumin to creatinine, mg/mg
Obese Zucker, 40 wks	6.1 ± 1.1	25.0 ± 13.6 (n=6) *
Lean Zucker, 40 wks	5.2 ± 0.7	2.6 ± 2.3 (n=3)
db/db, 8 wks	> 30	5.8 ± 2.2 (n=6) **
db/+, 8 wks	8.8 ± 0.8	0.6 ± 0.2 (n=4)

Table 1. Obese Zucker rats at 40 weeks had slightly higher blood glucose than controls, but this was not significant. The urinary albumin to creatinine ratio was elevated in the obese group, * $p < 0.05$. Diabetic db/db mice had significantly higher blood glucose than controls and also albuminuria (** $p < 0.005$) at the age of 8 weeks.

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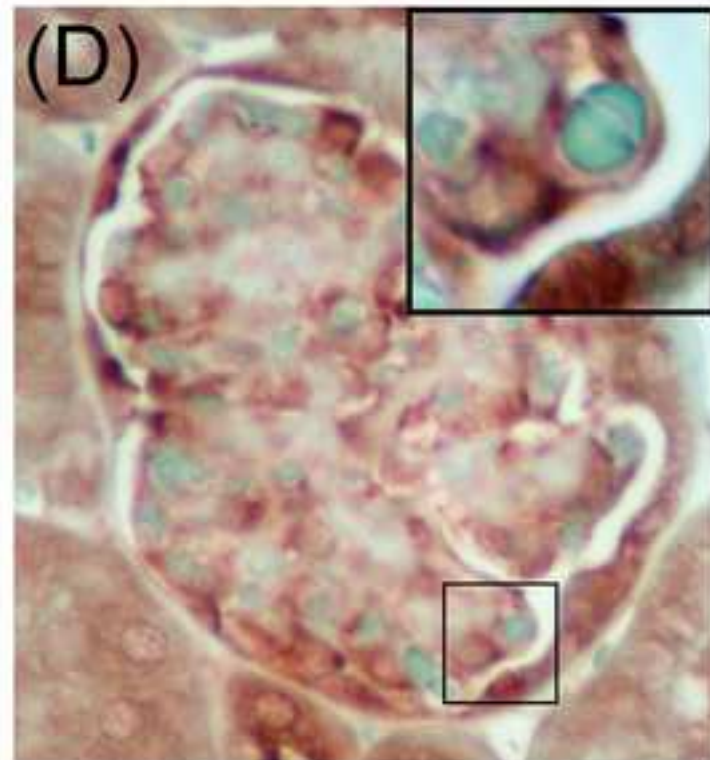
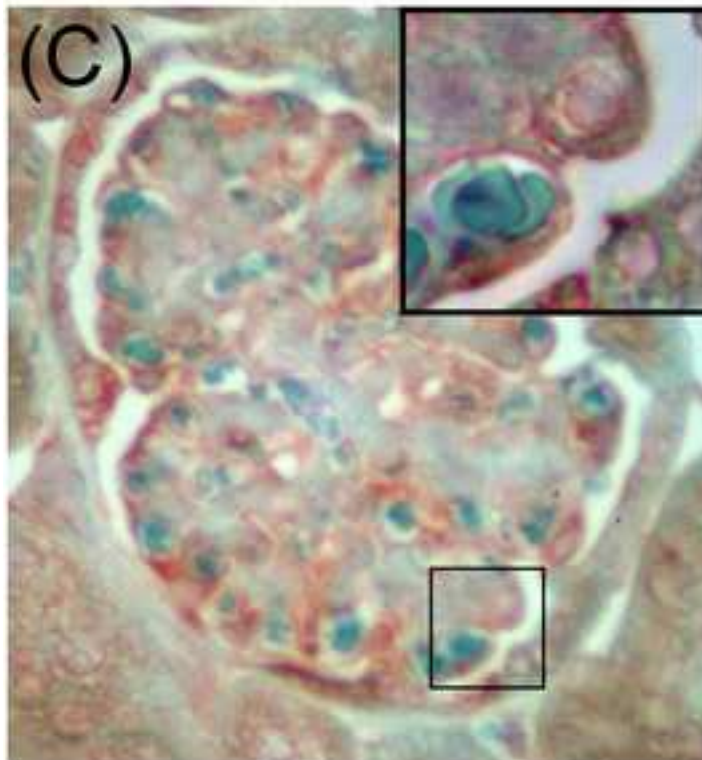
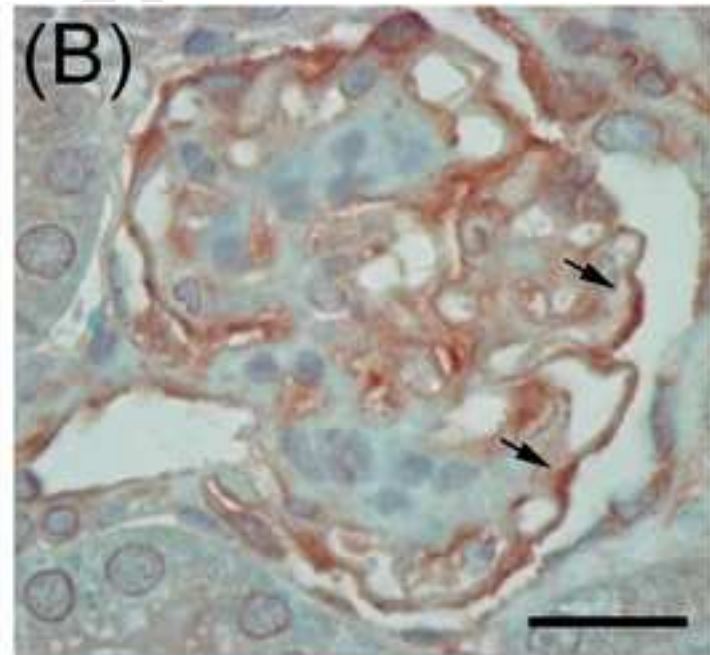
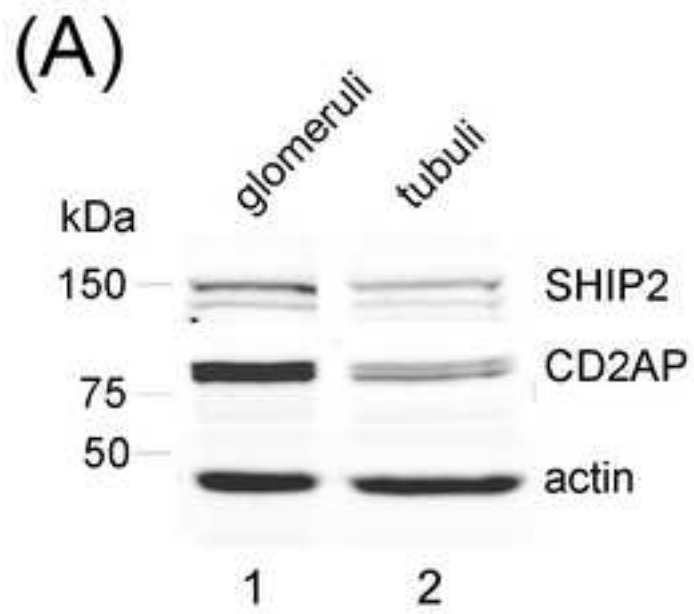
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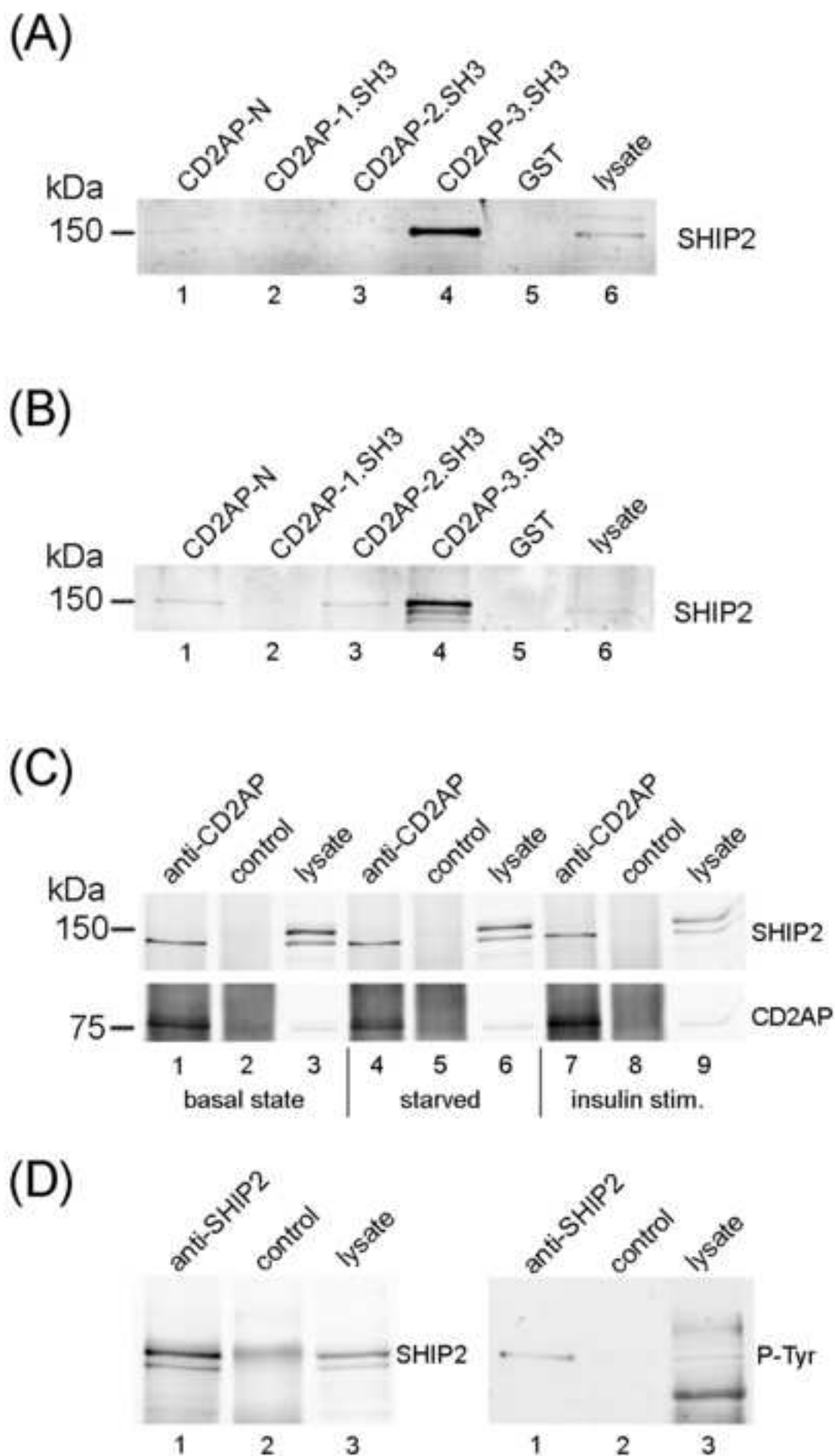
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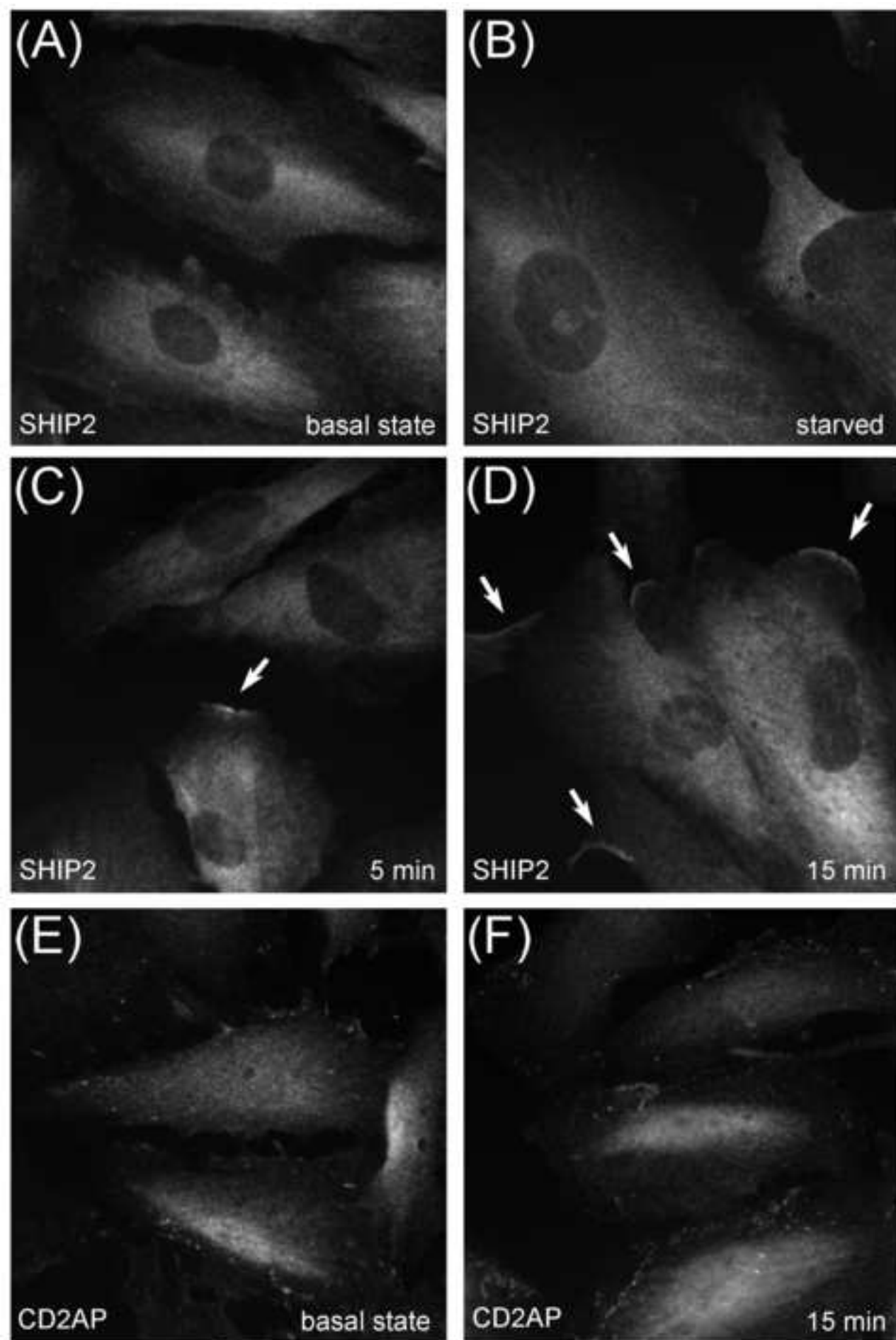
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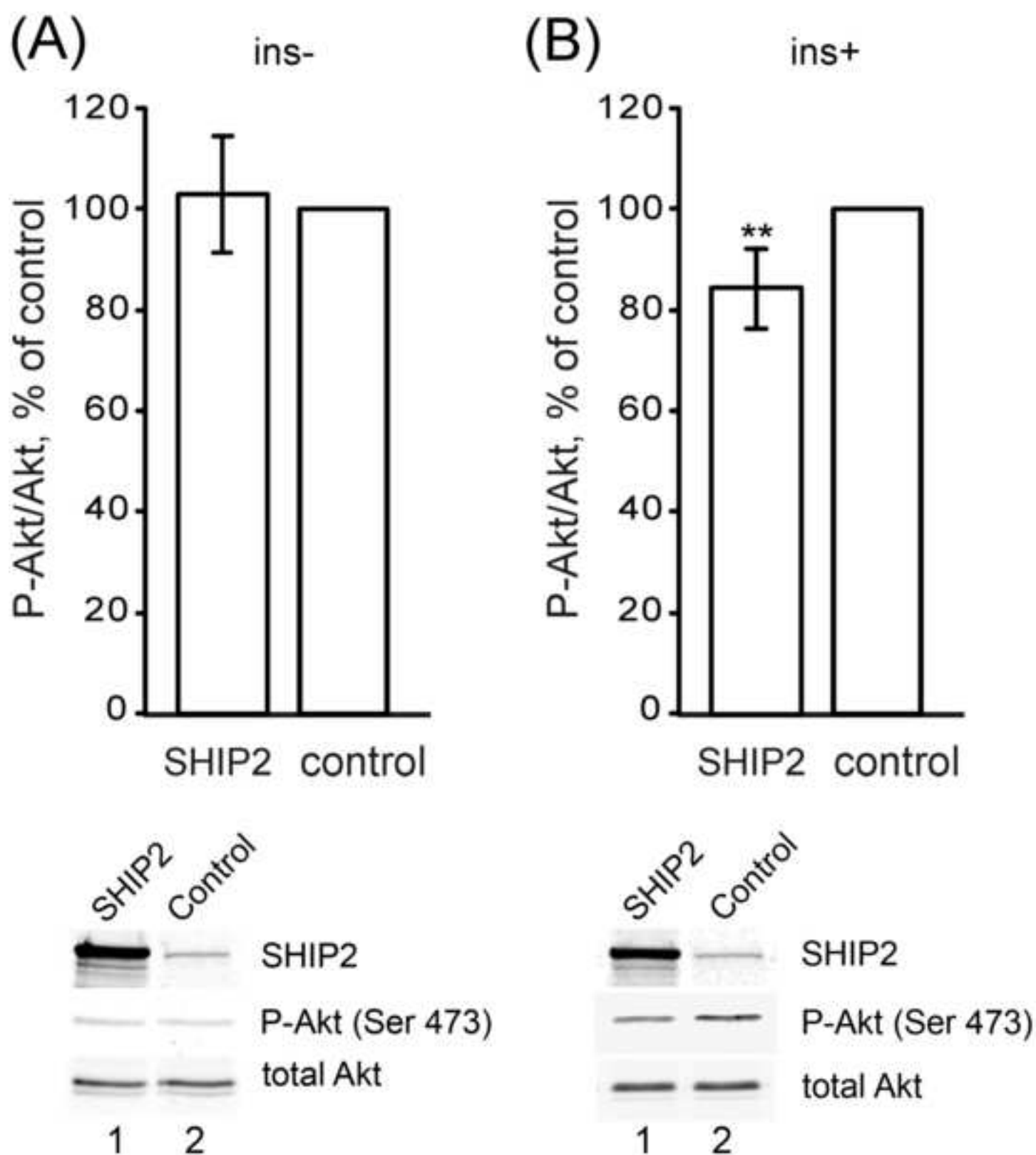
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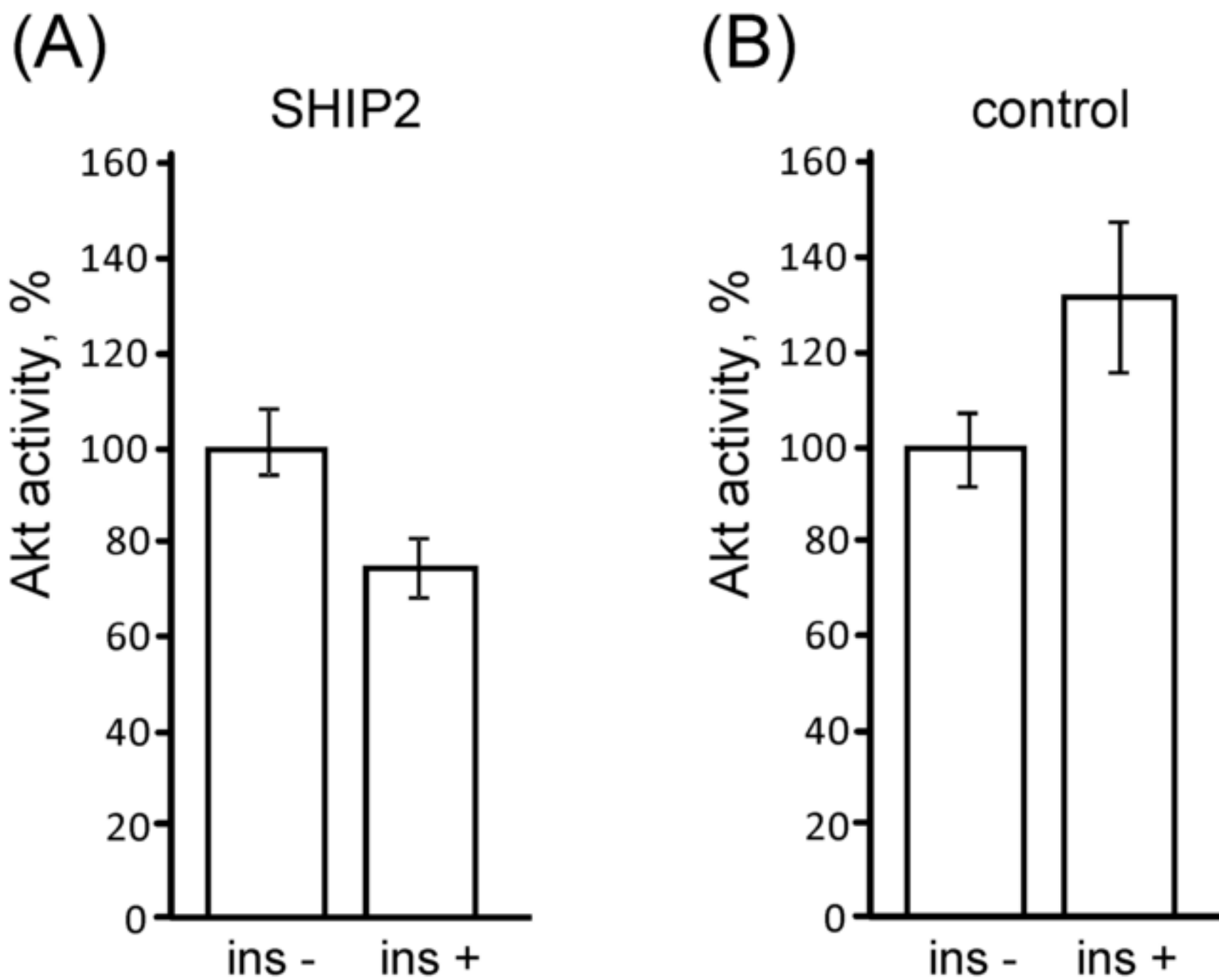
Figure 1



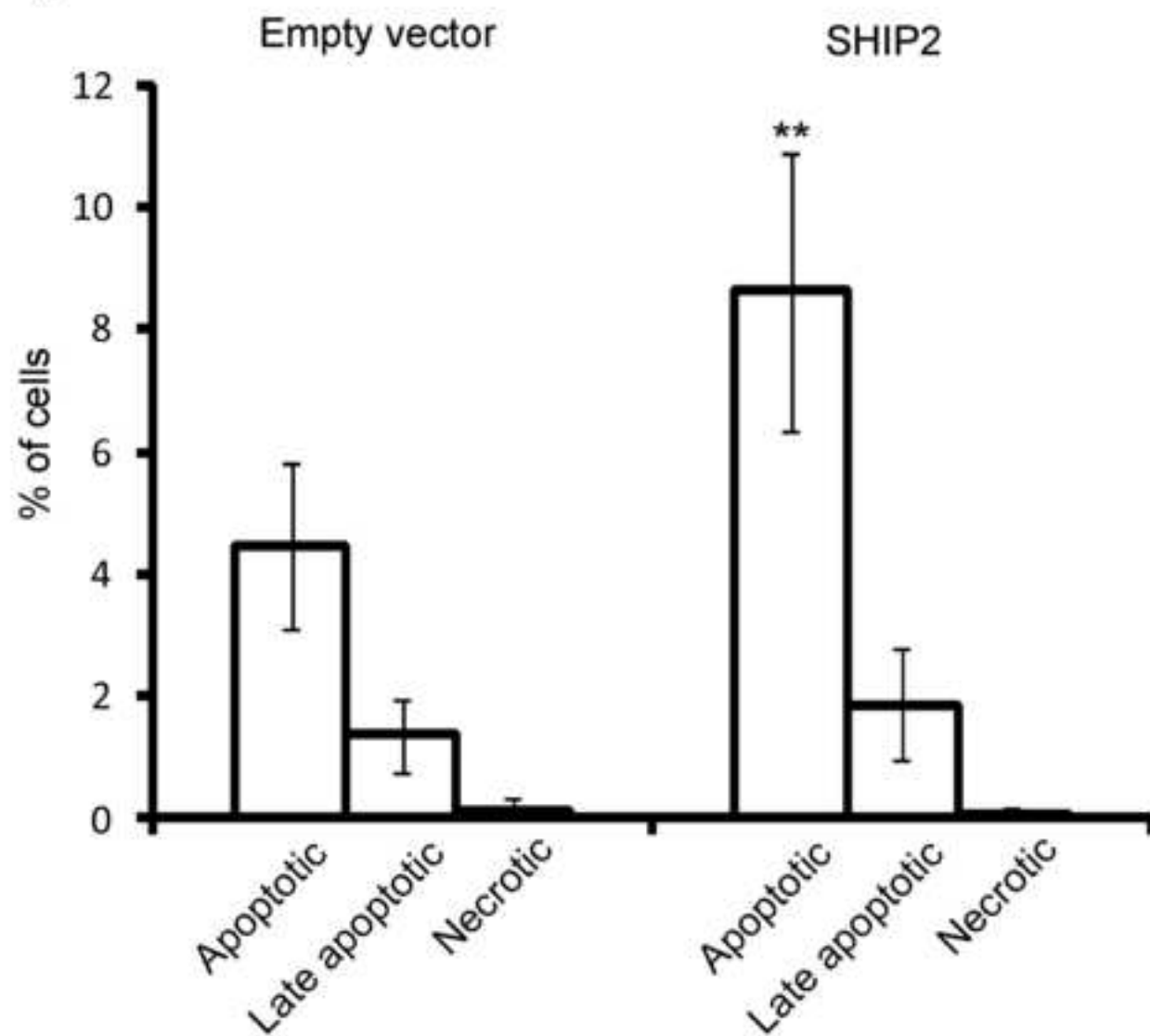




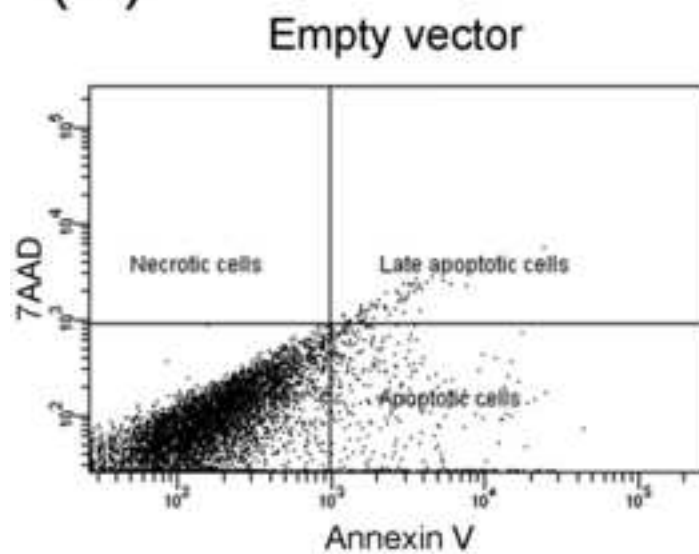




(A)



(B)



(C)

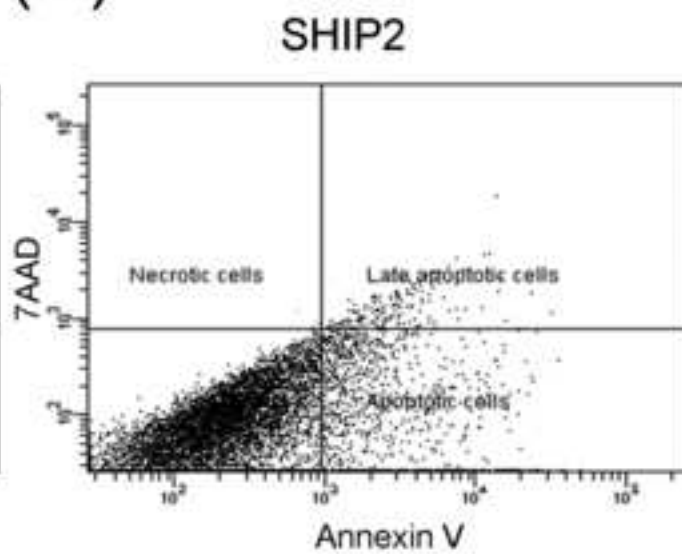


Figure 7

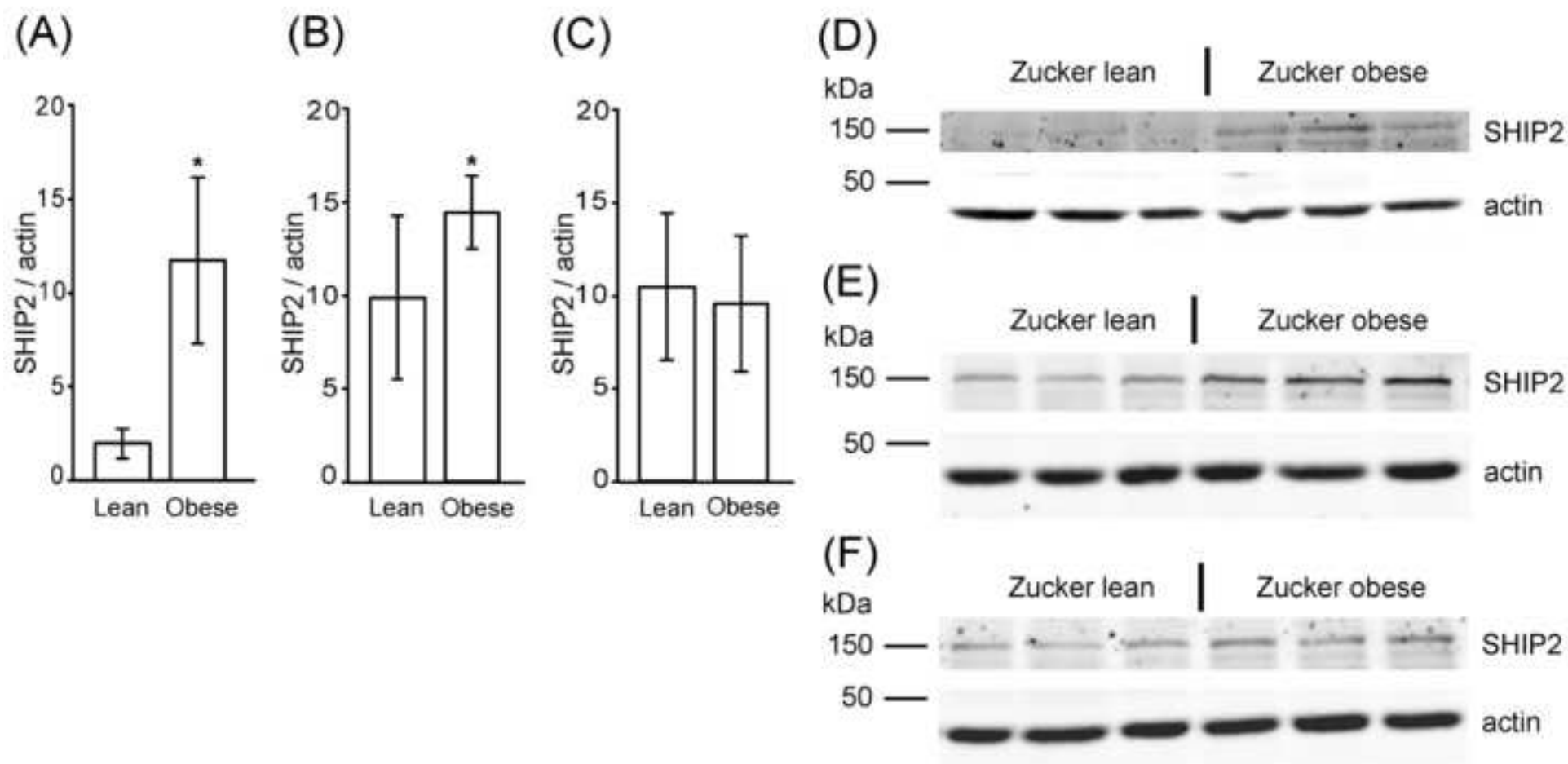


Figure 8

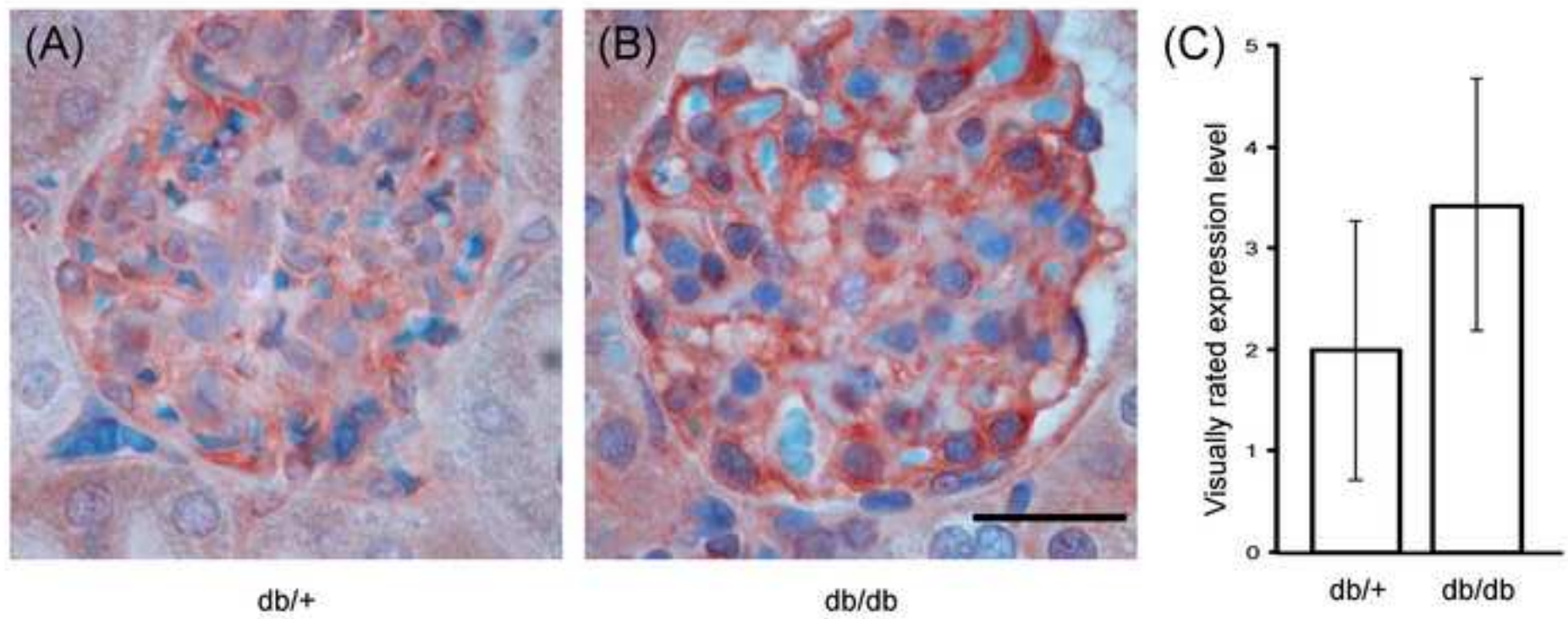


Figure 9

