Original Article

Nephrin Is Critical for the Action of Insulin on Human Glomerular Podocytes

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The leading causes of albuminuria and end-stage renal failure are secondary to abnormalities in the production or cellular action of insulin, including diabetes and hyperinsulinemic metabolic syndrome. The human glomerular podocyte is a critical cell for maintaining the filtration barrier of the kidney and preventing albuminuria. We have recently shown this cell to be insulin sensitive with respect to glucose uptake, with kinetics similar to muscle cells. We now show that the podocyte protein nephrin is essential for this process. Conditionally immortalized podocytes from two different patients with nephrin mutations (natural human nephrin mutant models) were unresponsive to insulin. Knocking nephrin down with siRNA in wild-type podocytes abrogated the insulin response, and stable nephrin transfection of nephrin-deficient podocytes rescued their insulin response. Mechanistically, we show that nephrin allows the GLUT1- and GLUT4-rich vesicles to fuse with the membrane of this cell. Furthermore, we show that the COOH of nephrin interacts with the vesicular SNARE protein VAMP2 in vitro and ex vivo (using yeast-2 hybrid and coimmunoprecipitation studies). This work demonstrates a previously unsuspected role of nephrin in vesicular docking and insulin responsiveness of podocytes. ***Diabetes*** **56:1127–1135, 2007**

The renal glomerulus is the region of the kidney forming the biological sieve that allows water and small solutes to freely pass from circulation into the urine but is relatively impermeable to macromolecules, such as albumin. Albumin loss into the urine (albuminuria) is prevented by the glomerular filtration barrier (GFB). The GFB consists of three layers: endothelial cells lining the capillary wall, the glomerular basement membrane, and, adjacent to the urinary space, podocytes. The importance of the podocyte in the development of albuminuria became obvious in the late 1990s through landmark discoveries of inherited human genetic conditions that resulted in congenital or early-onset nephrotic syndrome (1–3). These all coded for proteins (nephrin, podocin, and α-actinin–4) found exclusively in the podocyte in the kidney. Recently, two further inherited human proteinuric genetic conditions that exclusively affect the podocyte in the kidney have been described: mutations in Laminin-β2, which affect podocyte adhesion to the glomerular basement membrane (4), and TRPC6 mutations, which cause altered calcium flux into the podocyte (5,6).

The prevention of albuminuria is essential, because evidence suggests (7) if prolonged, it accelerates the progression to end-stage renal failure (ESRF). The largest proportion of patients with albuminuria and ESRF in the western world have abnormalities in the production of insulin (type 1 diabetes) or its cellular effects (type 2 diabetes) (8,9). Furthermore, even in nondiabetic normoglycemic subjects, microalbuminuria is associated with insulin resistance and is an independent cardiovascular risk factor (10). There is accumulating evidence that the podocyte is central in the development of diabetic nephropathy (11), although the mechanism is unclear. We have recently reported (12) the novel observation that wild-type (WT) podocytes are insulin-sensitive cells. Other cell types previously shown to respond in this way include adipocytes and muscle cells (13). In the GFB, insulin sensitivity is specific for podocytes, as conditionally immortalized human glomerular endothelial cells (14) are insulin unresponsive (12).

Nephrin (OMIM 602716) is a transmembrane protein of the immunoglobulin superfamily and consists of a large extracellular portion with eight C2-type IgG-like domains, a single fibronectin type-3–like motif, and a cytoplasmic domain with multiple tyrosine phosphorylation sites. Mutations in genes coding for this protein cause the most severe form of congenital nephrotic syndrome (Finnish-type congenital nephrotic syndrome), with children being born leaking massive amounts of protein into their urine. The most common genetic defect is coded for by the Fin major (FM) genotype, which results in complete truncation of the protein (1), although a large number of other mutations are also described (1,15). Children with this disorder used to succumb to the complications of prolonged severe nephrosis (infection and thrombosis) but now are surviving to adulthood through a program of therapy that includes therapeutic bilateral nephrectomy. The function of nephrin in preventing protein loss into the urine has been an area of intense research over the past 5 years and has been proposed to be due to its structural

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2-DOG, 2-deoxyglucose; ESRF, end-stage renal failure; GFB, glomerular filtration barrier; FM, Fin major; FM_{nt}, FM nephrin transfected; GFP, green fluorescent protein; GST, glutathione S-transferase; IF, immunofluorescence; MDCK, Madin-Darby canine kidney; NM, nephrin missense; WT, wild type.

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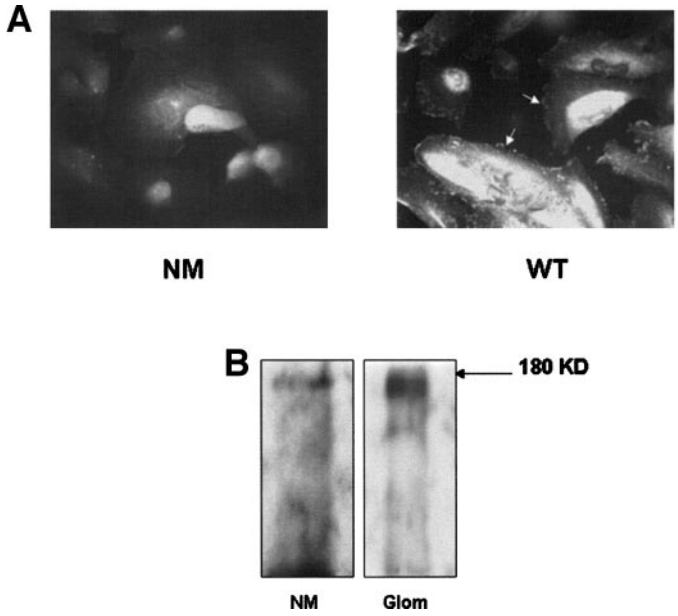

FIG. 1. Nephrin expression in NM mutant podocytes. *A*: IF of WT and NM podocytes. Both were thermoswitched for 14 days with the same antibody concentrations and incubation time. WT shows peripheral staining (arrowed). In NM, nephrin is located within the cytoplasm. *B*: NM quantitatively shows nephrin expression as demonstrated by Western blot. Single band corresponds with positive control from human glomeruli. Western blot and IF were performed with mouse monoclonal antibody 50A9.

properties in maintaining the slit diaphragm between podocyte foot processes (1,16) and as a signaling molecule through tyrosine phosphorylation of its COOH (17–20). Moreover, very recently, nephrin has been shown to be able to regulate the actin cytoskeleton of the podocyte through Nck adaptor proteins (21,22).

We have developed conditionally immortalized podocytes from children with mutations in the nephrin gene (natural human nephrin mutant models) and children who have normal WT podocytes with no detected podocyte protein mutations (23). Using these unique cell lines, this article demonstrates that the protein nephrin is critical for the insulin response in human podocytes.

RESEARCH DESIGN AND METHODS

Cells. Human podocytes were immortalized as previously described (23), and all contained the *tsSV40 T* gene and a gene encoding the catalytic domain of human telomerase (24). All expressed the podocyte markers Wilms' Tumor 1, synaptopodin, and podocin after 14 days of thermo-switching. The FM nephrin-deficient cell line characterization has previously been described (25). A cell line derived from a female child who had congenital nephrotic syndrome and her kidneys removed at 21 months of age was also examined. These podocytes had a homozygous missense mutation of the *NPHS1* gene (NM) with a resulting guanine to adenine nucleotide change at position 1379, causing a substitution at amino acid 460 of the protein of glutamate for arginine. This is predicted to cause protein trapping of nephrin in the cytoplasm of the cell, which was supported by immunofluorescence (IF) and Western blotting of the immortalized differentiated NM podocyte (Fig. 1). Different clones were studied for each cell type, and passages numbers between 5 and 20 were used for each. All experiments were performed on podocytes thermo-switched to 37° C for at least 14 days to silence the *SV40* gene.

2-Deoxyglucose uptake. The same methods as previously described (12) were used with paired basal and insulin-stimulated podocytes being studied. An insulin dose of 100 nmol/l for 15 min was used to stimulate the cells.

Small inhibition of nephrin RNA. The siRNA target sequence that was used to knock down nephrin was GUCGCUCAUCCUGAACGUA. A scrambled sequence of GUCGCUCUCACUGAACGUA (a 3-bp difference) was used as a control. The same methodology as previously described (12) was used.

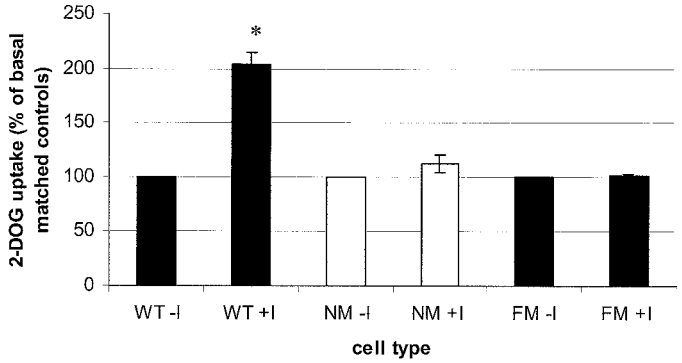

FIG. 2. Insulin-stimulated 2-DOG uptake in human nephrin mutant podocyte cell lines. 2-DOG uptake in WT and NM and FM immortalized podocytes. Basal wells (−) were compared with 15 min of stimulation with 100 nmol/l insulin (+). Seven to 14 independent experiments were performed for each condition (SEM shown). Significant difference between groups, $P < 0.001$ (ANOVA). Post hoc Bonferroni reveals a significant increase in WT insulin response (*) compared with either nephrin mutant cell lines (FM or NM); $P < 0.01$.

Podocytes were incubated for 48 h of siRNA before assessing protein levels and performing 2-deoxyglucose (2-DOG) uptake.

Stable transfection of nephrin into FM podocytes. Full-length nephrin cDNA was incorporated into a Zeocin-resistant vector pcDNA3.1/Zeo$^+$(Invitrogen, Paisley, U.K.). Transfection was achieved using the cationic lipid-based transfection reagent Lipofectamine 2000 CD (Invitrogen) with Optimem (Gibco) and selection with 1 mg/ml Zeocin. Transfection was achieved in the permissive 33°C podocytes, but after transfection the cells were allowed to differentiate after thermo-switching for 14 days. Passages 1–3 of the FM nephrin-transfected (FM_{nt}) cells were studied.

Immunofluorescence. Nephrin immunofluorescence (IF) was achieved using the monoclonal mouse antibodies 48E11 or 50A9, which were a kind gift from Prof. K. Trygvasson (Karolinska Institute, Stockholm, Sweden). GLUT4 was visualized using the monoclonal antibody 1F8 (R&D Systems). Secondary fluoroscein isothiocyanate anti-mouse antibodies (Jackson) were used in all IF. The same protocol as previously described was used (12)

Bis-glucose photolabeling. WT, FM, and FM_{nt} cells were studied for functional GLUT1 at the plasma membrane in their basal state and after 15 min of 100 nmol/l insulin as previously described (12).

Western blotting. A standard Western blot protocol was followed (25). For nephrin, the monoclonal mouse antibody 50A9 or rabbit polyclonal K2737 was used (kind gifts from Prof. Trygvasson), and for GLUT1, a polyclonal rabbit antibody was used (a kind gift from Prof. G. Holman). Where necessary, densitometry was performed using a Bio-Rad gel doc 1000 mini transilluminator and processed using Quantity One software package (Bio-Rad, Hercules, CA).

Yeast 2 hybrid analysis: identification of an interaction between cytosolic nephrin and VAMP2 by kidney cDNA library screening

Formation of a pGBDU-nephrin (amino acids 1085–1160) construct. A cDNA corresponding to cytosolic nephrin amino acids 1085–1160 was PCR amplified from Quickclone human kidney cDNA (Clontech) using *Pfu* Polymerase (Stratagene) and gene-specific primers: 5′ aaagaattcctctggcagcggagac tcaggc, 3′ aaagtcgacttatcgggaataagacacctcctcc. This was subcloned into the *Gal4* DNA–binding domain vector pGBDU-C3 (gift from Dr. P. James, University of Wisconsin) to generate an in-frame *Eco*R1/*Sal*I fragment. Inserts were confirmed by *Eco*R1/*Sal*I digest and directly sequenced using ABI 377 Automated Technology.

Yeast 2 hybrid screening. A Gal4-based yeast 2 hybrid system was used. Competent yeast cells were prepared using a freshly genotyped *pJ69–4A* yeast strain (MATa, *gal4*Δ, *gal80*Δ, *trp*1-901, *leu*2-3, *ura*3-52, *his*3-200, GAL2-ADE2, LYS2::GAL1-HIS3, and met2::GAL7-LacZ). These were sequentially transformed using lithium acetate with 100 ng pGBDU-nephrin (amino acids 1085–1160) DNA followed by 250 μg pYES-Trp-human adult kidney cDNA library DNA. Transformed cells were recovered by shaking at 225 rpm at 30°C in selective media for ~9 h, harvested, and then resuspended in minimal media. For serial dilutions and plating on -Trp plates, 100 μl was removed to determine library transformation efficiency. To test histidine reporter activation, 500 μl aliquots were plated onto -Ura -Trp -His + 2 mmol/l 3AT and incubated at 30°C for 4 days. Colonies rising from histidine reporter activation were streaked in duplicates onto -Ura, -Trp, -Ade plates to test adenine reporter activation. One set of duplicate

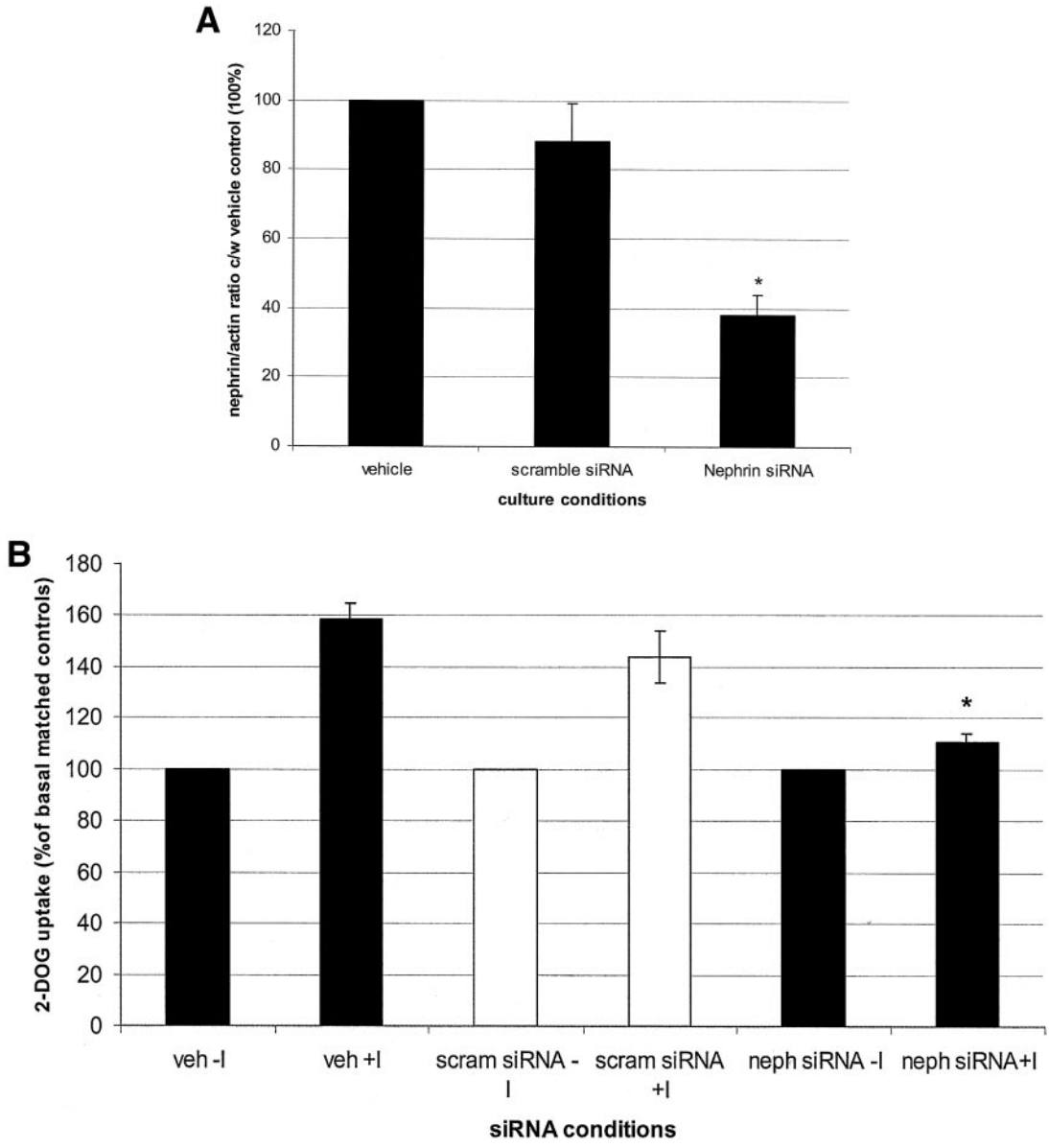

FIG. 3. Nephrin knockdown and glucose uptake in WT human podocytes. *A*: Densitometry of three independent experiments showing the nephrin/actin ratio between vehicle, nephrin, and scramble siRNA. Nephrin knockdown results in a 62% reduction in nephrin/actin signal (SEM shown, analyzed using ANOVA). Significant difference between groups, $P < 0.05$. Post hoc Bonferroni analysis; *$P < 0.05$ vs. vehicle. *B*: 2-DOG uptake in WT podocytes with nephrin-specific siRNA (nephrin siRNA), scrambled siRNA (scram siRNA), and vehicle-only treated podocytes (veh); five to seven independent experiments for each condition. SEM shown. ANOVA, $P = 0.01$ for all groups. Post hoc Bonferroni analysis with significant decrease in nephrin siRNA-treated cells (*). $P < 0.05$ in comparison to WT podocytes. No significant difference in scramble siRNA-treated cells. In this set of experiments, vehicle alone resulted in an insulin-stimulated increased glucose uptake of 59% compared with 105% seen in previous work (12).

colonies was also tested by β-Gal Filter Lift Assay to test *LacZ* reporter activation. Plasmids were recovered from pJ69 initially by culture in -Trp minimal media, followed by suspension of harvested cells in 200 μl plasmid rescue buffer (100 μl of Tris-saturated phenol [pH 8.0]) and 100 μl 24:1 chloroform:isoamyl alcohol) and vortex in conjunction with acid-washed glass beads (Sigma). Supernatants were collected and bound to Qiagen spin columns by further centrifugation. The bound plasmid DNA was washed with 750 μl phycoethrin (Qiagen) and eluted by incubation of the column with 50 μl EB (10 mmol/l Tris, [pH 8.0]) (Qiagen) for 1 min followed by centrifugation at 13,000 rpm for 1 min.

Analysis of yeast clones. Five microliters of DNA was used as template in a PCR using pYES-TRP vector-specific primers (5′ gatgttaacgataccagcc, 3′ gcgtgaatgtaagcgtgac). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and directly sequenced using ABI 377 automated technology. Sequenced clones were determined for frame and orientation, and putative interactors were identified through screening against computer databases using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST).

Immunoprecipitation of the nephrin-VAMP2 complex

Cellular transfections. Madin-Darby canine kidney (MDCK) epithelial cells were transfected with 1 μg pcDNA3-glutathione S-transferase (GST)-nephrin (amino acids 1057–1241; pCDNA3-GST–tagged vector generated in house) and pGFP (green fluorescent protein)-VAMP2 (full-length) (a kind gift from Prof. G. Rutter, University of Bristol) using Lipofectamine 2000, according to the manufacturers' protocol. Cells were incubated at 37°C for 48 h before harvesting in ice-cold NETN lysis buffer (100 mmol/l NaCl, 1 mmol/l EDTA, 20 mmol/l Tris-HCl [pH 8.0] 0.5% NP40). GST-nephrin and GFP-VAMP2 protein expression was confirmed by SDS-PAGE and Western blot using specific nephrin and VAMP2 antibodies (Abcam). Protein concentration of extracts was confirmed by Bradford assay. These cells were used because they were easily transfectable with the two constructs, which was not the case for immortalized podocytes.

Glutathionine precipitation. Glutathione-Sepharose beads (Amersham/Pharmacia) were preblocked in 1% BSA then washed in NETN and stored as 50% slurry; 400 μg cell lysate was added to 50 μl of beads per incubation, which was conducted at 4°C for 3 h. Glutathione precipitates were collected

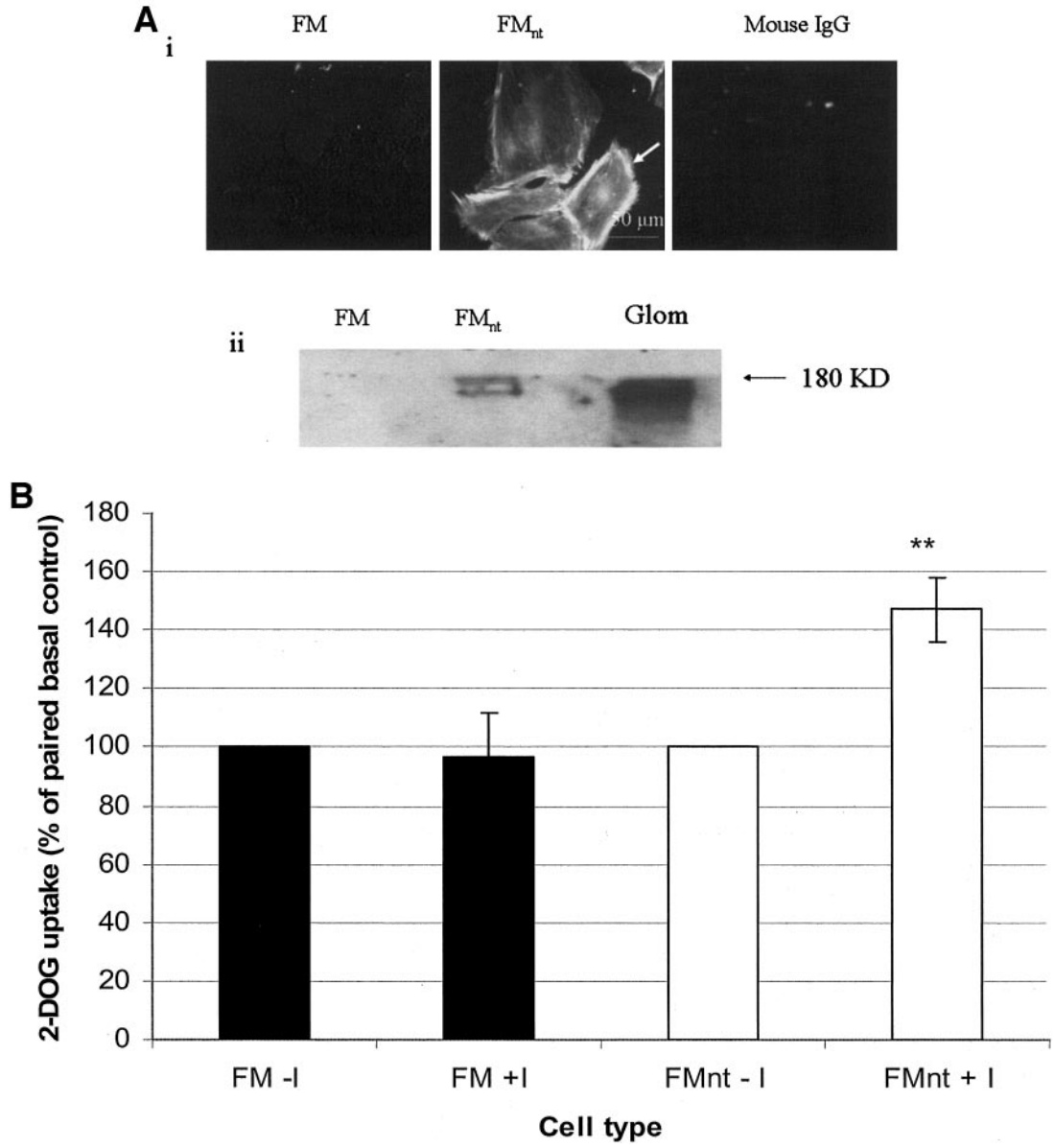

FIG. 4. *A*: Stable nephrin transfection in FM podocytes. Nephrin IF using monoclonal antibody 48E11 (*i*). Peripheral nephrin staining (indicated by arrow) shown in FM_{nt}. Mouse monoclonal isotype control and FM show minimal staining for this antibody. Western blot for nephrin (*ii*). Monoclonal 50A9 antibody used signal in human glomerular positive control and FM_{nt} lanes. FM lane negative for nephrin. Equal amounts of protein were loaded for each (90 μg). *B*: 2-DOG uptake in nephrin-transfected FM podocytes. 2-DOG uptake after 15 min of 100 nmol/l insulin (+) stimulation or in basal state (−). Comparison of nephrin-transfected FM_{nt} cells and nontransfected FM cells. Significant increase in glucose uptake in response to insulin in FM_{nt} cells. **$P < 0.001$ using a paired two-tailed Student's *t* test; $n \geq 6$ experiments for each condition studied.

by pulse centrifugation, the supernatant was discarded, and the pellets were washed four times with NETN lysis buffer. The pellet was resuspended in 20 μl of 2 × Laemmeli and boiled and analyzed by SDS-PAGE and Western blot. Membranes were immunoblotted with GST (Clontech; BD Biosciences) and GFP (Vector Labs) antibodies.

Nephrin-VAMP2 immunoprecipitation from human glomerular extracts. Glomeruli were studied from two patients. One patient had a kidney removed for a renal tumor, and the normal pole was sieved for glomeruli. The other patient was a deceased donor for a kidney transplant, but the kidney vasculature was unsuitable for transplantation. This kidney was offered for research. Both kidneys were obtained with full ethical consent. Glomeruli were sieved as previously described (23). The transplant kidney was received and immediately put in serum-free RPMI media for 2 h after sieving the glomeruli. One aliquot was then insulin stimulated with 100 nmol/l insulin, and this was compared with another aliquot of glomeruli under basal conditions.

VAMP2 was immunoprecipitated from glomeruli lysed in a Triton-based lysis buffer (1% Triton X, 50 mmol/l Tris [pH 7.5] containing 1 mmol/l EDTA, 120 mmol/l NaCl, 50 mmol/l NaF, 40 mmol/l β-glycerophosphate, 1 mmol/l benzamidine, 1% NP40, 1 μm microcystin, 5 mmol/l orthovanadate, and 1μg/ml each of pepstatin, leupeptin, and antipain) using Protein A/G beads (Santa Cruz) and 5 μg monoclonal antibody (SYSY systems). Western blot was then performed using polyclonal anti-nephrin antibody. A negative control of normal mouse immunoglobulin was used to immunoprecipitate an equimolar aliquot of glomeruli.

To identify if nephrin pull-down resulted in VAMP2 precipitation in human glomeruli, we used the Profound system (Pierce), which limits contamination with immunoglobulin light chains. The manufacturers' instructions were followed, and polyclonal nephrin (K2737) was used to immunoprecipitate the glomerular lysate. After washing and eluting polyclonal VAMP2 (Abcam) was used in the Western blot. An equal amount of glomerular lysate was immunoprecipitated against normal rabbit immunoglobulin (Sigma) (same concentration as nephrin antibody) as a negative control.

Statistical analysis. For comparisons among multiple groups, ANOVA was used followed by a post hoc Bonferroni test. The Prism 2 program was used for this analysis. When paired samples were analyzed, a two-tailed Student's *t* test was used. $P < 0.05$ was taken to demonstrate statistical significance.

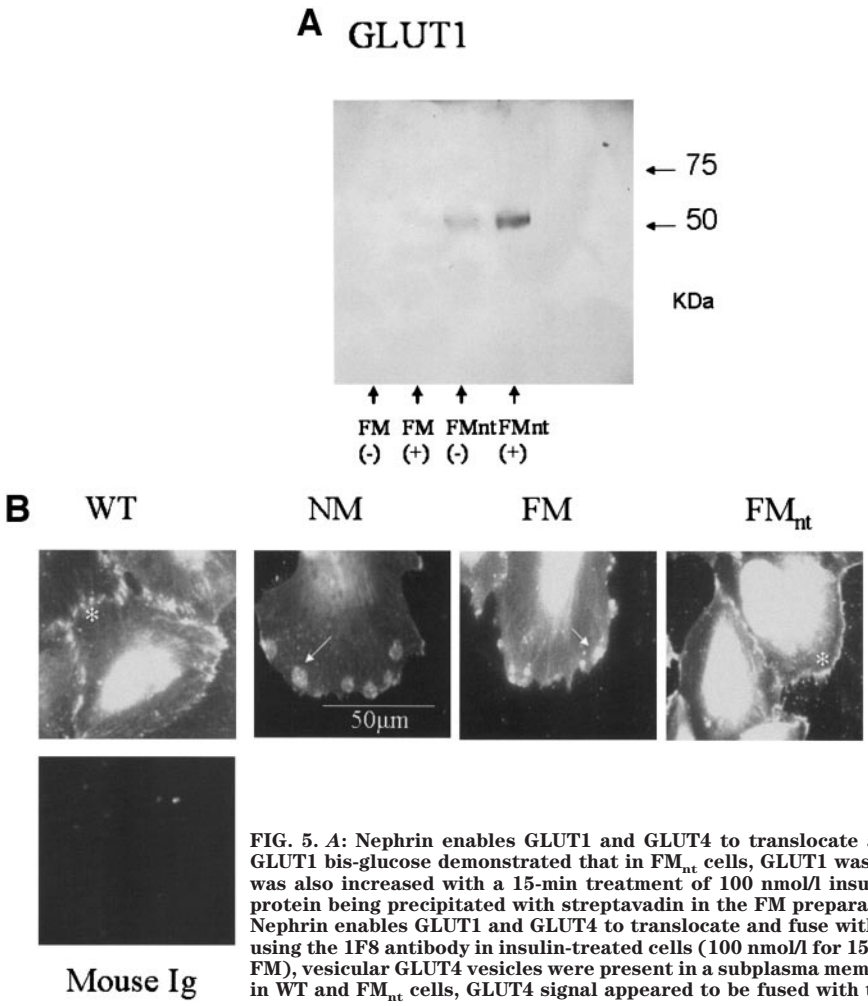

FIG. 5. ***A*****: Nephrin enables GLUT1 and GLUT4 to translocate and fuse with the plasma membrane of the podocyte. GLUT1 bis-glucose demonstrated that in FM_{nt} cells, GLUT1 was present at the plasma membrane of the podocyte and was also increased with a 15-min treatment of 100 nmol/l insulin. FM demonstrated no GLUT1 signal, despite more protein being precipitated with streptavadin in the FM preparation (288 μg) compared with FM_{nt} cells (166.5 μg).** ***B*****: Nephrin enables GLUT1 and GLUT4 to translocate and fuse with the plasma membrane of the podocyte. IF for GLUT4 using the 1F8 antibody in insulin-treated cells (100 nmol/l for 15 min) showed that in the nephrin mutant cells (NM and FM), vesicular GLUT4 vesicles were present in a subplasma membrane location (arrowed) after insulin stimulation, but in WT and FM_{nt} cells, GLUT4 signal appeared to be fused with the plasma membrane (*).**

RESULTS

Nephrin is essential for insulin-induced glucose uptake in human podocytes. We studied the insulin response on 2-DOG uptake in fully differentiated immortalized podocytes derived from subjects with no renal disease (WT), FM, or NM nephrin mutations. Both of the nephrin mutant cell lines were unresponsive to insulin; there was an approximate doubling of insulin-stimulated glucose uptake in WT cells (Fig. 2). All cell types had comparable basal uptake of 2-DOG per milligram protein, so the insulin effect was not due to basal saturation of cell-surface glucose transporters in nephrin mutant cells (data not shown). To ensure this was not a clonal effect, we tested a number of different clones for each cell type, all of which gave similar results (data not shown). We also knocked down nephrin in WT podocytes with nephrin-specific siRNA, achieving ~60% protein knockdown after 48 h (Fig. 3*A*). Doing so abrogated the effect of insulin. This was not the case in cells treated with scrambled siRNA, which differed by 3 bp to the nephrin siRNA (Fig. 3*B*).

Reconstitution of nephrin in FM podocytes rescues their insulin responsiveness. We generated a stable nephrin-expressing FM cell line (FM_{nt}) to address if nephrin was the critical factor that resulted in a loss of insulin responsiveness (in respect to glucose transport). Nephrin protein was expressed in the transfected cells in abundant amounts and in the correct location (at the plasma membrane) as assessed by Western blotting and IF (Fig. 4*A*). When stimulated with insulin, we found that the insulin-responsive phenotype of the cells could be rescued (Fig. 4*B*).

Nephrin is required for the fusion of GLUT1 and GLUT4 with the plasma membrane of the podocyte. Using bis-glucose photolabeling for GLUT1 and IF for GLUT4, we studied the effect of nephrin on the functional plasma membrane incorporation of these glucose transporters in response to insulin. We compared WT, NM, FM, and the FM_{nt} podocytes. Using GLUT1 bis-glucose photolabeling, we found that FM was unable to functionally incorporate GLUT1 into the plasma membrane of the cell under basal conditions and was not upregulated by insulin as occurs in WT podocytes (12). Furthermore, using IF in the nephrin-mutant cells, GLUT4 vesicles were shown to move to the periphery of the cell in response to insulin but did not appear able to fuse with the membrane. The appearance of vesicle fusion was rescued after transfecting nephrin back into the cells, giving a response similar to wild-type podocytes (Fig. 5). The bis-glucose assay is not sensitive enough to detect GLUT4 in podocytes (12); however, we speculate from the GLUT4 IF observations that nephrin is required to incorporate GLUT4 (as well as GLUT1) into the plasma membrane of the podocyte in response to insulin.

The COOH-terminus of nephrin forms a protein-protein interaction with VAMP2. Initially we performed a yeast- 2 hybrid screen using the COOH-terminus of nephrin (amino acids 1085–1160) as the bait. A transformation efficiency of 1.5×10^6 was observed for yeast 2 hybrid screening using pGBDU-Gal4-BD-cytosolic nephrin (amino acids 1057–1217) and the pYES-Trp-human adult

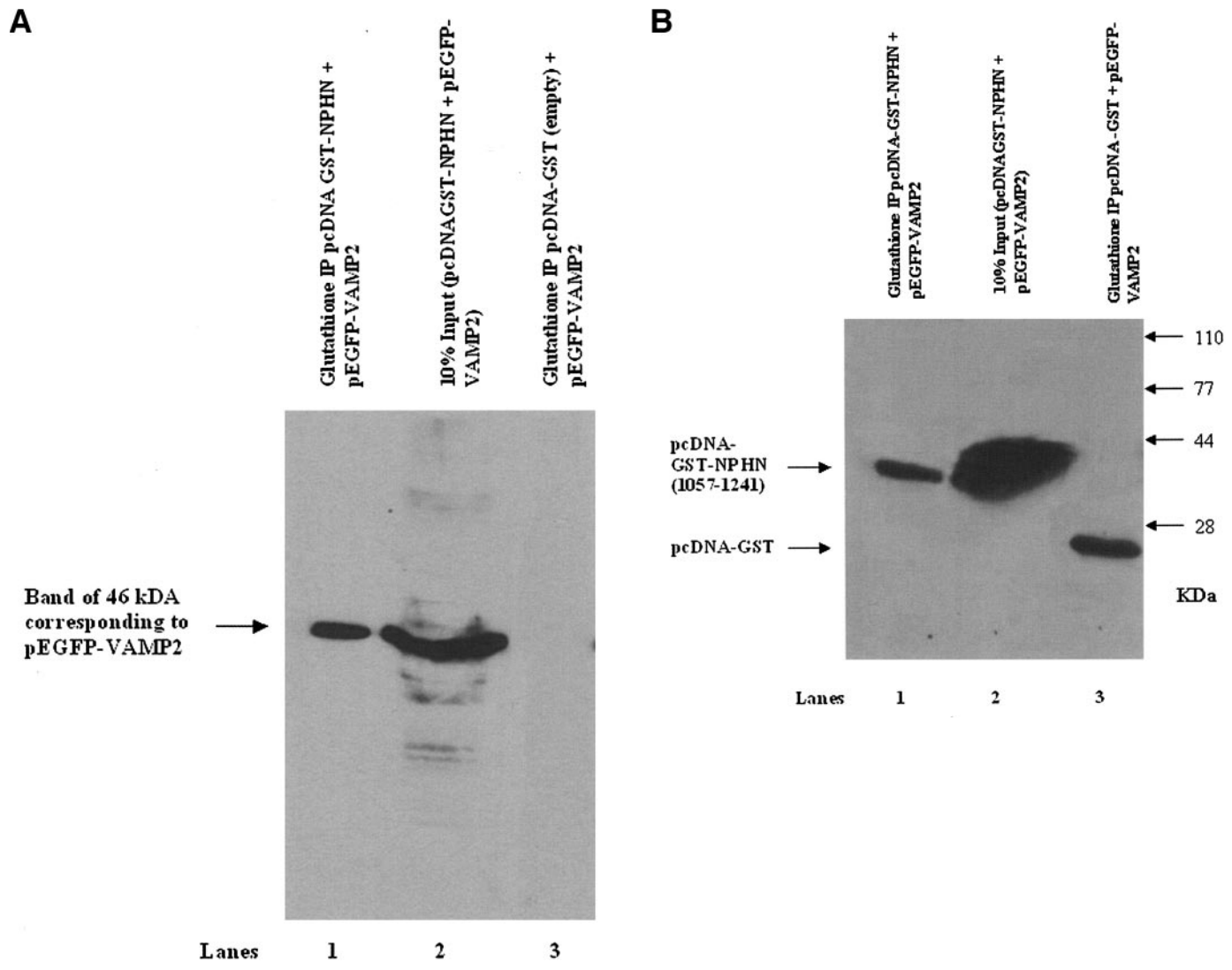

FIG. 6. *A*: Glutathionine precipitation of the COOH of nephrin with VAMP2. Glutathione precipitation of pEGFP-VAMP2 by pcDNA-GST-COOH nephrin (NPHN) (*lane 1*) and pEGFP-VAMP2 by pcDNA-GST (*lane 3*) using glutathione-Sepharose beads (Amersham/Pharmacia). Precipitated samples were subjected to SDS-PAGE and then immunoblotted with GFP antibody (Vector Labs). No band was detected in *lane 3*, which indicates that no precipitation occurred between pcDNA-GST alone and pEGFP-VAMP2. Conversely, a band of 46 kDa is detected by the GFP antibody in *lane 1*. This verifies the presence and therefore successful precipitation of pEGFP-VAMP2 by nephrin. *Lane 2* demonstrates 10% input. *B*: Glutathione precipitation of pEGFP-VAMP2 by pcDNA-GST-NPHN (1057–1241) (*lane 1*) and pEGFP-VAMP2 by pcDNA-GST (empty) (*lane 3*) subsequently immunoblotted with GST antibody (Clontech; BD Biosciences) to confirm presence of GST-tagged reagents. The detection of bands of 26 kDa, corresponding to the size of pcDNA-GST (empty) (*lane 3*), and of 42kDa, corresponding to the size of pcDNA-GST-NPHN (*lane 1*), verifies the presence of pcDNA-GST alone and pcDNA-GST-NPHN within the precipitation. *Lane 2* shows 10% input.

kidney cDNA library (Invitrogen). Two overlapping nucleotide sequences in the correct orientation were identified through screening against computer databases using BLAST searches as VAMP2 (or vesicle-associated membrane protein, synpatobrevin). Translation of both sequences confirmed that both were in frame, and the identity was further verified by sequencing. The VAMP2 primers used were designed over intron-exon boundaries to distinguish PCR products generated by genomic contamination or from mRNA. We subsequently isolated VAMP2 mRNA from conditionally immortalized WT podocytes using reverse transcriptive PCR and VAMP2 protein using Western blotting (data not shown).

After this screening experiment, we went on to prove a protein-protein interaction in vitro using coimmunoprecipitation between GST-tagged nephrin COOH-terminus and GFP-tagged VAMP2 in MDCK cells (Fig. 6). This demonstrated a protein-protein interaction between VAMP2 and the COOH-terminus of nephrin. Finally, we examined human glomerular extract and found that nephrin and VAMP2 interact ex vivo (Fig. 7) and that insulin stimulation increased this interaction.

DISCUSSION

This paper demonstrates that nephrin is critical in vitro to enable insulin-stimulated glucose uptake into podocytes. Furthermore, after a yeast 2 hybrid screen we have discovered a novel protein interaction of nephrin with VAMP2 that we have confirmed in vitro in MDCK cells and that this interaction occurs ex vivo in humans by immunoprecipitation of glomerular extracts.

Nephrin is a critical molecule in maintaining the GFB of the kidney. Mutations in this protein in humans cause the most severe phenotype of the early-onset nephrotic syndromes (congenital nephrotic syndrome of the Finnish

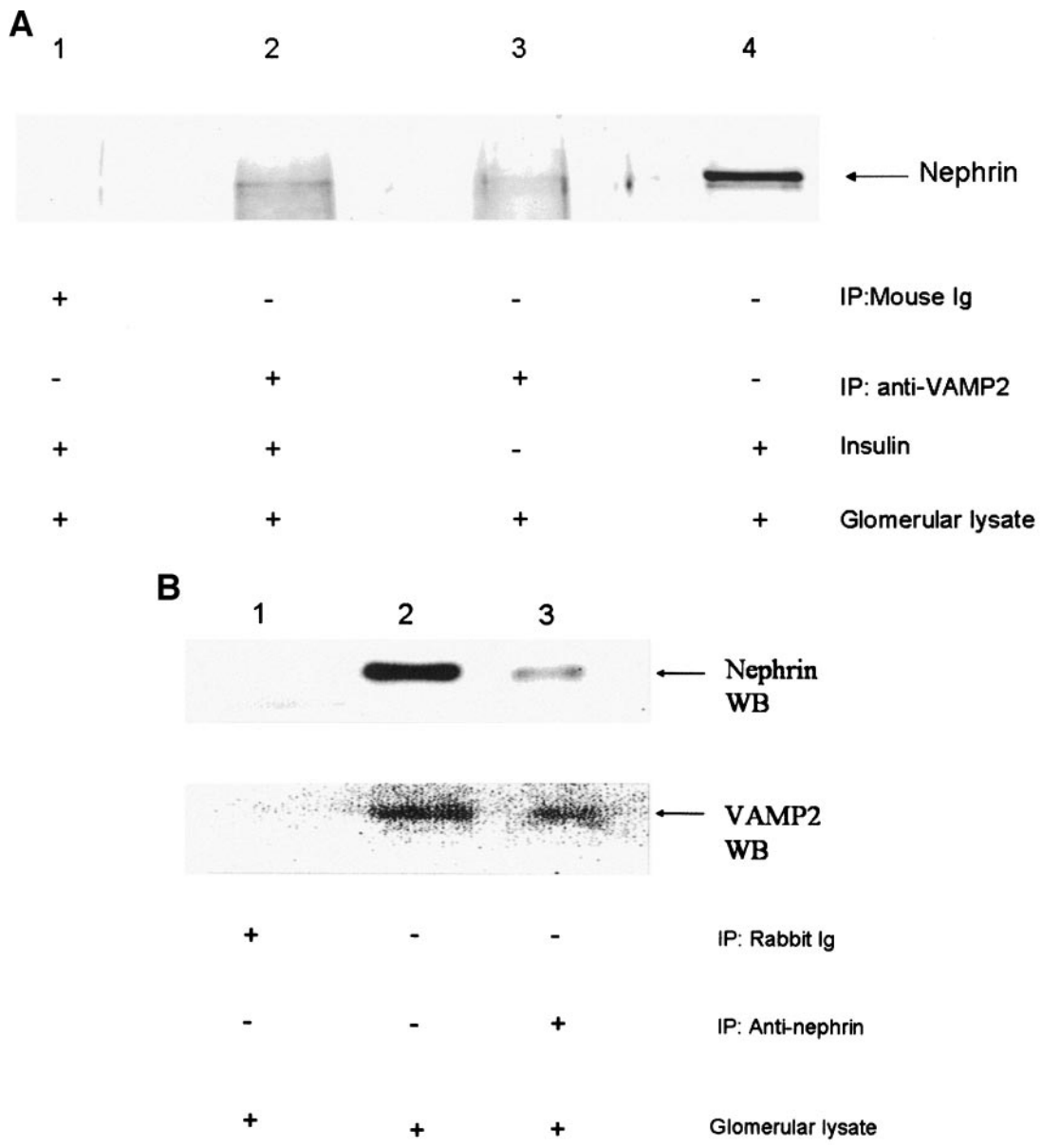

FIG. 7. Nephrin–VAMP2 coimmunoprecipitation in human glomeruli. *A*: VAMP2 immunoprecipitation with monoclonal mouse anti-VAMP2 antibody and probing of Western blot with anti-nephrin rabbit antibody. A negative control of normal mouse antibody (Sigma) was used for pull-down in *lane 1*. Positive glomerular whole cell lysate in *lane 4*. *Lane 2* is insulin-stimulated glomeruli, which gave increased signal compared with basal glomeruli (*lane 3*). Equal amounts of protein were immunoprecipitated in *lanes 1–3* (750 μg) *B*: Nephrin pull-down of human glomeruli followed by VAMP2 Western blot. The Profound system was used for this (RESEARCH DESIGN AND METHODS). Whole glomerular lysate positive control (*lane 2*) and normal rabbit immunoglobulin negative control (*lane 1*) are shown. Equal amounts of antibody were used for immunoprecipitation (100 μg) and equal amounts of glomeruli were immunoprecipitated (700 μg). Pull-down of nephrin–VAMP2 complex is shown by probing the blot with anti-VAMP2 antibody *(bottom panel)*. Then blot was stripped and reprobed for nephrin (*top panel*). Nephrin IP results in pull-down of VAMP2 (*lane 3*).

type) (1). There is also evidence that a reduction in nephrin quantity and/or location (from a plasma membrane to a cytoplasmic distribution) occurs in the more common acquired human nephrotic syndromes (25–27). In diabetic nephropathy, it has been shown that nephrin expression decreases as albuminuria progresses (28–30); this occurs independent of expression of other molecules located at the slit diaphragm of the podocyte such as podocin and CD2AP, suggesting that decreased nephrin expression is not a nonspecific marker of generalized podocyte dysfunction (31). At a cellular level, this effect is similar to knocking down nephrin in the podocyte, which, as we show in this article, induces podocyte insulin resistance in respect to glucose uptake in vitro. We hypothesize that one reason insulin responsiveness of the podocyte is important is to enable a physiological response allowing the cell to rapidly increase easily metabolized carbohydrate to facilitate structural remodelling required to withstand an increase in the glomerular blood flow that occurs after a meal. This adaptive response is lost in diabetic nephropathy, resulting in podocyte dysfunction and contributing to breakdown of the GFB and albuminuria. Recent intriguing evidence (32) strengthens the link between insulin sensitivity, nephrin, and proteinuria. The peroxisome proliferator–activated receptor γ agonist, pioglitazone, which increases the insulin sensitivity of cells, reduces proteinuria in a Heymann nephritis model and results in increased nephrin expression.

The expression of nephrin in the body is controversial. It is agreed that within the kidney, it is exclusively located in the podocyte; however, its extra-renal distribution is contentious. Some groups suggest that it is only expressed in the podocyte (33), while others have described it in the brain (34) and testes (35) and within the β-cells (34) and endothelium (36) of the pancreas. Intriguingly, in relation to the role of nephrin in insulin sensitivity of other tissues, a homolog of nephrin called Hibris has been found in muscle in *Drosophila* (37), which is important in myoblast fusion. If such a homolog is present in human muscle, then the action of nephrin could have implications for the insulin sensitivity of muscle, which is the main tissue responsible for insulin-stimulated glucose deposition after a meal (38) and the major tissue responsible for the development of type 2 diabetes.

We have observed the lack of insulin sensitivity (see above) in two different nephrin mutant podocyte cell lines, one with a complete truncation of nephrin (FM) and another with a missense mutation (NM), resulting in a failure of nephrin protein to traffic to the plasma membrane of the podocyte. This suggests not only the quantity but the location of nephrin (at the plasma membrane) is important for its effect on insulin's action. We have gone on to knock nephrin down in WT podocytes (which abrogates the effect of insulin) and, most powerfully of all, rescue the insulin-responsive phenotype by stably reconstituting nephrin back (in both quantity and location) into the nephrin-deficient FM cells. This demonstrates in vitro the importance of nephrin in insulin-stimulated glucose uptake in podocytes. This observation suggests in vivo that insulin-stimulated glucose uptake occurs predominantly in the foot process of the podocyte where nephrin is located (39). Podocytes are unique, highly specialized cells that depend on their foot processes to maintain the filtration barrier in the glomerulus, which is the main function of this cell. Being able to absorb glucose in response to insulin would intuitively seem beneficial, as soon after a meal it would facilitate glucose entry into this part of the cell. The absorbed glucose would be an easily metabolized energy source to remodel the actin cytoskeleton in preparation for the increased glomerular filtration load the meal would produce. A similar adaptive process for cellular remodelling occurs in cardiac and skeletal muscle where glucose is absorbed in response to contractility (40).

Using a yeast 2 hybrid screen, we identified VAMP2 as an interactor with nephrin and went on to show that this occurs at the protein level in vitro using MDCK cells and ex vivo in human glomerular specimens. Target SNARE-proteins form complexes with proteins found on the vesicles (vesicular SNARES) (41). VAMP2 is an important vesicular SNARE that is involved in the targeting and docking of vesicles with cognate target SNARES in cells. It is predominantly involved in the docking of neurotransmitter secretary vesicles in neurones and insulin-responsive GLUT4-rich vesicles in muscle and adipocytes (42). We presume that nephrin is acting as a modifying protein in this SNARE complex in the podocyte. It is unlikely a target SNARE, as its predicted crystal structure is not helical, which is required for SNARE proteins (43). Podocytes have a number of similarities with neurones (44) that extensively use SNARE complexes for inside out signaling of neurotransmitters. This supposition is supported by the recent reports that podocytes contain neurone-like synaptic vesicles and express molecules such as Rab3a (45), synaptotagmin 1, synapsion 1, synaptophysin (46), and synaptic vesicle 2b (47). In neurones, there are similar nephrin-like immunoglobulin superfamily-like adhesion molecules (48) that are important in the targeting and docking of vesicles (49), supporting our findings.

Finally, we have shown that nephrin is important in the insulin-stimulated fusion of GLUT4 and GLUT1 to the plasma membrane of the cell. This may be through the interaction of the COOH-terminus of nephrin with VAMP2; however, this has not been shown to be the case for GLUT1 in adipocytes (50). Alternatively, nephrin may have its effect on the fusion of GLUT1 and/or GLUT4 through its involvement in signaling pathways; it is known to signal through Phosphoinositide 3-OH Kinase (17) and Mitogen-Activated Protein Kinase (18), both of which are involved in insulin signaling in other cells. Another possibility is that it is nephrin's interaction with the actin cytoskeleton (21,22) that is required to translocate glucose transporters to the plasma membrane of the cell.

In conclusion, we have shown a previously unsuspected role for nephrin in the insulin-sensitivity pathway in podocytes and its role in the docking of GLUT-rich vesicles with the plasma membrane of the cell. Understanding the molecular basis of the effect of insulin on the podocyte, we hope, will lead to the development of novel treatment strategies for the leading causes of albuminuria and ESRF in the world—diabetes and the hyperinsulinemic metabolic syndrome.

NOTE ADDED IN PROOF

Recently, phospholipase C-ε gene (*PLCEI*) mutations have been shown to cause early-onset nephrotic syndromes. *PLCEI* is predominantly expressed in the podocyte in the glomerulus.

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