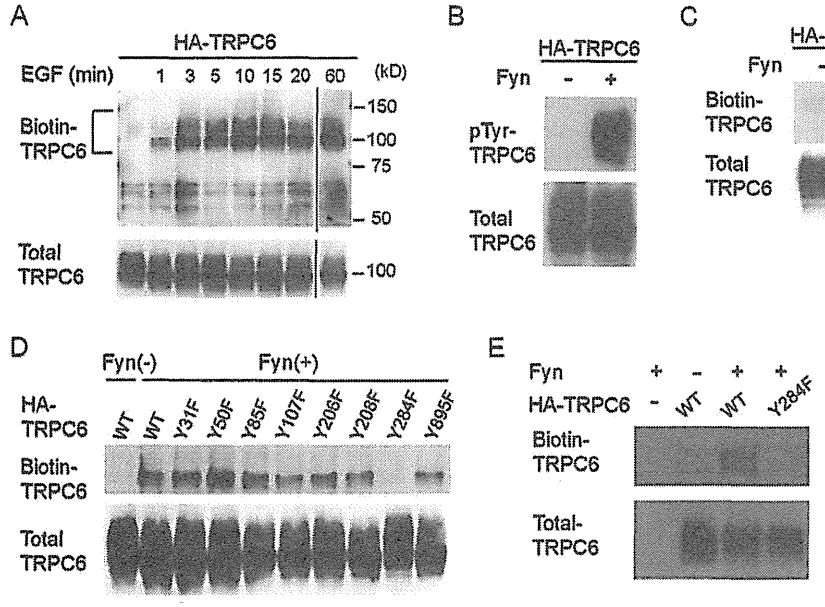

FIGURE 1: Phosphorylation of TRPC6 Y284 is necessary for its trafficking to the plasma membrane. (A) Induction of membrane trafficking of TRPC6 by EGF. HEK293T cells expressing HA-TRPC6 were stimulated by EGF (200 ng/ml) for the indicated times. The cells were surface biotinylated with Sulfo-NHS-SS-Biotin, and the streptavidin-agarose–bound proteins were analyzed by Western blotting with α-TRPC6 antibody. The positions of the molecular-weight-marker proteins in kilodaltons are shown on the right side of the panel. A portion of each lysate (2%) was examined by Western blot to confirm the expression level of TRPC6 (bottom panel). (B) HA-TRPC6 expressed in HEK293T cells with or without constitutively active Fyn was immunoprecipitated and probed with anti-phosphotyrosine antibody. (C) HEK293T cells expressing HA-TRPC6 with or without Fyn were processed as in A. (D and E) Fyn and each of a series of phenylalanine substitution mutants of HA-TRPC6 were expressed in HEK293T cells (D) or in cultured podocytes (E), and the surface biotinylation assay for TRPC6 was performed as in A. Representative data from three to five independent experiments are shown.

staining. As shown in Figure 2A, a protein with an apparent molecular weight of 140,000 specifically bound to the phosphorylated TRPC6 Y284 peptide (marked by an asterisk). Mass spectrometric analysis of tryptic digest of the protein identified it as PLC-γ1. The score for PLC-γ1 was 60.65, and peptides with a greater than 95% confidence covered 30.9% of the total PLC-γ1 sequence (Supplemental Figure S1). siRNA-mediated depletion of endogenous PLC-γ1 in HEK293T cells markedly reduced the phosphorylation-induced surface expression of TRPC6 on the plasma membrane, and this reduction was rescued by adding rat PLC-γ1, demonstrating that PLC-γ1 is a prerequisite for Fyn-induced TRPC6 trafficking (Figure 2B). The coimmunoprecipitation analysis shown in Figure 2C demonstrates that TRPC6 binds to PLC-γ1 in a Fyn-dependent manner. Whereas TPRC6 mutants with substitution (Y50F, Y85F, Y107F, Y206F, Y208F, Y284F, Y895F) bound to PLC-γ1 to the same extent as did wild-type TRPC6 (unpublished data), Y31F mutation partially abrogated the binding to PLC-γ1, and TRPC6 mutated both at Y31 and Y284 did not interact with PLC-γ1. Therefore, the TRPC6–PLC-γ1 interaction requires not only the phosphorylation of Y284, but also that of Y31. Indeed, a TRPC6 peptide around Y31 also bound to PLC-γ1 upon phosphorylation as did phosphorylated Y284 (Figure 2D). PLC-γ1 has two SH2 domains, N-SH2 and C-SH2, which are located side-by-side at the center of the molecule. Both pY31 and pY284 peptides preferentially bound to PLC-γ1 N-SH2, because point mutation (N*) (Plattner *et al.*, 2003) or deletion (Δ N) (Bae *et al.*, 2000) of N-SH2 strongly impaired the binding (Figure 2E). The phosphorylation-dependent TRPC6 trafficking was not affected by a PLC-γ inhibitor, U73122 (Supplemental Figure S2A), indicating that catalytic activity of PLC-γ1 is not necessary for the TRPC6 translocation.

PLC-γ1 binds to the N-terminal amino acids 40–46 of TRPC3 (that correspond to residues 98–104 of TRPC6) to form a functional pleckstrin homology (PH) domain that binds to phosphatidylinositol bisphosphate, which is required for the agonist-induced surface expression of TRPC3 (van Rossum *et al.*, 2005). We evaluated the role of these residues in TRPC6 by coimmunoprecipitation and biotinylation assays using TRPC6 mutant lacking residues 98–104. Because this mutant (Δ98–104) still bound to PLC-γ1 (Figure 2F) and translocated to the plasma membrane (Figure 2G) in a Fyn-dependent manner, as did the wild type, we conclude that Y31 and Y284, but not residues 98–104, are critical for the interaction with PLC-γ1 and trafficking to the plasma membrane.

TRPC6 Y31 and Y284 are phosphorylated by Src family kinase in isolated glomeruli

We raised phosphospecific anti-TRPC6 antibodies against pY31 and pY284 (Figure 3). Specificity of these antibodies was shown using Y31F and Y284F mutants of TRPC6 (Figure 3A). EGF also induced the phosphorylation of these sites (Figure 3B). In isolated rat kidney glomeruli, Y284 phosphorylation of endogenous TRPC6 was significantly augmented by treatment with vanadate, a nonspecific protein phosphatase inhibitor (Figure 3C). This phosphorylation was suppressed when glomeruli were pretreated with either SU6656 or PP2, specific Src family kinase inhibitors, before vanadate treatment. This finding suggests that Src family kinase is responsible for the reaction.

Tyrosine-phosphorylated TRPC6 interacts with nephrin

Given that TRPC6 is a slit diaphragm component (Reiser *et al.*, 2005; Huber *et al.*, 2006), we next sought to determine whether binding to other slit diaphragm components affected its phosphorylation-dependent membrane translocation. First, we performed coimmunoprecipitation assays using HEK293T cells expressing nephrin, Neph1, Neph2, or podocin, in addition to TRPC6 and Fyn. We found that nephrin bound to TRPC6 only when coexpressed with Fyn (Figure 4A). We did not detect any interaction between TRPC6 and other slit diaphragm components (unpublished data). Because nephrin is also tyrosine phosphorylated by Src family kinase (Verma *et al.*, 2003, 2006; Jones *et al.*, 2006), we examined which phosphorylation is necessary for the TRPC6-nephrin interaction. Upon phosphorylation nephrin Y1204 binds to PLC-γ1 (Harita *et al.*, 2009). This phosphorylation site, however, was not involved in the binding with TRPC6, because Y1204F mutant also bound to TRPC6 (Figure 4A). We next performed a pull-down assay using glutathione *S*-transferase (GST)-nephrin-cytoplasmic domain (nephrin-CD, residues 1102–1252) with or without prior in vitro phosphorylation by Fyn (Harita *et al.*, 2008, 2009). As shown in Figure 4B, TRPC6 from HEK293T cells coexpressing Fyn, but not from cells without Fyn, bound to GST-nephrin-CD, irrespective of nephrin-CD phosphorylation by Fyn in vitro. Thus, phosphorylation of TRPC6, but not that of

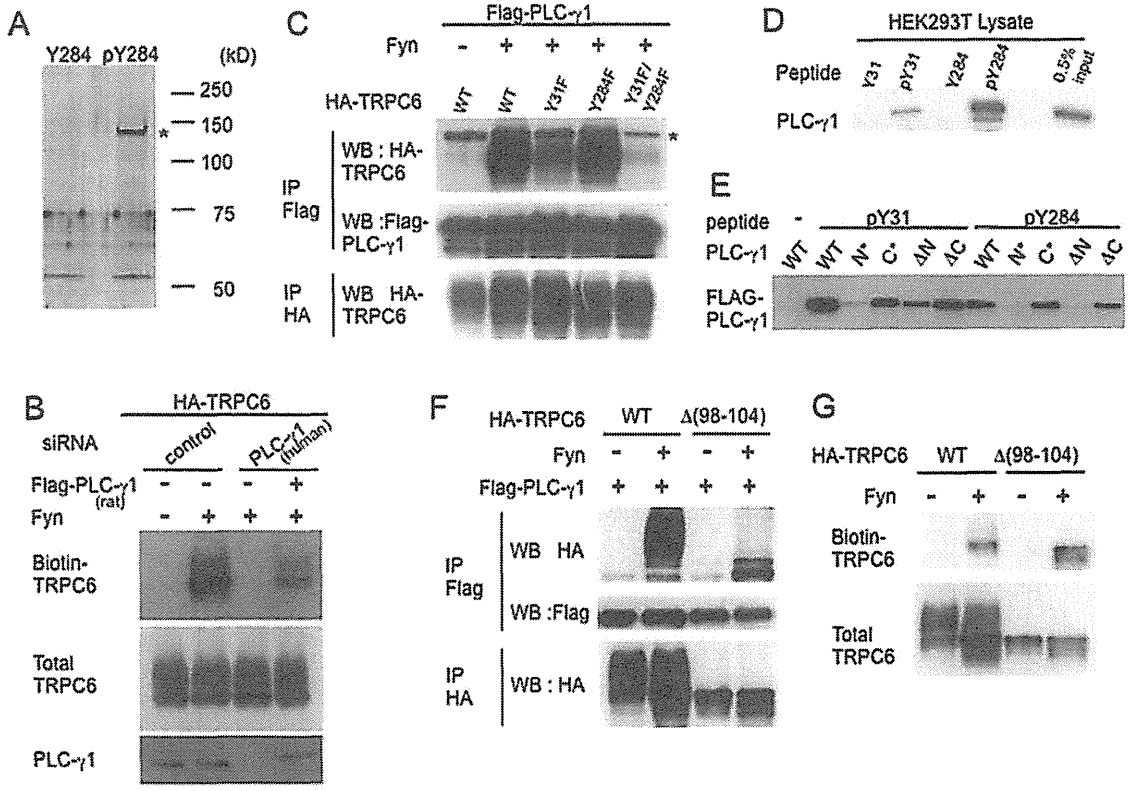

FIGURE 2: PLC-γ1 binds to phosphorylated TRPC6 Y31/Y284 and controls the surface expression of TRPC6. (A) Phosphorylated or nonphosphorylated TRPC6 Y284 peptide immobilized on a coupling gel was incubated with HEK293T cell lysates. The bound proteins were analyzed by SDS–PAGE followed by silver staining. A protein band (marked by an asterisk) was excised and subjected to tryptic digestion followed by analysis by LC-MS/MS. (B) Scrambled siRNA (control) or hPLC-γ1 siRNA was transfected into HEK293T cells, then HA-TRPC6, Fyn, and Flag-PLC-γ1 (rat) were transfected on the next day. One day after the transfection, the surface expression of TRPC6 was examined as in Figure 1A. (C) Flag-PLC-γ1, Fyn, and HA-TRPC6 (wild type, Y31F, Y284F, Y31F/Y284F) were transfected into HEK293T cells. Immunoprecipitation (IP) and Western blot (WB) were performed with the indicated antibodies. The protein band marked by an asterisk is a cross-reaction with overexpressed PLC-γ1. (D) Phosphorylated or nonphosphorylated peptides surrounding TRPC6 Y31 or Y284 were used to pull down PLC-γ1 from HEK293T cell lysates. A portion of the lysates (0.5% input) was applied for a recovery marker. (E) Peptide pull-down assays were performed as in C using wild type (WT) or SH2 point mutants (N*: R586K, C*: R694K) or SH2 deletion mutants (ΔN : Δ550–657, ΔC : Δ667–756) of PLC-γ1. (F) Coimmunoprecipitation was performed using HEK293T cells transfected with Flag-PLC-γ1, Fyn, and TRPC6 (wild type or Δ98–104). (G) The surface expression of TRPC6 lacking 98–104 (Δ98–104) was examined.

nephrin, is required for the TRPC6–nephrin interaction. nephrin phosphorylation could also be induced by clustering its extracellular domain (Lahdenpera *et al.*, 2003; Jones *et al.*, 2006; Verma *et al.*, 2006). A fusion protein construct in which the CD8 extracellular domain and the transmembrane domain were coupled to nephrin-CD (CD8/nephrin-CD) was expressed in HEK293T cells, and a mouse anti-CD8 antibody and a secondary anti–mouse IgG antibody were added to the culture medium (Harita *et al.*, 2009). TRPC6 phosphorylation and surface expression, however, were not observed by cross-linking experiments (unpublished data), suggesting, that clustering-induced nephrin phosphorylation may not be enough for TRPC trafficking. Temporal or spatial regulation of SFK activation may be required.

Phosphorylation of Y31 and Y284 of TRPC6 is necessary for the interaction with PLC-γ1 (Figure 2C). Similarly, we evaluated the ability of a series of TRPC6 mutants with a phenylalanine substitution to bind to nephrin, to determine the critical tyrosine residue for the TRPC6–nephrin interaction (Figure 4C). We found that Y284 is the critical residue, because Y284F mutation almost completely abolished the interaction between TRPC6 and nephrin, whereas other substitutions including Y31F did not affect the interaction. The role of Y284 phosphorylation was further confirmed by a pull-down analysis using GST-nephrin-CD (Figure 4D). Although not as efficient as full-length TPRC6, a TRPC6 peptide surrounding Y284, when phosphorylated, could pull down nephrin from HEK293T cells (Figure 4E, left) and GST-nephrin-CD (Figure 4E, right), demonstrating the direct binding of nephrin to TRPC6.

To define the region in nephrin-CD that is required for the binding to TRPC6, we constructed a series of deletion mutants of GST-nephrin-CD. A GST pull-down assay using these deletion mutants showed that residues 1216–1227 are necessary for the interaction (Figure 4F) and that the deletion of this region from nephrin abolished the binding (Δ(1216–1227) in Figure 4G).

Nephrin suppresses the phosphorylation-dependent surface expression and channel activation of TRPC6

Because both PLC-γ1 and the nephrin-CD bind to the same tyrosine residue on TRPC6 (Y284), they may compete with each other for

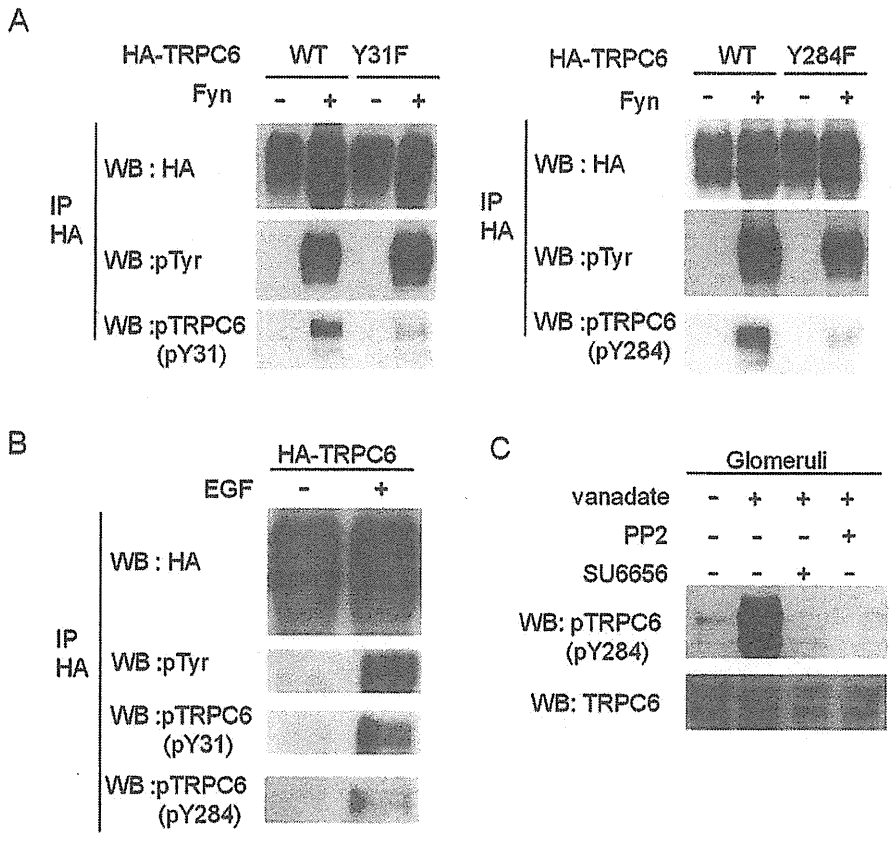

FIGURE 3: Tyrosine phosphorylation of TRPC6 in living cells and kidney glomeruli. (A) Lysates from HEK293T cells transfected with the indicated plasmids were probed with α-pY31 or α-pY284 antibody. (B) HEK293T cells expressing HA-TRPC6 were stimulated by EGF. α-HA immunoprecipitates were probed with α-phosphospecific TRPC6 antibodies. (C) Isolated rat glomeruli treated with or without 1 mM sodium vanadate for 30 min were blotted with α-pY284. For the indicated samples, glomeruli were pretreated with 10 mM PP2 for 15 min or 5 mM SU6656 for 60 min before treatment with vanadate.

binding. As shown in Figure 5A, coexpression of nephrin in HEK293T cells completely inhibited the TRPC6–PLC-γ1 interaction. Because phosphorylated nephrin Y1204 provides the binding site for PLC-γ1 (Harita *et al.*, 2009), and Y1204 is close to the TRPC6 binding region on nephrin (1216–1227), it is possible that the nephrin inhibition of TRPC6–PLC-γ1 complex formation is due to the binding of PLC-γ1 to phosphorylated nephrin Y1204. The nephrin Y1204F mutant that does not bind to PLC-γ1 still could abrogate the TRPC6–PLC-γ1 interaction (Figure 5A), however, indicating that nephrin interferes with the TRPC6–PLC-γ1 interaction irrespective of the nephrin–PLC-γ1 interaction.

Because PLC-γ1 has a key role in membrane trafficking of TRPC6 (Figure 2B), nephrin may inhibit the trafficking of TRPC6 by interfering with the TRPC6–PLC-γ1 interaction. We performed a surface biotinylation assay, and found that coexpression of nephrin in cultured podocytes reduced the Fyn-induced surface expression of TRPC6 (Figure 5B). This result was also reproduced with HEK293T cells (Figure 5C). The phosphorylation status of TRPC6 was not attenuated by the addition of nephrin, and vice versa (Supplemental Figure S3), indicating that this inhibition did not result from altered Fyn kinase activity. A nephrin deletion mutant (Δ1216–1227), which does not bind to TRPC6, showed a much lower suppression compared to wild-type nephrin (Figure 5C). These results demonstrate that nephrin inhibition of TRPC6 trafficking is due to the inhibition of the TRPC6–PLC-γ1 interaction. A PLC-γ inhibitor U73122 did not affect the nephrin inhibition of TRPC6 trafficking (Supplemental Figure S2B).

Nephrin also suppressed TRPC6 channel activity when monitored by Ca^{2+} influx in HEK293T cells (Figure 5, D and E) and cultured podocytes (Figure 5F) expressing TRPC6 and Fyn. Fyn augmented Ca^{2+} influx in cells expressing TRPC6, whereas Fyn-dependent increase in the Ca^{2+} entry was significantly suppressed when nephrin was coexpressed ($p < 0.001$, Mann–Whitney test), consistent with its inhibitory effect on TRPC6 surface expression. Fyn-induced increase of Y284F mutant was marginal in the Ca^{2+} assays.

A nephrin cytoplasmic peptide mimics the inhibitory effect of nephrin on TRPC6 activity

Given that nephrin competitively inhibits TRPC6–PLC-γ1 complex formation and that residues 1216–1227 of nephrin are necessary for the interaction with TRPC6, we then introduced a nephrin peptide (residues 1216–1227) into HEK293T cells by a commercially available delivery kit and assessed Fyn-induced translocation of TRPC6 (Figure 6A). Introduction of the nephrin peptide (1216–1227) blocked the Fyn-dependent membrane trafficking of TRPC6 in HEK293T cells. PLC-γ1 was detected in the biotinylated samples from cells cotransfected with Fyn, and the nephrin peptide (1216–1227) also inhibited PLC-γ1 from targeting to the plasma membrane (Figure 6A). As an alternative and convenient peptide-delivery approach, polyarginine-fusion (11R) peptides were transduced into cultured podocytes (Figure 6B). Almost all the cells incorporated the peptides. Treatment with the 11R-nephrin peptide (1216–1227) strongly inhibited the Fyn-induced membrane expression of TRPC6 in podocytes (Figure 6C). In agreement with its ability to suppress the membrane trafficking of TRPC6, the nephrin peptide (1216–1227) was able to mimic the inhibitory effect of nephrin on TRPC6 channel activity ($p < 0.001$, Figure 6, D and E).

The FSGS mutants of TRPC6 escape the inhibitory effect of nephrin

Since the identification of TRPC6 mutations as the cause of FSGS, the activity of mutated TRPC6 channels has been measured in cultured cells (Reiser *et al.*, 2005; Winn *et al.*, 2005). The results, however, do not clearly demonstrate a relationship between channel activity and the pathogenesis of FSGS: Some TRPC6 mutations (P112Q, R895C, E897K) enhance channel activity, whereas others (N143S, S270T, K874X) do not. These apparently contradictory results led us to hypothesize that there may be a podocyte-specific regulation of TRPC6.

On the basis of this hypothesis, we analyzed the interaction between disease-causing TRPC6 mutants (P112Q, N143S, S270T, K874X, R895C, and E897K) and nephrin by coimmunoprecipitation (Figure 7, A and B). Five disease-causing mutations (all except K874X, $p < 0.001$) decreased the phosphorylation-dependent interaction between TRPC6 and nephrin. Then, we analyzed the

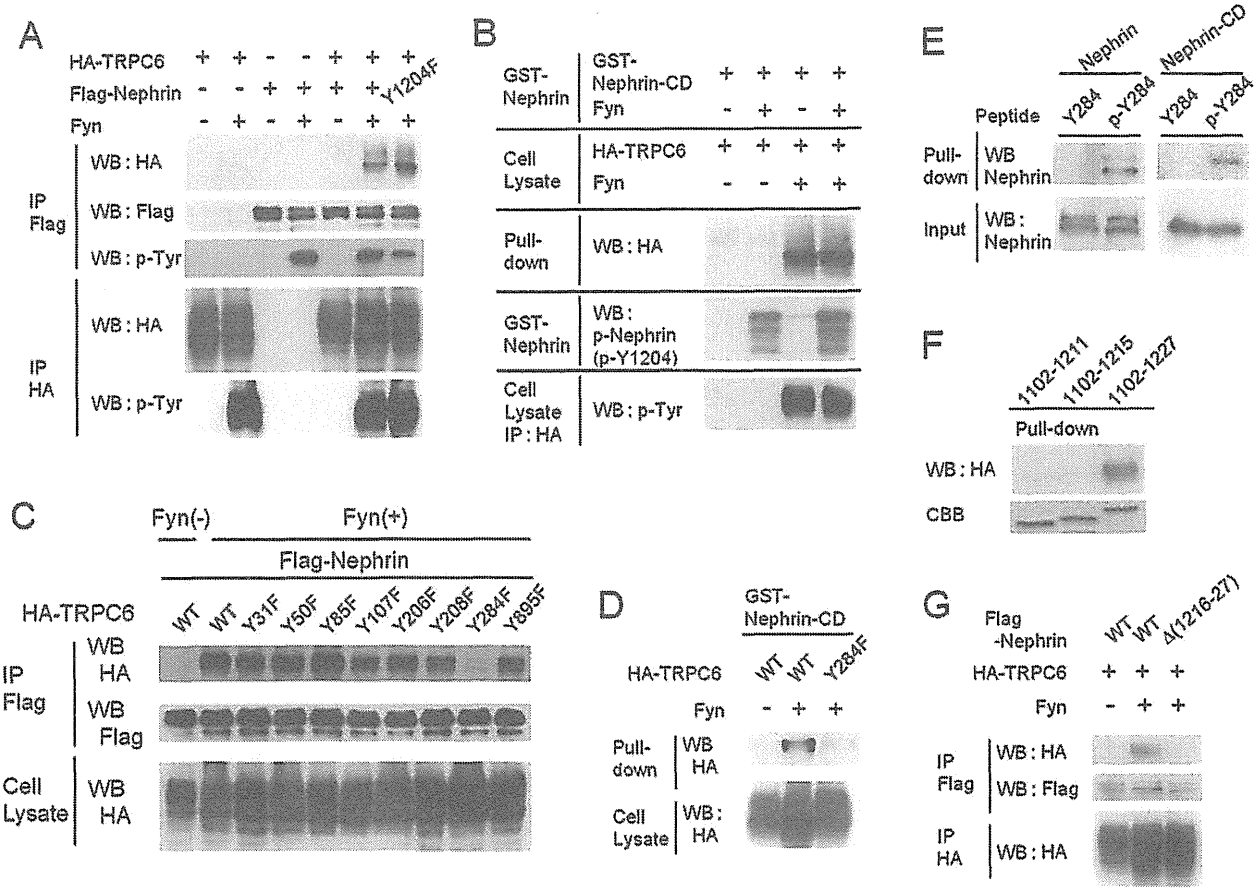

FIGURE 4: TRPC6 Y284 and nephrin 1216–1227 are critical for their tyrosine-phosphorylation–dependent interaction. (A) Lysates from HEK293T cells expressing the indicated plasmids were processed for immunoprecipitation and Western blotting with indicated antibodies. (B) Lysates from HEK293T cells expressing HA-TRPC6 with or without Fyn were pulled down with GST-nephrin-CD (residues 1102–1252) or GST-nephrin-CD prephosphorylated in vitro by Fyn. Bound proteins were probed with α-HA antibody. Phosphorylation of GST-nephrin and TRPC6 was confirmed (bottom two panels). (C) Flag-nephrin, Fyn, and HA-TRPC6 (wild type or one of a series of single phenylalanine mutants) were expressed in HEK293T cells. Immunoprecipitates and total cell lysates were blotted with indicated antibodies. (D) Lysates from HEK293T cells transiently expressing wild-type HA-TRPC6 or TRPC6 (Y284F) with or without Fyn were pulled down with GST-nephrin-CD, and probed with α-HA. (E) Lysates from HEK293T cells expressing nephrin or recombinant nephrin-CD were pulled down with phosphorylated/nonphosphorylated TRPC6 Y284 peptide immobilized on beads, and bound proteins were analyzed by Western blot with α-nephrin. (F) A series of deletion mutants of GST-nephrin-CD (residues 1102–1211, 1102–1215, 1102–1227) was used to pull down tyrosine-phosphorylated HA-TRPC6 from lysates of HEK293T cells expressing HA-TRPC6 and Fyn. CBB, Coomassie Brilliant Blue staining showing nephrin-CD. (G) HEK293T cells were transfected with HA-TRPC6, Fyn, and Flag-nephrin (wild type or nephrin lacking 1216–1227), and HA/Flag immunoprecipitates were blotted with indicated antibodies.

inhibitory effect of nephrin on the TRPC6–PLC-γ1 interaction and on the surface expression of TRPC6 mutants. Interestingly, nephrin did not suppress the complex formation between TRPC6 mutants and PLC-γ1, contrasting with wild-type TRPC6 (Figure 7C). Fyn-induced membrane translocation was observed in all TRPC6 mutants as well as in wild-type TRPC6 in the absence of nephrin (unpublished data). Surprisingly, nephrin did not inhibit the surface expression of any disease-causing TRPC6 mutant, in striking contrast to wild-type TRPC6 (Figure 7D).

Finally, we investigated the impact of nephrin on the Fyn-dependent channel activation of the TRPC6 disease-causing mutants (Figure 7, E and F). Consistent with the membrane localization results, nephrin suppressed the Fyn-induced augmentation of the wild-type channel, but the TRPC6 N143S mutant was almost insensitive to nephrin. The other five TRPC6 mutants also escaped the inhibitory effects of nephrin (Figure 7F). The result that nephrin could not suppress the activity of all the FSGS-associated mutants could lead to uncontrolled TRPC6 surface expression and exaggerated TRPC6 activation in patients' podocytes.

DISCUSSION

Recently PLC-γ has been shown to regulate cell-surface expression of ion channels and transporters through protein–protein interaction, independent of its catalytic activity (Patterson *et al.*, 2002). For example, the PH-c domain of PLC-γ1 binds to TRPC3 (amino acids 40–46), which functions in the membrane trafficking of the PLC-γ1-TRPC3 complex (van Rossum *et al.*, 2005). The PH-c domain of PLC-γ1 also binds to a Na^+/H^+ antiporter, NHE3, mediating calcium regulation of Na^+/H^+ exchange activity (Zachos *et al.*, 2009). In this study, we have shown TRPC6 to be another TRPC

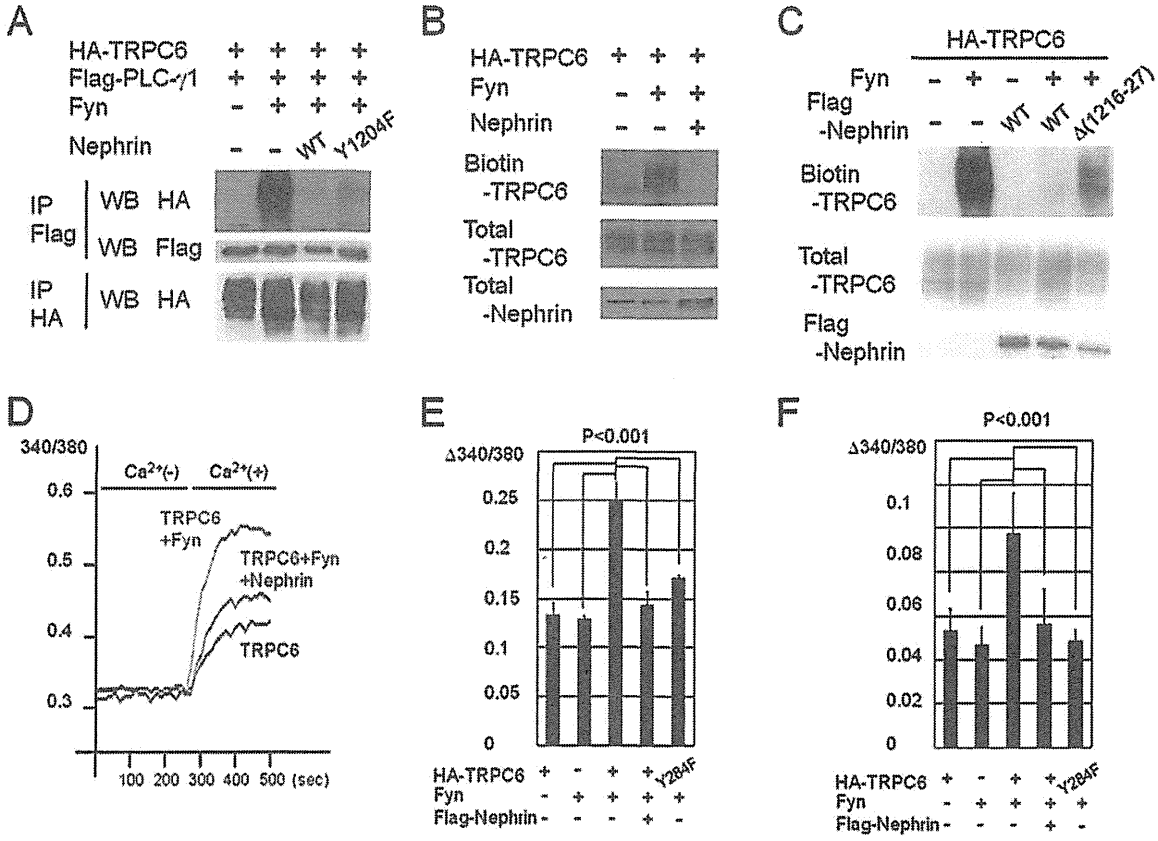

FIGURE 5: Nephrin negatively regulates the phosphorylation-dependent surface expression and channel activation of TRPC6. (A–C) Indicated plasmids were transfected into HEK293T cells (A and C) or cultured podocytes (B), and a coimmunoprecipitation (A) or surface biotinylation (B and C) assay was performed. (D–F) Fura-2–loaded HEK293T cells (D and E) and cultured podocytes (F) transfected with the indicated plasmids were superfused in succession with Ca^{2+}-free HBS and HBS with $CaCl_2$. $[Ca^{2+}]_i$ was recorded from a cell population ($n > 400$) as F340/F380 ratio of Fura-2 fluorescence, and mean values are plotted (D). The peak ratio amplitude from baseline was calculated for each cell, and the bar graphs show the mean of the data from five independent experiments, with standard errors (E and F).

member for which the membrane trafficking is controlled by a tyrosine phosphorylation–dependent interaction with PLC-γ1. The critical role of PLC-γ1 is shown by siRNA-mediated depletion of PLC-γ1 abolishing membrane translocation of TRPC6 and by the rescue control (Figure 2B). This phosphorylation-dependent membrane insertion of TRPC6 was not mediated by the interaction through the PH domain of PLC-γ1, because the deletion TRPC6 mutation (Δ98–104, which correspond to residues 40–46 of TRPC3) did not affect the phosphorylation-dependent interaction with PLC-γ1 (Figure 2F) and membrane trafficking of TRPC6 (Figure 2G). Rather, the N-terminal SH-2 domain of PLC-γ1 was necessary for the binding with TRPC6 through the critical tyrosine residue essential for its surface localization (Figure 2, D and E). Taken together, our results indicate that PLC-γ1 regulates TRPC channels through multiple interactions depending on a variety of stimulations.

Nephrin is highly expressed in podocytes and plays a major role in the slit diaphragm (Wartiovaara *et al.*, 2004). Nephrin directly binds to TRPC6 upon phosphorylation of Y284 and inhibits the TRPC6–PLC-γ1 interaction, leading to suppression of TRPC6 surface expression. Although PLC-γ1 interacts with phosphorylated Y1204 of nephrin (Harita *et al.*, 2009), this interaction is not involved in the suppression of TRPC6 membrane insertion by nephrin, because nephrin mutant (Y1204F) suppressed TRPC6 trafficking to the same extent as did the wild type (Figure 5A). Recently a similar inhibitory mechanism for TRPC3 has been reported. A transcription factor TFII-I binds to the SH2 and PH-c domains of PLC-γ1 and displaces TRPC3 from PLC-γ1, which prevents membrane expression of the channel and associated calcium entry (Caraveo *et al.*, 2006). This interference by TFII-I or nephrin on the TRPC–PLC-γ1 interaction reveals a multitiered regulatory mechanism for TRPC activity. The mode of regulation of TRPC6 trafficking by PLC-γ1 and nephrin is schematically summarized in Figure 8.

Nephrin is thought to continuously turn over by endocytosis and exocytosis in podocytes to maintain constant reorganization of the foot process (Quack *et al.*, 2006; Qin *et al.*, 2009; Tossidou *et al.*, 2010). In contrast, TRPCs are known to reside on vesicles beneath the plasma membranes, and these vesicles fuse with the plasma membranes to expose the channel upon growth factor stimuli (Bezzerides *et al.*, 2004). TRPC3, which is a close member of TRPC6, is known to undergo constitutive recycling in and out of the plasma membranes (Smyth *et al.*, 2006). Although we do not have quantitative data on the relative amount of nephrin on plasma membranes and endocytic vesicles in vivo, nephrin–TRPC6 interaction may occur at various membrane compartments.

Recent findings that mutations in *TRPC6* cause FSGS highlight the importance of Ca^{2+} signaling in podocytes (Reiser *et al.*, 2005; Winn *et al.*, 2005). How the channel activity of mutated TRPC6 is involved in the pathogenesis remains unclear, however, because the

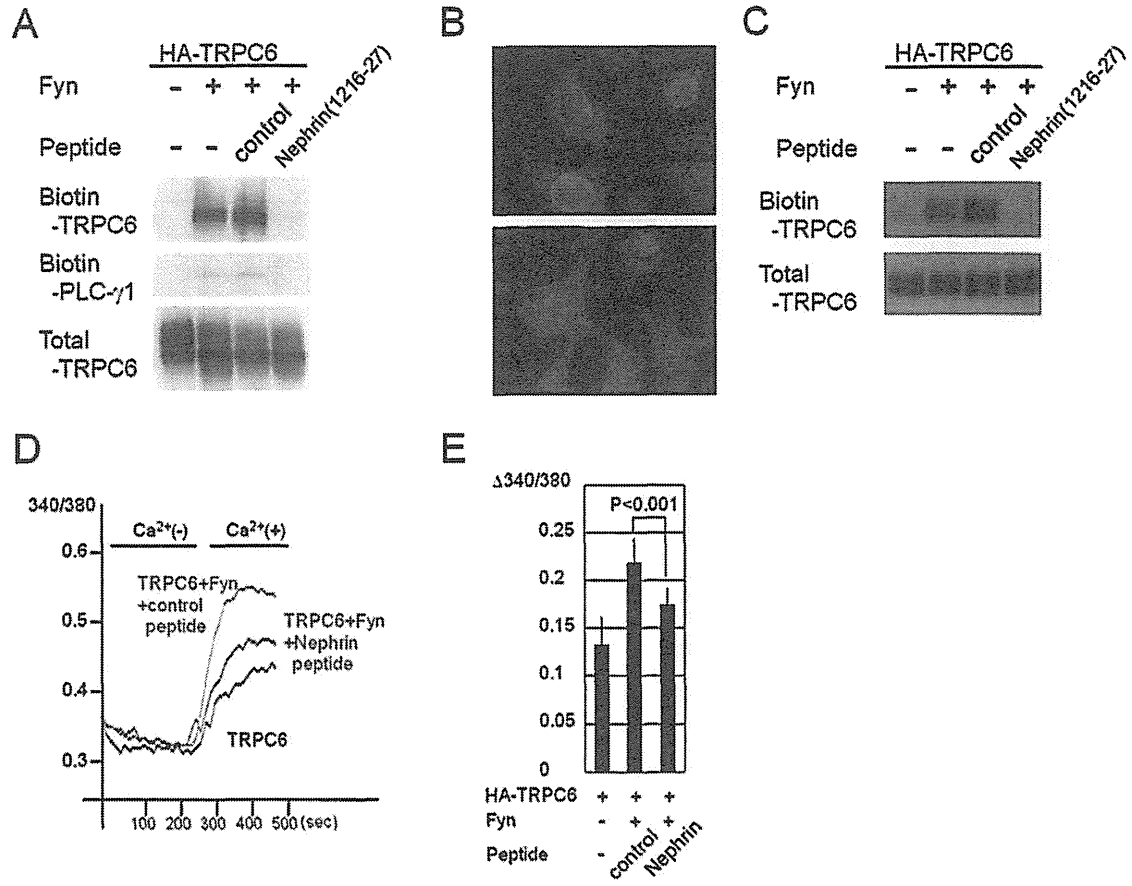

FIGURE 6: A nephrin peptide mimics the inhibitory effect of nephrin on TRPC6 activity. (A) A nephrin peptide (1216–1227) or a control peptide was transfected into HEK293T cells 4 h after the transfection of the indicated plasmids, and a surface biotinylation assay was performed 24 h later. (B) Cultured podocytes were incubated with an FITC-conjugated 11R-nephrin peptide (top panel) or an 11R-control peptide (bottom panel) and examined with a fluorescence microscope. (C) An 11R-nephrin peptide or an 11R-control peptide was delivered into cultured podocytes expressing HA-TRPC6 with or without Fyn, and the surface biotinylation assay was performed. (D) Indicated plasmids and peptides were introduced in HEK293T cells. Changes in $[Ca^{2+}]_i$ were recorded as in Figure 5D. The representative data from five independent experiments are shown. (E) The data from five experiments were processed as in Figure 5E.

channel activity measured in cultured cells is increased by some mutations (P112Q, R895C, E897K) or unchanged by others (S270T, N143S, K874X) (Reiser *et al.*, 2005; Winn *et al.*, 2005). In HEK293T, the surface expression of wild-type TRPC6 and all the disease-causing mutant TRPC6s was equally increased by tyrosine phosphorylation by Fyn. Whereas the Fyn-induced increase in wild-type TRPC6 was significantly suppressed by nephrin, that of the mutant TRPC6s was insensitive to nephrin inhibition (Figure 7D). This insensitivity may be due to the much lower binding affinity of mutant TRPC6s (P112Q, N143S, S270T, R885C, E897K) to nephrin (Figure 7, A and B). Because of its decreased affinity, nephrin could not inhibit mutant TRPC6s from binding to PLC-γ1 (Figure 7C) and being expressed at the plasma membrane (Figure 7D). The channel activity of wild type and mutant TRPC6s (Figure 7, E and F) correlated well with the results obtained by the biotinylation assay (Figure 7D). Again, Fyn stimulated the Ca^{2+} channel activity of both the wild type and the mutants; the former stimulation was suppressed by nephrin, but the latter was not. These results suggest increased calcium signaling even in the patients' podocytes with mutations that do not enhance the channel activity when measured in usual cell culture systems. In this regard, whereas one mutation (K874X) bound to nephrin, Fyn-induced translocation of the K874X mutant was insensitive to nephrin. The K874X mutant may have a structural defect such that interaction with nephrin somehow cannot suppress its interactions with molecules that promote its trafficking, including PLC-γ1 (Figure 7C).

Phosphorylated Y284 and the amino acids at the mutated positions (P112, N143, S270, R885, E897) of TRPC6 are both necessary for the interaction with nephrin (Figure 7A), indicating that these residues constitute the binding site. On nephrin itself, a 12-amino-acid sequence (residues 1216–1227) is essential for the binding to TRPC6, and this sequence is sufficient to suppress TRPC6 activation. It has been shown that TRPC channels form homo- or hetero-tetramers with other TRPC channels (Hofmann *et al.*, 2002). Indeed, biochemical (Lepage *et al.*, 2006) and cryo-electron microscopic (Mio *et al.*, 2007) analyses have demonstrated that the N-terminal region is in close contact with the C-terminal region. In such quaternary structures, the N-terminal (including P112, N143, S270, Y284) and C-terminal (R885, E897) regions of TRPC6 may be in close proximity to each other, and these amino acids may be necessary for the interaction with nephrin. Nephrin and nephrin peptide may inhibit the membrane trafficking of

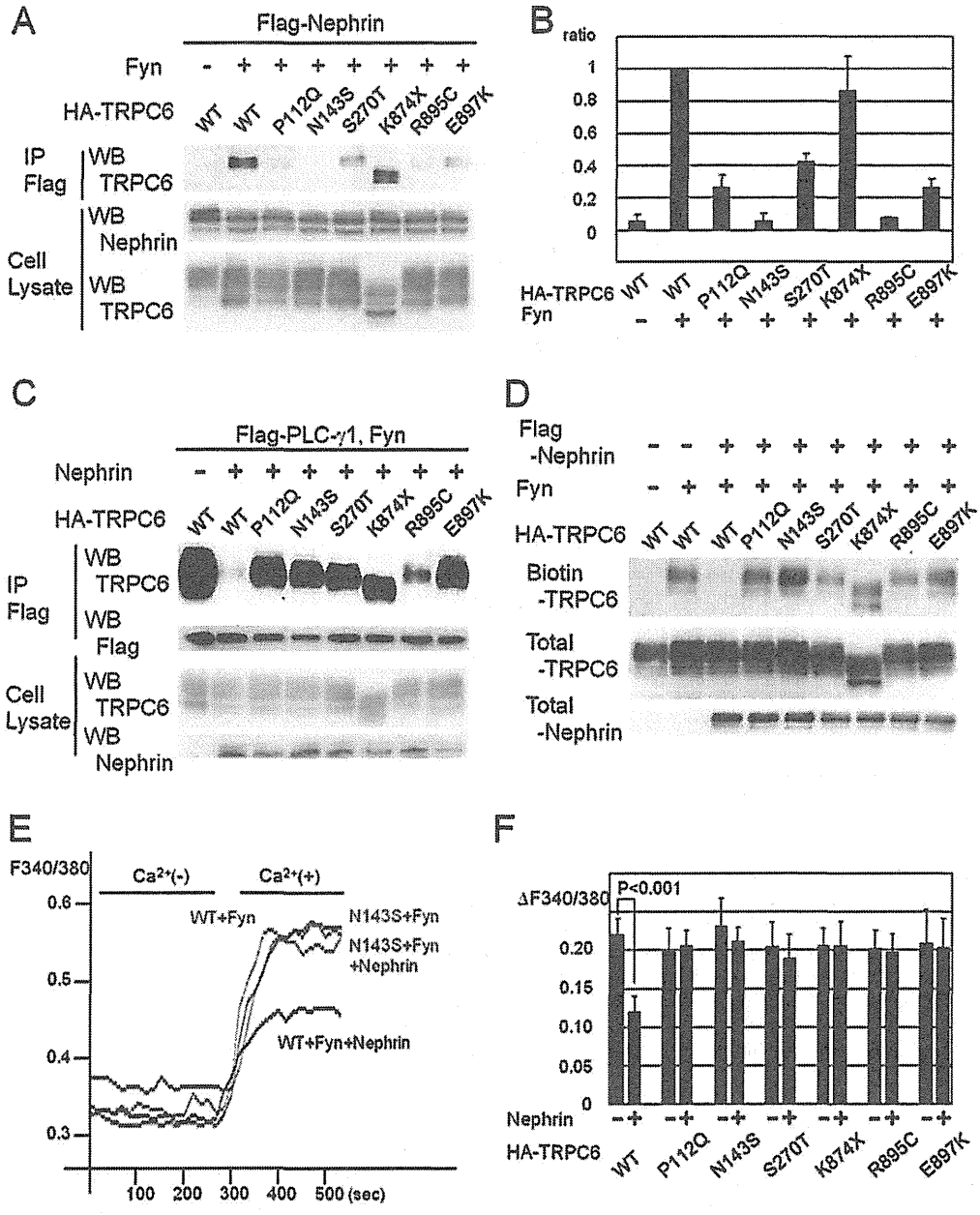

FIGURE 7: Impairment of nephrin interaction and dysregulated surface expression of TRPC6 by FSGS-associated TRPC6 mutations. (A) Flag-nephrin, Fyn, and each of the HA-TRPC6 mutants were coexpressed in HEK293T cells, and α-Flag or α-HA immunoprecipitates were analyzed by Western blot with indicated antibodies. (B) TRPC6-nephrin binding was quantified by densitometric tracing of A (WB: TRPC6), and the mean values were shown in the bar graph, with standard errors (n = 3). Values were normalized to wild-type TRPC6. (C) The effect of nephrin expression on the TRPC6–PLC-γ1 interaction was examined with HEK293T cells expressing wild type or each of the TRPC6 mutants, in addition to Flag-PLC-γ1 and Fyn. (D) Surface expression of TRPC6 was analyzed as in Figure 1A by using HEK293T cells transfected with a series of disease-causing TRPC6 mutants, with or without nephrin/Fyn. (E) Nephrin, Fyn, and either TRPC6 or TRPC6 N143S mutant were expressed in HEK293T cells. Changes in $[Ca^{2+}]_i$ were recorded as in Figure 5D. The representative data from five independent experiments are shown. (F) The channel activities of FSGS-associated TRPC6 mutants were evaluated as in E, and the data from five experiments were processed as in Figure 5E.

TRPC6 by changing the tertiary structure through binding to TRPC6.

Whereas TRPC6 Y31F mutant decreased the levels of TRPC6–PLC-γ1 complex (Figure 2C), its membrane translocation was not significantly impaired (Figure 1D). In contrast, Y284F mutation robustly reduced the membrane trafficking with a marginal decrease in TRPC6–PLC-γ1 complex level. We interpret these results as follows: Because both the pY31 and pY284 peptides bind preferentially to N-SH2 of PLC-γ1 (Figure 2E), and C-SH2 of PLC-γ1 binds to pY783 of PLC-γ1 intramolecularly (Poulin *et al.*, 2005), there may be two kinds of TRPC6–PLC-γ1 complexes, the interaction of which is mediated by N-SH2 of PLC-γ1 and either by pY31 or pY284

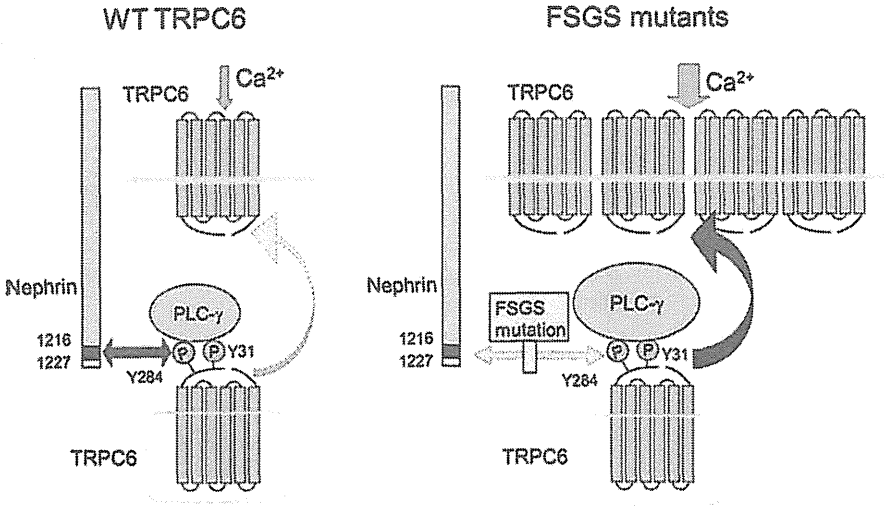

FIGURE 8: Schematic illustration of the interaction among nephrin, TRPC6, and PLC-γ1.

of TRPC6. Only the latter complex may adopt a conformation that is necessary for membrane trafficking. Although the majority of the complex is mediated by pY31, the membrane trafficking may shift the equilibrium between the two complexes in the cytoplasm to reproduce the complex mediated by pY284. Nephrin competitively binds to pY284 and may inhibit TRPC6–PLC-γ1 complex formation. Because nephrin does not have an SH2 or phosphotyrosine binding domain, its mode of interaction is currently unknown.

The mode of regulation of TRPC6 presented here may also have relevance to nonrenal diseases, because TRPC6 has been linked to pulmonary hypertension, cardiac hypertrophy, cardiac fibrosis, neuronal outgrowth, and eryptosis in human or animal models (Dietrich *et al.*, 2005; Nilius *et al.*, 2007; Abramowitz and Birnbaumer, 2009). Modulation of TRPC6 activity by small molecules or peptides may be an effective therapeutic strategy for treating proteinuric diseases or a wide variety of diseases.

MATERIALS AND METHODS

Antibodies and reagents

Rabbit α-phospho-TRPC6 antibodies (α-pY31, α-pY284) were raised against synthetic oligopeptides of 12 amino acids, CNESQDpYLLMDEL (pY31) and CLASPApYLSLSSE (pY284) (the first cysteine residues are not part of the TRPC6 sequence), respectively. The antisera were affinity purified using the immunogen coupled to a SulfoLink (Pierce, Rockford, IL) and absorbed with nonphosphorylated peptides. α-FLAG, 0.5 μg/ml (M2; Sigma, St. Louis, MO), α-HA, 50 ng/ml (3F10; Roche Diagnostics, Indianapolis, IN), α-phosphotyrosine, 0.5 μg/ml (4G10; Upstate, Lake Placid, NY), rabbit α-TRPC6, 0.5 μg/ml (Alomone Labs, Jerusalem, Israel), and rabbit α-PLC-γ1, 0.1 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were obtained commercially. Rabbit α-nephrin and α-phospho-nephrin (pY1204) were previously described (Harita *et al.*, 2009). Western blotting was performed with these antibodies diluted at 1:2000. PP2 (Merck KGaA, Darmstadt, Germany), SU6656 (Merck KGaA), and EGF (BD Biosciences, Bedford, MA) were purchased.

Cell culture, transfection, and RNAi

Human embryonic kidney HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. A temperature-sensitive rat podocyte cell line, 2DNA1D7, was described previously (Harita *et al.*, 2008). Transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The sequences of siRNA used were nontargeting control (D-001210-0120; Dharmacon, Lafayette, CA) and human PLC-γ1 (5'- CCUUGUUGACCUCAUCAGCUACUAU -3') containing two mismatches with rat PLC-γ1. These siRNA duplexes were transfected into HEK293T cells using Lipofectamine RNAiMAX (Invitrogen).

Eukaryotic expression constructs

Mammalian expression plasmids encoding full-length rat nephrin, full-length nephrin-Flag, Y1204F nephrin, and Y1204F nephrin-Flag were previously described (Harita *et al.*, 2008, 2009). Expression vectors for Fyn (a gift from T. Tezuka, Tokyo University) (Tezuka *et al.*, 1999), mouse TRPC6-HA (a gift from C. Hisatsune, RIKEN Brain Science Institute, Japan) (Hisatsune *et al.*, 2004), and human Flag-PLC-γ1 (a gift from P. G. Suh, Pohang University, Korea) (Bae *et al.*, 2000) were described. SH2 mutants of PLC-γ1 were made according to the literature (Bae *et al.*, 1998; Plattner *et al.*, 2003). Mammalian expression plasmids encoding mouse TRPC6 mutants, Y31F, Y50F, Y85F, Y107F, Y206F, Y208F, Y284F, Y895F, P112Q, N143S, S270T, K874X, R895C, E897K and deletion (98–104) mutant, and a nephrin-Flag deletion mutant (1216–27) were prepared using standard PCR methods. DNA sequencing was performed to validate all the constructs. Oligonucleotides used in PCR are described in the Supplementary Material.

Cell-surface biotinylation

Surface expression of TRPC6 was assayed according to Winn et al. (2005). Briefly, HEK293T cells or cultured podocytes transfected with indicated plasmids were surface biotinylated for 30 min at 4°C with 2 mg/ml Sulfo-NHS-SS-Biotin (Pierce). The biotinylated proteins were recovered with streptavidin beads (Pierce) and were analyzed by Western blot analyses.

Immunoprecipitation

Cells were lysed with lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40 [NP40], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 50 mM NaF, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium vanadate) for 15 min on ice. Lysates were clarified by centrifugation and incubated with beads conjugated with M2 α-Flag antibody or with 3F10 α-HA antibody for 1 h at 4°C. Beads were washed three times with Tris-buffered saline–1% NP40, and bound proteins were eluted with 100 mM glycine–HCl (pH 2.6) and analyzed by Western blotting.

Bacterial fusion protein expression

GST-tagged nephrin cytoplasmic region (nephrin-CD: amino acids 1102–1252) was previously described (Harita *et al.*, 2009). GST-nephrin-CD deletion mutants (1102–1211, 1102–1215, 1102–1227) were prepared using standard PCR methods. Bacterial pellets were resuspended and sonicated in a solution containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP40, 2 mM dithiothreitol, 1 mM PMSF, 10 μg/ml antipain, 10 μg/ml leupeptin, and insoluble material was removed by centrifugation. GST fusion protein was purified on a glutathione–Sepharose column (GE Healthcare, Buckinghamshire,

UK) and eluted with free glutathione. Oligonucleotides used to make GST-nephrin-CD deletion mutants are listed in the Supplementary Material.

Pull-down assay

GST-nephrin-CD immobilized on glutathione-Sepharose beads was phosphorylated with Fyn as described previously (Harita *et al.*, 2008, 2009). HA-TRPC6 expressing HEK293T whole-cell lysates were incubated with phosphorylated GST-nephrin-CD or nonphosphorylated GST-nephrin-CD immobilized on beads at 4°C overnight. Beads were washed extensively with wash buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP40). Nephrin and bound proteins were eluted with free glutathione and analyzed by Western blotting and Coomassie Brilliant Blue staining. For pull-down analysis using peptides, peptides were fixed on SulfoLink Coupling Gel (Pierce).

Determination of proteins using mass spectrometry

Proteins of interest were excised from silver-stained gel and digested with 1 pmol of Achromobacter protease I in 40 µl of digestion buffer (10 mM Tris, pH 8.5) at 37°C overnight. Peptides were purified and subjected to peptide mass fingerprinting using liquid chromatograph-mass/mass spectrometry (LC-MS/MS) (Q-STAR Elite; Applied Biosystems, Carlsbad, CA). Peptide ions were analyzed with ProteinPilot (version 3.0; Applied Biosystems) software.

Isolation of rat kidney glomeruli

The glomeruli were isolated from the rat renal cortexes using a graded sieving technique and were saved for protein extraction. All experiments were carried out according to the guidelines set by the Animal Center of the Institute of Medical Science, the University of Tokyo. Wistar rats were purchased from Charles River Laboratories Japan (Atsugi, Japan).

Measurement of $[Ca^{2+}]_i$

HEK293T cells or cultured podocytes grown on coverslips were loaded with 4 µM Fura-2/AM (Dojindo, Kumamoto, Japan) and bathed in HEPES-buffered saline (HBS; 115 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, pH 7.4) for 30 min before Ca^{2+} measurements. The cells were successively perfused at room temperature with Ca^{2+}-free HBS (115 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 2 mM $MgCl_2$, 10 mM glucose, 0.05 mM EGTA, pH 7.4) and HBS. The fluorescence of Fura-2–loaded cells was monitored under an Olympus IX70 microscope with a 20× objective lens (N.A. 0.75). Fluorescence of Fura-2 was excited alternately by 340- and 380-nm wavelength light every 10 s, and images were recorded with a cooled CCD camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan). The time course of ratio of fluorescence intensity (F340/F380) was calculated from all the cells (400~500) in each view field and averaged. Image acquisition and data analysis were performed with custom-made TI Workbench software written by T. I on a Macintosh computer (Bannai *et al.*, 2004).

Peptide delivery

A nephrin peptide (amino acids 1216–1227, CWPEVQCEDPRGI) or a control peptide (nephrin C terminus; amino acids 1241–1252, CSSLPFELRGHLV) was delivered into HEK293T cells using Transport Protein Delivery Reagent (Takara, Tokyo, Japan). Cultured podocytes were incubated with 3 µM fluorescein isothiocyanate (FITC)-conjugated 11R-nephrin peptide (1216–1227, RRRRRRRRRRRGGG-WPEVQCEDPRGI) or FITC-conjugated 11R-control peptide (nephrin C terminus; amino acids 1241–1252, RRRRRRRRRRRGGGSSLPFEL-RGHLV) for 24 h. The cells were washed three times with phosphate-buffered saline, and the fluorescence of FITC was monitored under an Olympus IX71 microscope with a 60× objective lens (N.A. 0.7).

Statistics

Values are presented as means ± SEM. Comparison between two groups of $[Ca^{2+}]_i$ was assessed with the Mann–Whitney *U* test. The unpaired Student's *t* test was used to compare the band intensities in Western blot analyses. Values of $p < 0.05$ were considered significant.

ACKNOWLEDGMENTS

We thank H. Kosako for valuable comments on the manuscript. We also thank T. Tezuka (Tokyo University, Japan), C. Hisatsune (RIKEN, Japan), and J. K. Kim and P. G. Suh (Pohang University, Korea) for plasmids. This study was partially supported by the Molecular Nephrology Forum and the Japan Foundation for Pediatric Research. This study was also supported by a Grant-in-Aid from the Japan Society for the Promotion of Science (JSPS) Fellows from the JSPS (to K.S.), a Grant-in-Aid for Young Scientists (B) (20790719, 22790991) (to H.Y.), and a Grant-in-Aid for Scientific Research (B) (22390204) (to H.Y., H.S., and I.T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

Abramowitz J, Birnbaumer L (2009). Physiology and pathophysiology of canonical transient receptor potential channels. FASEB J 23, 297–328.

Bae SS, Perry DK, Oh YS, Choi JH, Galadari SH, Ghayur T, Ryu SH, Hannun YA, Suh PG (2000). Proteolytic cleavage of phospholipase C-gamma1 during apoptosis in Molt-4 cells. FASEB J 14, 1083–1092.

Bae YS, Cantley LG, Chen CS, Kim SR, Kwon KS, Rhee SG (1998). Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 273, 4465–4469.

Bannai H, Inoue T, Nakayama T, Hattori M, Mikoshiba K (2004). Kinesin dependent, rapid, bi-directional transport of ER sub-compartment in dendrites of hippocampal neurons. J Cell Sci 117, 163–175.

Berridge MJ, Lipp P, Bootman MD (2000). The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1, 11–21.

Bezzerides VJ, Ramsey IS, Kotecha S, Greka A, Clapham DE (2004). Rapid vesicular translocation and insertion of TRP channels. Nat Cell Biol 6, 709–720.

Birnbaumer L *et al.* (1996). On the molecular basis and regulation of cellular capacitative calcium entry: roles for Trp proteins. Proc Natl Acad Sci USA 93, 15195–15202.

Bush EW, Hood DB, Papst PJ, Chapo JA, Minobe W, Bristow MR, Olson EN, McKinsey TA (2006). Canonical transient receptor potential channels promote cardiomyocyte hypertrophy through activation of calcineurin signaling. J Biol Chem 281, 33487–33496.

Caraveo G, van Rossum DB, Patterson RL, Snyder SH, Desiderio S (2006). Action of TFII-I outside the nucleus as an inhibitor of agonist-induced calcium entry. Science 314, 122–125.

Cayouette S, Lussier MP, Mathieu EL, Bousquet SM, Boulay G (2004). Exocytotic insertion of TRPC6 channel into the plasma membrane upon Gq protein-coupled receptor activation. J Biol Chem 279, 7241–7246.

Dietrich A, Kalwa H, Rost BR, Gudermann T (2005). The diacylglycerol-sensitive TRPC3/6/7 subfamily of cation channels: functional characterization and physiological relevance. Pflugers Arch 451, 72–80.

Harita Y, Kurihara H, Kosako H, Tezuka T, Sekine T, Igarashi T, Hattori S (2008). Neph1, a component of the kidney slit diaphragm, is tyrosine-phosphorylated by the Src family tyrosine kinase and modulates intracellular signaling by binding to Grb2. J Biol Chem 283, 9177–9186.

Harita Y, Kurihara H, Kosako H, Tezuka T, Sekine T, Igarashi T, Ohsawa I, Ohta S, Hattori S (2009). Phosphorylation of nephrin triggers Ca2+ signaling by recruitment and activation of phospholipase C-gamma 1. J Biol Chem 284, 8951–8962.

Hisatsune C, Kuroda Y, Nakamura K, Inoue T, Nakamura T, Michikawa T, Mizutani A, Mikoshiba K (2004). Regulation of TRPC6 channel activity by tyrosine phosphorylation. J Biol Chem 279, 18887–18894.

Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G (1999). Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature 397, 259–263.
Hofmann T, Schaefer M, Schultz G, Gudermann T (2002). Subunit composition of mammalian transient receptor potential channels in living cells. Proc Natl Acad Sci USA 99, 7461–7466.
Huber TB *et al.* (2006). Podocin and MEC-2 bind cholesterol to regulate the activity of associated ion channels. Proc Natl Acad Sci USA 103, 17079–17086.
Inoue R, Okada T, Onoue H, Hara Y, Shimizu S, Naitoh S, Ito Y, Mori Y (2001). The transient receptor potential protein homologue TRP6 is the essential component of vascular alpha(1)-adrenoceptor-activated Ca(2+)-permeable cation channel. Circ Res 88, 325–332.
Jones N *et al.* (2006). Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. Nature 440, 818–823.
Kawasaki BT, Liao Y, Birnbaumer L (2006). Role of Src in C3 transient receptor potential channel function and evidence for a heterogeneous makeup of receptor- and store-operated Ca2+ entry channels. Proc Natl Acad Sci USA 103, 335–340.
Kiselyov K, Patterson RL (2009). The integrative function of TRPC channels. Front Biosci 14, 45–58.
Krall P *et al.* (2010). Podocyte-specific overexpression of wild type or mutant trpc6 in mice is sufficient to cause glomerular disease. PLoS One 5, e12859.
Kuwahara K, Wang Y, McAnally J, Richardson JA, Bassel-Duby R, Hill JA, Olson EN (2006). TRPC6 fulfills a calcineurin signaling circuit during pathologic cardiac remodeling. J Clin Invest 116, 3114–3126.
Lahdenpera J, Kilpelainen P, Liu XL, Pikkarainen T, Reponen P, Ruotsalainen V, Tryggvason K (2003). Clustering-induced tyrosine phosphorylation of nephrin by Src family kinases. Kidney Int 64, 404–413.
Lepage PK, Lussier MP, Barajas-Martinez H, Bousquet SM, Blanchard AP, Francoeur N, Dumaine R, Boulay G (2006). Identification of two domains involved in the assembly of transient receptor potential canonical channels. J Biol Chem 281, 30356–30364.
Lin MJ, Leung GP, Zhang WM, Yang XR, Yip KP, Tse CM, Sham JS (2004). Chronic hypoxia-induced upregulation of store-operated and receptor-operated Ca2+ channels in pulmonary arterial smooth muscle cells: a novel mechanism of hypoxic pulmonary hypertension. Circ Res 95, 496–505.
Mio K, Ogura T, Kiyonaka S, Hiroaki Y, Tanimura Y, Fujiyoshi Y, Mori Y, Sato C (2007). The TRPC3 channel has a large internal chamber surrounded by signal sensing antennas. J Mol Biol 367, 373–383.
Moller CC *et al.* (2007). Induction of TRPC6 channel in acquired forms of proteinuric kidney disease. J Am Soc Nephrol 18, 29–36.
Nakayama H, Wilkin BJ, Bodi I, Molkentin JD (2006). Calcineurin-dependent cardiomyopathy is activated by TRPC in the adult mouse heart. FASEB J 20, 1660–1670.
Nilius B, Owsianik G, Voets T, Peters JA (2007). Transient receptor potential cation channels in disease. Physiol Rev 87, 165–217.
Nilius B, Voets T, Peters J (2005). TRP channels in disease. Sci STKE 2005, re8.
Nishida M, Watanabe K, Sato Y, Nakaya M, Kitajima N, Ide T, Inoue R, Kurose H (2010). Phosphorylation of TRPC6 channels at Thr69 is required for anti-hypertrophic effects of phosphodiesterase 5 inhibition. J Biol Chem 285, 13244–13253.
Odell AF, Scott JL, Van Helden DF (2005). Epidermal growth factor induces tyrosine phosphorylation, membrane insertion, and activation of transient receptor potential channel 4. J Biol Chem 280, 37974–37987.
Okada T *et al.* (1999). Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. Ca(2+)-permeable cation channel that is constitutively activated and enhanced by stimulation of G protein-coupled receptor. J Biol Chem 274, 27359–27370.
Onohara N, Nishida M, Inoue R, Kobayashi H, Sumimoto H, Sato Y, Mori Y, Nagao T, Kurose H (2006). TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy. EMBO J 25, 5305–5316.
Patrakka J, Tryggvason K (2009). New insights into the role of podocytes in proteinuria. Nat Rev Nephrol 5, 463–468.
Patterson RL, van Rossum DB, Ford DL, Hurt KJ, Bae SS, Suh PG, Kurosaki T, Snyder SH, Gill DL (2002). Phospholipase C-gamma is required for agonist-induced Ca2+ entry. Cell 111, 529–541.
Plattner R, Irvin BJ, Guo S, Blackburn K, Kazlauskas A, Abraham RT, York JD, Pendergast AM (2003). A new link between the c-Abl tyrosine kinase and phosphoinositide signalling through PLC-gamma1. Nat Cell Biol 5, 309–319.
Poulin B, Sekiya F, Rhee SG (2005). Intramolecular interaction between phosphorylated tyrosine-783 and the C-terminal Src homology 2 domain activates phospholipase C-gamma1. Proc Natl Acad Sci USA 102, 4276–4281.
Putney JW (2009). Capacitative calcium entry: from concept to molecules. Immunol Rev 231, 10–22.
Putney JW Jr (1986). A model for receptor-regulated calcium entry. Cell Calcium 7, 1–12.
Qin XS, Tsukaguchi H, Shono A, Yamamoto A, Kurihara H, Doi T (2009). Phosphorylation of nephrin triggers its internalization by raft-mediated endocytosis. J Am Soc Nephrol 20, 2534–2545.
Quack I, Rump LC, Gerke P, Walther I, Vinke T, Vonend O, Grunwald T, Sellin L (2006). beta-Arrestin2 mediates nephrin endocytosis and impairs slit diaphragm integrity. Proc Natl Acad Sci USA 103, 14110–14115.
Reiser J *et al.* (2005). TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. Nat Genet 37, 739–744.
Schlondorff J, Del Camino D, Carrasquillo R, Lacey V, Pollak MR (2009). TRPC6 mutations associated with focal segmental glomerulosclerosis cause constitutive activation of NFAT-dependent transcription. Am J Physiol Cell Physiol 296, C558–C569.
Smyth JT, Lemonnier L, Vazquez G, Bird GS, Putney JW Jr (2006). Dissociation of regulated trafficking of TRPC3 channels to the plasma membrane from their activation by phospholipase C. J Biol Chem 281, 11712–11720.
Tezuka T, Umemori H, Akiyama T, Nakanishi S, Yamamoto T (1999). PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the *N*-methyl-D-aspartate receptor subunit NR2A. Proc Natl Acad Sci USA 96, 435–440.
Tossidou I, Teng B, Drobot L, Meyer-Schwesinger C, Worthmann K, Haller H, Schiffer M (2010). CIN85/RukL is a novel binding partner of nephrin and podocin and mediates slit diaphragm turnover in podocytes. J Biol Chem 285, 25285–25295.
Trebak M, St JBird G, McKay RR, Birnbaumer L, Putney JW Jr (2003). Signaling mechanism for receptor-activated canonical transient receptor potential 3 (TRPC3) channels. J Biol Chem 278, 16244–16252.
Tryggvason K, Patrakka J, Wartiovaara J (2006). Hereditary proteinuria syndromes and mechanisms of proteinuria. N Engl J Med 354, 1387–1401.
van Rossum DB, Patterson RL, Sharma S, Barrow RK, Kornberg M, Gill DL, Snyder SH (2005). Phospholipase Cgamma1 controls surface expression of TRPC3 through an intermolecular PH domain. Nature 434, 99–104.
Verma R, Kovari I, Soofi A, Nihalani D, Patrie K, Holzman LB (2006). Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. J Clin Invest 116, 1346–1359.
Verma R, Wharram B, Kovari I, Kunkel R, Nihalani D, Wary KK, Wiggins RC, Killen P, Holzman LB (2003). Fyn binds to and phosphorylates the kidney slit diaphragm component Nephrin. J Biol Chem 278, 20716–20723.
Wang J, Weigand L, Lu W, Sylvester JT, Semenza GL, Shimoda LA (2006). Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular Ca2+ in pulmonary arterial smooth muscle cells. Circ Res 98, 1528–1537.
Wartiovaara J *et al.* (2004). Nephrin strands contribute to a porous slit diaphragm scaffold as revealed by electron tomography. J Clin Invest 114, 1475–1483.
Winn MP *et al.* (2005). A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. Science 308, 1801–1804.
Woudenberg-Vrenken TE, Bindels RJ, Hoenderop JG (2009). The role of transient receptor potential channels in kidney disease. Nat Rev Nephrol 5, 441–449.
Zachos NC, van Rossum DB, Li X, Caraveo G, Sarker R, Cha B, Mohan S, Desiderio S, Patterson RL, Donowitz M (2009). Phospholipase C-gamma binds directly to the Na+/H+ exchanger 3 and is required for calcium regulation of exchange activity. J Biol Chem 284, 19437–19444.

Article

Two-Year Follow-Up of a Prospective Clinical Trial of Cyclosporine for Frequently Relapsing Nephrotic Syndrome in Children

Kenji Ishikura, Norishige Yoshikawa,† Hitoshi Nakazato,‡ Satoshi Sasaki,§ Kazumoto Iijima,‖ Koichi Nakanishi,† Takeshi Matsuyama,¶ Shuichi Ito,** Nahoko Yata,†† Takashi Ando,‡‡ and Masataka Honda,* for the Japanese Study Group of Renal Disease in Children*

Summary

Background and objectives Although the safety and efficacy of cyclosporine in children with frequently relapsing nephrotic syndrome (FRNS) have been confirmed, no prospective follow-up data on relapse after cyclosporine have appeared. This study is a prospective follow-up trial after 2-year treatment with cyclosporine to investigate cyclosporine dependency after its discontinuation.

Design, setting, participants, & measurements Participants who had undergone 2-year protocol treatment with microemulsified cyclosporine for FRNS between January 2000 and December 2005 were followed for an additional 2 years. The primary end point was relapse-free survival after the complete discontinuation of cyclosporine, and the secondary end point was regression-free survival (time to regression to FRNS).

Results After exclusion of 7 patients who showed regression to FRNS during the 2-year treatment period, 49 children (median age, 6.5 years) were followed, and classified as children without (n=32; group A) and with (n=17; group B) relapse during the initial cyclosporine treatment. Overall, relapse-free survival probability at 24 months after cyclosporine discontinuation was 15.3% and regression to FRNS-free survival probability was 40.8%. By group, the probability of relapse-free survival was significantly higher in group A (17.9%) than in group B (8.3%) (P<0.001).

Conclusions Children with FRNS who receive cyclosporine are at high risk of relapse after discontinuation, particularly those who experience relapse during cyclosporine treatment.

Clin J Am Soc Nephrol 7: 1576–1583, 2012. doi: 10.2215/CJN.00110112

Departments of *Nephrology and ††Pediatrics, Tokyo Metropolitan Children's Medical Center, Fuchu, Japan; †Department of Pediatrics, Wakayama Medical University, Wakayama, Japan; ‡Department of Pediatrics, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan; §Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan; ‖Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan; ¶Department of Pediatrics, Fussa Hospital, Fussa, Japan; **Division of Nephrology and Rheumatology, National Center for Child Health and Development, Setagaya, Japan; and ‡‡Japan Clinical Research Support Unit, Tokyo, Japan

Correspondence: Dr. Kenji Ishikura, Department of Nephrology, Tokyo Metropolitan Children's Medical Center, 2-8-29 Musashidai, Fuchu, Tokyo 183-8561, Japan. Email: kenji_ishikura@tmhp.jp

Introduction

The safety and efficacy of cyclosporine in children with frequently relapsing nephrotic syndrome (FRNS) and steroid-dependent nephrotic syndrome (SDNS) have been demonstrated in a number of studies, including a randomized controlled trial (1–8). We previously showed that microemulsified cyclosporine administered according to our treatment protocol is safe and effective in children with FRNS (2). In that study, the probability of relapse-free survival at month 24 was 58.1% and the probability of regression (to frequently relapsing nephrotic syndrome)–free survival at month 24 was 88.5%. Cyclosporine nephrotoxicity was detected in only 8.6% of patients who underwent renal biopsy after 2 years of treatment. Nevertheless, an important limitation of this treatment is cyclosporine dependency, namely the frequent relapse of nephrosis after discontinuation (9–12). Most studies of relapse after cyclosporine to date have been retrospective, however, and no prospective evaluation in these patients has been reported.

Several factors have been linked to a prolonged disease course in children with FRNS, including age at the onset of nephrotic syndrome (13,14) and number of relapses or steroid dependency (13,15,16). However, factors associated with relapse after cyclosporine have not been clearly established. Furthermore, it is unclear whether infrequent relapse during cyclosporine treatment is associated with disease activity. Identification of risk factors would allow better treatment decisions, particularly in predicting patients at highest risk of regression of FRNS.

Here, we conducted a prospective follow-up study of the participants of our previous clinical trial to evaluate the rate of relapse of nephrosis and FRNS after the complete discontinuation of cyclosporine. We also evaluated factors associated with relapse.

Materials and Methods

Previous Trial

This study was a prospective follow-up analysis of a previous multicenter trial that evaluated the efficacy

and safety of 2-year treatment with cyclosporine in children with FRNS between January 2000 and December 2005. Entry criteria and protocol have been detailed elsewhere (2). Briefly, children aged 1–18 years with FRNS who had idiopathic nephrotic syndrome were eligible. Patients with a history of cyclosporine treatment were excluded. Microemulsified cyclosporine (Neoral; Novartis, Basel, Switzerland) was administered for 2 years under trough control. For the first 6 months, all patients were administered a dose that maintained a whole-blood trough level between 80 and 100 ng/ml of cyclosporine. The dose was adjusted over the next 18 months to maintain a trough level between 60 and 80 ng/ml. Maintenance prednisolone was not prescribed. After 2 years of treatment, all patients were scheduled to undergo renal biopsy, and cyclosporine was stopped by dose tapering at a rate of 0.5–1.0 mg/kg per week.

Follow-Up Study

This study was conducted under a prospective, follow-up design in 21 participating institutions. Eligibility was restricted to patients who completed 2-year treatment with cyclosporine in the previous trial. Follow-up was conducted for 2 years, beginning from the time of complete cessation of cyclosporine. Patients who experienced relapse of nephrosis during the study period were administered prednisolone at 2 mg/kg per day in three divided doses (maximum, 80 mg/d) until remission, and then a single dose of prednisolone at 2 mg/kg in the morning on alternate days for 2 weeks, 1 mg/kg on alternate days for the next 2 weeks, and 0.5 mg/kg on alternate days for a final 2 weeks. Children who then met the criteria for FRNS received cyclosporine by the same protocol as in the original trial (2), or cyclophosphamide (2.5 mg/kg per day) for 8–12 weeks if cyclosporine nephrotoxicity was found on renal biopsy. Use of antihypertensive drugs was not restricted, including angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers. Data were collected from the participating institutions for analysis at 1-year intervals, and included body weight and height, BP, medications given, blood analysis (complete blood cell count, blood chemistry), and urine tests (urinalysis, quantitative proteinuria).

The design and execution of this study were in accordance with the ethical standards of the Declaration of Helsinki. The protocol was approved by the ethics committee of Tokyo Metropolitan Kiyose Children's Hospital (predecessor of Tokyo Metropolitan Children's Medical Center). Informed consent was obtained from all patients or their parents. This study was registered in the University Hospital Medical Information Network public trials registry (ID C000000010; http://www.umin.ac.jp/ctr/index.htm).

Criteria and Definitions

The criteria for and definitions of nephrotic syndrome, remission, and relapse were in accordance with the International Study of Kidney Disease in Children (17). FRNS was defined as ≥2 relapses of nephrotic syndrome within 6 months after the initial episode, ≥3 within any 6-month period, or ≥4 within any 12-month period. Furthermore, FRNS was defined as including SDNS and any case requiring the administration of an immunosuppressant (cyclosporine or cyclophosphamide). Steroid dependence was defined as the occurrence of two consecutive relapses during tapering of the steroid dosage or within 14 days after the cessation of administration. Renal function was evaluated as estimated GFR (eGFR) calculated using the Chronic Kidney Disease in Children study equation [0.413 × (height/serum creatinine)] (18).

Statistical Analyses

Kaplan–Meier analyses for relapse from the complete discontinuation of cyclosporine were conducted in children who had discontinued cyclosporine without the occurrence of FRNS during tapering. Analyses included time to first relapse and time to the regression to FRNS. Differences between groups were compared using the log-rank test. Risk ratio and 95% confidence interval (CI) for first recurrence and for regression to FRNS after discontinuation of cyclosporine were evaluated by Poisson regression analysis, using the explanatory variables of group (patients without [group A] and with [group B] relapse during cyclosporine treatment), age (<6 years and ≥6 years), and history of steroid dependence before the start of cyclosporine treatment. Sandwich variance was used to handle the overdispersion problem. Relapses occurring during tapering were excluded from the primary analysis of relapse-free survival, with only those occurring after the complete discontinuation of cyclosporine counted as events in the Kaplan–Meier and Poisson analyses. Patients administered cyclosporine or cyclophosphamide by their attending physician were considered to have regressed to FRNS regardless of whether they fulfilled the criteria of FRNS. All statistical analyses were performed using the SAS software package for Windows (release 9.13; SAS Institute Inc, Cary, NC). A two-sided P value <0.05 was considered statistically significant in all analyses.

Results

Data Set

Of the 62 children with FRNS in our previous study who received cyclosporine for 2 years (2), 7 regressed to FRNS during protocol treatment. Cyclosporine in these children was discontinued and their data were excluded from the present analyses. After exclusion of 6 other children, 4 due to loss to follow-up and 2 due to protocol violation (refusal to discontinue cyclosporine), this study followed and analyzed a total of 49 children (median age at complete discontinuation of cyclosporine, 6.5 years; 39 male patients) (Figure 1). Although follow-up was set at 24 months, one patient each was censored at 16, 19, and 21 months due to loss to follow-up, and six patients completed 2-year follow-up at 23 months. The participants were classified into two groups, with group A consisting of patients who had not experienced relapse during the 2-year cyclosporine treatment (n=32) and group B consisting of those who had (n=17). Basic characteristics of these children are shown in Table 1.

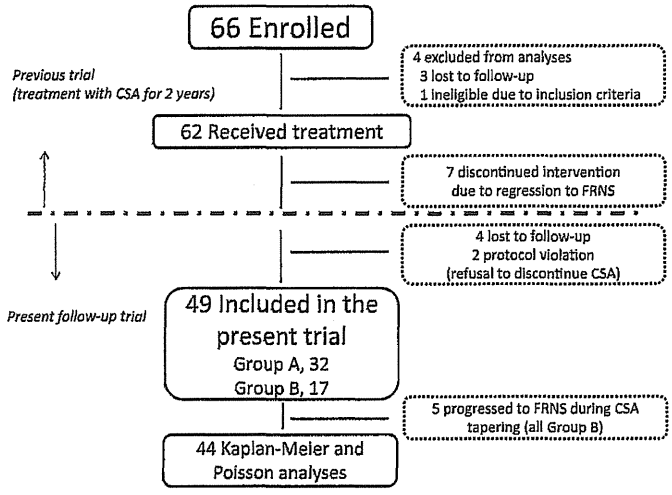

Figure 1. | Flow diagram. Group A included children without relapse during the initial cyclosporine treatment, whereas group B included children with relapse during the initial cyclosporine treatment. CSA, cyclosporine; FRNS, frequently relapsing nephrotic syndrome.

Table 1. Background characteristics of study participants

	All Participants			Participants Succeeding in Discontinuing CSA		
	Group		All	Group		All
	A	B		A	B	
Number of participants	32	17	49	32	12	44
Sex						
Male	24	15	39	24	10	34
Female	8	2	10	8	2	10
Age (yr)						
0<6	12	5	17	12	4	16
6–12	17	6	23	17	6	23
≥12	3	6	9	3	2	5
Median (IQR)	6.4 (5.3–8.5)	7.4 (5.9–12.1)	6.5 (5.5–9.6)	6.4 (5.3–8.5)	6.5 (5.8–10.3)	6.4 (5.4–8.5)
Steroid dependence						
No	16	7	23	16	6	22
Yes	16	10	26	16	6	22
Discontinuation of CSA						
Successful	32	12	44	32	12	44
Failed	0	5	5			
CSA does tapering period (d)						
Median (IQR)				68 (45–94)	77 (63–112)	69 (48–97)
Relapse in the tapering period						
No				31	9	40
Yes				1	3	4

CSA, cyclosporine; IQR, interquartile range.

Probability of Relapse-Free and Regression-Free Survival

The median (interquartile range [IQR]) cyclosporine dose tapering period was 68 (45–94) days in group A and 77 (63–112) days in group B (Table 1). Discontinuation of cyclosporine failed in five participants due to the regression of FRNS during tapering, all of whom belonged to group B. Kaplan–Meier and Poisson analyses were conducted in the remaining 44 children (group A, n=32; group B, n=12).

Thirty-seven children experienced relapse during the follow-up period. The median (IQR) time from the complete cessation of cyclosporine to relapse was 4.3 (1.5–15.6) months for group A and 0.5 (0.0–1.1) months for group B

(Table 2). In group A, 6 patients (19%) did not experience a relapse, 9 (28%) had infrequent relapses, and 17 (53%) regressed to FRNS after the discontinuation of cyclosporine. In group B, one patient (8.3%) did not experience a relapse, two (16.7%) had infrequent relapses, and nine (75%) regressed to FRNS after discontinuation. Time to regression to FRNS was 16.6 months for group A and 3.8 months for group B. The probability of relapse-free survival at 24 months from complete discontinuation was 15.3% in all children (Figure 2) and that of regression to FRNS-free survival was 40.8% (Figure 3). By group, the probability of relapse-free survival was significantly higher in group A (17.9%) than in group B (8.3%) ($P<0.001$, Figure 2).

Factors Associated with Relapse

The recurrence rates were 0.089 per month (26 of 292.7 months) for group A and 0.30 per month (11 of 36.3 months) for group B, whereas the rate of regression to FRNS was 0.034 per month (17 of 506.2 months) and 0.074 per month (9 of 122.4 months), respectively. Results of Poisson regression are shown in Table 3. Controlling for age and steroid dependence at the beginning of cyclosporine treatment, the risk ratio of group A to B for first recurrence was 0.17 (95% CI, 0.04–0.72; $P=0.02$). Similar results were obtained for the risk of regression to FRNS. The risk ratio for group A compared with group B was 0.35 (95% CI, 0.15–0.83; $P=0.02$).

Patient Survival, Renal Survival, and Growth

Information regarding patient survival and renal survival was available for 58 of 62 patients who received protocol treatment with cyclosporine. No lethal event was seen. Mean eGFR was 140.9±31.6 ml/min per 1.73 m^2 ($n=62$, no patient with eGFR <90 ml/min per 1.73 m^2) at

Table 2. Number of participants who experienced relapse and time to relapse

Group	Total, *n*	Children Who Experienced Relapse, *n* (%)	Time to First Relapse (mo), Median (IQR)	Children Who Regressed to FRNS, *n* (%)	Time to FRNS (mo), Median (IQR)
A	32	26 (81)	4.3 (1.5–15.6)	17 (53)	16.6 (7.5)
B	12	11 (92)	0.5 (0.0–1.1)	9 (75)	3.8 (3.1–11.1)
All	44	37 (84)	2.0 (0.7–13.3)	26 (59)	10.2 (4.4)

FRNS, frequently relapsing nephrotic syndrome; IQR, interquartile range.

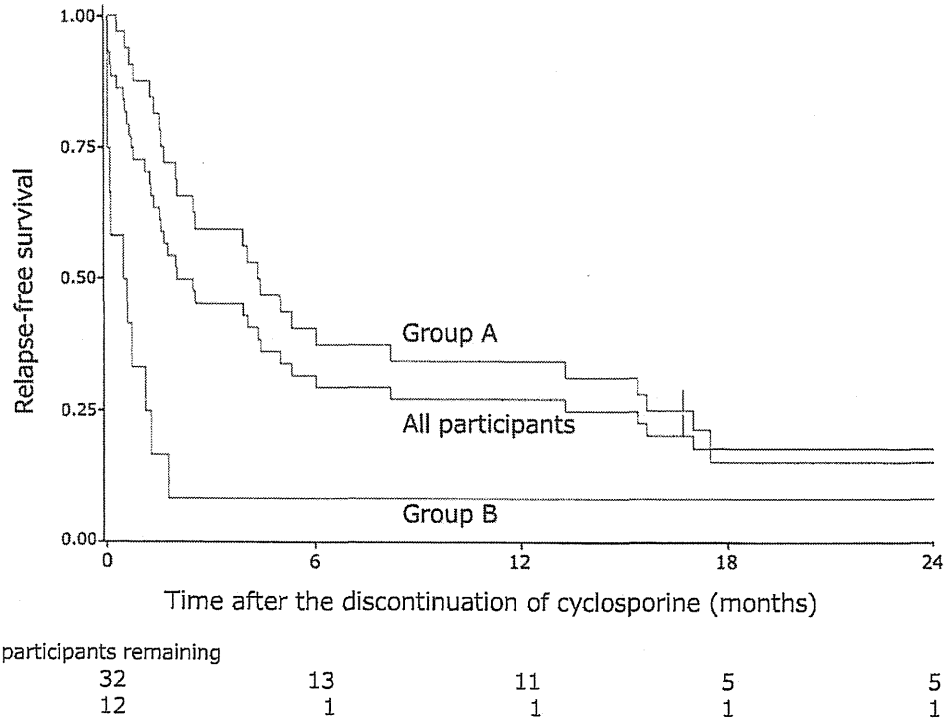

Figure 2. | Relapse after the discontinuation of cyclosporine. Relapse-free survival probability at 2 years was 15.3% in all participants, and 17.9% in those without (group A) and 8.3% in those with (group B) relapse during cyclosporine treatment ($P<0.001$, log-rank test).

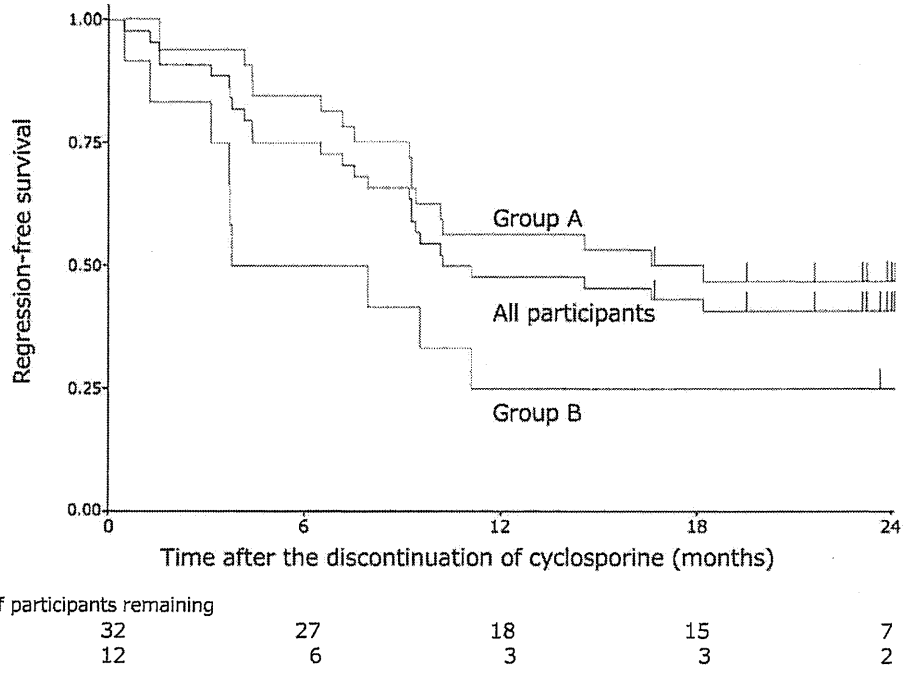

Figure 3. | Regression to FRNS after discontinuation of cyclosporine. Regression (to FRNS)–free survival probability at 2 years was 40.8% in all participants, and 46.7% in those without (group A) and 25.0% in those with (group B) relapse during cyclosporine treatment (P=0.05, log-rank test). FRNS, frequently relapsing nephrotic syndrome.

Table 3. Poisson regression analyses for first recurrence (n=44)

Parameter	Risk Ratio	95% Confidence Interval	P
Group			
A	0.17	0.04–0.72	0.02
B	1.00	—	
Steroid dependence			
Yes	0.31	0.11–0.88	0.03
No	1.00	—	
Age at the start of follow-up (yr)			
≤6	1.23	0.44–3.40	0.70
>6	1.00	—	

initial study entry (beginning of cyclosporine treatment), 143.5±27.7 ml/min per 1.73 m^2 (n=58, again no patient with eGFR <90) at the end of cyclosporine treatment, and 132.1±23.9 ml/min per 1.73 m^2 (n=36, eGFR of one patient in group A was <90 [87.8 ml/min per 1.73 m^2]) in the children analyzed in this study. One patient (group A) progressed to SRNS during the follow-up period, but finally remitted with cyclosporine. Mean (±SD) score for body height was −0.27±1.01 (n=62) at initial study entry, 0.27±0.97 (n=58) at the end of cyclosporine treatment, and 0.15±1.09 (n=42) at the end of follow-up.

Adverse Events of Steroids and Immunosuppressants

Other adverse events attributable to corticosteroids and immunosuppressants at the end of follow-up are shown in Table 4. Hypertension, defined as a requirement for antihypertensive agents during the trial, was seen in 3 of 49 (6.1%) patients but was manageable, and no severe sequelae of hypertension such as encephalopathy, seizures, or cardiac dysfunction were detected.

Discussion

In this follow-up study of patients who had completed 2-year protocol treatment with cyclosporine for the treatment of FRNS, we found that the relapse rate was high, with approximately 60% of patients regressing to FRNS within 2 years of cessation. Risk of relapse of nephrotic syndrome and regression to FRNS were higher in those who had experienced relapse during the 2-year treatment with cyclosporine. These findings indicate that patients experiencing relapse of nephrotic syndrome during treatment with cyclosporine are at high risk of relapse after discontinuation of the drug. To our knowledge, this is the first prospective study to identify the risk of relapse in children with nephrotic syndrome treated with cyclosporine.

The effectiveness and limitations of cyclosporine in children with FRNS have been reported. Several regimens have been tried, which differed with regard to target blood cyclosporine concentration and duration of administration (5–8,19), and although these showed certain efficacy for controlling relapse with varying degrees of adverse events, including nephrotoxicity, these studies did not provide

Table 4. Adverse events

	All Participants			Participants Succeeding in Discontinuing CSA		
	Group		All	Group		All
	A	B		A	B	
Number of participants	32	17	49	32	12	44
Adverse reaction						
Yes	2	4	6	2	4	6
No	30	13	43	30	8	38
Gastrointestinal discomfort	1	2	3	1	2	3
Hypertension	0	3	3	0	3	3
Osteoporosis	0	2	2	0	2	2
Cataract	0	1	1	0	1	1
Obesity	1	0	1	1	0	1
Adrenal suppression	0	1	1	0	1	1

CSA, cyclosporine.

definitive information on treatment dosage or duration. To better define the optimal dosage and duration of treatment with cyclosporine, we conducted a randomized controlled trial with cyclosporine (Sandimmune; Novartis, Basel, Switzerland) (1), followed by a prospective multicenter trial with microemulsified cyclosporine (Neoral) (2), on which the present follow-up study was based. These two trials helped establish that 2-year treatment with cyclosporine under trough control is effective and safe in children with FRNS. Nevertheless, the frequent relapse seen in patients after the cessation of cyclosporine remained of concern.

In this study, most children with FRNS experienced relapse within a few months after the 2-year protocol treatment with cyclosporine was discontinued. These findings strongly suggest that the current 2-year administration period for cyclosporine in the long-term management of children with FRNS is insufficient. The 2-year period was originally established on the basis of previous findings of an increased risk of nephrotoxicity when administration was extended beyond 2 years (20). However, our more recent prospective finding that only 5 of 58 children experienced biopsy-proven nephrotoxicity (mostly mild arteriolar hyalinosis and mild interstitial fibrosis) indicates the good safety of cyclosporine for 2 years (2). In addition, the present high prevalence of relapse after complete discontinuation indicates that the initial treatment period may be extended beyond 2 years, with consideration to the advisability of repeated renal biopsy at 2- to 3-year intervals (9) and relatively lower doses of cyclosporine (8).

Risk factors for relapse after the complete discontinuation of cyclosporine were also evaluated. Comparison of relapse-free survival between those who did (group B) and did not (group A) relapse during the 2-year treatment showed a significantly greater risk in those who did relapse. Similarly, group B had a significantly greater risk of regression to FRNS than group A. These results indicate that the experience of relapse during cyclosporine treatment predicts the course after its discontinuation. Furthermore, they may also suggest the necessity of extending the cyclosporine treatment period beyond 2 years, or a change in immunosuppressive therapy after 2-year treatment with cyclosporine.

We also analyzed other factors with a possible association with relapse, namely age at the beginning of observation and steroid dependency before treatment with cyclosporine. Contrary to our expectation and the results of previous reports (13,14,16), age at the beginning of observation was not significantly associated with either relapse or the regression of FRNS. Similarly, nonsignificant results were also obtained using age at the onset of nephrotic syndrome instead of age at the beginning of observation (data not shown). Moreover, steroid dependency was not a risk factor for relapse or regression to FRNS. The trend is nevertheless consistent with our previous finding that relapse during cyclosporine treatment was unrelated to steroid dependence (2). To note, however, four of five children who were excluded from analysis due to relapse during tapering were steroid dependent; inclusion of these children would have strengthened the association between steroid dependency and risk. In six patients (three each in group A and B), mizoribine, an agent in the same antimetabolite class as mycophenolate mofetil whose efficacy is mild and limited to patients aged ≤10 years (21), was administered during follow-up to prevent regression to FRNS (data not shown). On inclusion in Poisson analyses, administration of mizoribine was positively associated with relapse. Administration was at the discretion of the physician in charge and may have been administered early in those at risk of relapse.

Consistent with a previous report (15), the life expectancy and renal prognosis of our patients were excellent, supporting cyclosporine's role as first-line treatment in children with FRNS. No lethal events occurred during the approximately 4 years from the beginning of cyclosporine treatment in any participant, and only one patient developed a very mild decrease in renal function. Growth in terms of height was also maintained or slightly improved during the 4 years in spite of relapses and the

administration of prednisolone. Other adverse events attributable to corticosteroids and immunosuppressants were also acceptable. This may have been partly due to our prednisolone protocol for relapse, which moves relatively quickly from daily administration to administration every second day.

FRNS has been shown to be a chronic condition (22,23) which requires a long-term strategy with several immunosuppressants and prednisolone (24). Whereas the efficacy of cyclosporine remains clear, we speculate that in addition to prolonged or repeated administration of cyclosporine, the combination of several immunosuppressants given in sequence should also be considered. Recent attention has focused on the efficacy of several emerging immunosuppressants, including tacrolimus (24,25) and rituximab (24,26), in controlling relapse, and both are now under evaluation in children with FRNS in a multicenter, prospective, randomized fashion in Japan. In addition to the control of relapse, long-term management must also consider any adverse effects of therapy as well as the growth and quality of life of children.

Several strengths of this study warrant mention. First, the study is the first multicenter, prospective trial to evaluate the clinical course of children with FRNS after the discontinuation of cyclosporine. Second, the number of participants was relatively large. Several limitations also warrant mention. First, although protocols for the administration of immunosuppressants and prednisolone after treatment with cyclosporine were provided, treatment was at the discretion of the physician in charge. Immunosuppressants were occasionally started before criteria for the diagnosis of FRNS or SDNS had been met, and mizoribine to prevent relapse was sometimes started very early, even before the first relapse, owing to its low incidence of adverse events (21). Second, several data points were missing, including some measurements of height and serum creatinine, particularly among stable children. Third, because of the small sample size, several factors potentially associated with relapse were not examined in Poisson regression analysis. Fourth, five patients in group B who failed in tapering were excluded from analysis. Findings for group B would have been worse if these cases had been included.

In conclusion, we found that children with FRNS who received cyclosporine were at high risk of relapse after its discontinuation. This effect was particularly large in those who experienced relapse during cyclosporine treatment. Given that overall prognosis in terms of life expectancy and renal survival in these children was excellent, long-term strategies should be formulated in consideration of not only relapse, but also adverse effects of treatment, as well as growth and quality of life.

Acknowledgments

The authors thank Drs. Y. Akioka (Tokyo), M. Awazu (Tokyo), A. Furuse (Kumamoto), S. Fujinaga (Saitama), M. Goto (Tokyo), R. Hamada (Tokyo), K. Hamahira (Hyogo), Y. Hamasaki (Tokyo), T. Harada (Kanagawa), K. Hatae (Fukuoka), H. Hataya (Tokyo), M. Hattori (Tokyo), S. Hattori (Kumamoto), M. Hiramatsu (Oita), T. Igarashi (Tokyo), M. Ikeda (Tokyo), S. Kagami (Tokushima), Y. Kaku (Fukuoka), K. Kamei (Tokyo), S. Kodama (Kagoshima), T. Konomoto (Miyazaki), Y. Mishuku (Kanagawa), N. Nakanishi (Osaka), F. Niimura (Kanagawa), K. Nozu (Hyogo), R. Ochiai (Tokyo), Y. Otsuka (Saga), Y. Owada (Tochigi), M. Sako (Tokyo), T. Sato (Saga), H. So (Kagoshima), R. Tanaka (Hyogo), Y. Tanaka (Saitama), H. Wakaki (Tokyo), and K. Yoshidome (Kagoshima) of the Japanese Study Group of Renal Disease in Children for their contributions to this study.

This study was supported in part by the Kidney Foundation, Japan.

The results presented in this article have not been published previously in whole or part, except in abstract form at the 42nd Annual Meeting of the American Society of Nephrology, October 27–November 1, 2009, in San Diego, California.

Disclosures

N.Y. has received research grants from Novartis, Japan.

References

1. Ishikura K, Ikeda M, Hattori S, Yoshikawa N, Sasaki S, Iijima K, Nakanishi K, Yata N, Honda M: Effective and safe treatment with cyclosporine in nephrotic children: A prospective, randomized multicenter trial. *Kidney Int* 73: 1167–1173, 2008
2. Ishikura K, Yoshikawa N, Hattori S, Sasaki S, Iijima K, Nakanishi K, Matsuyama T, Yata N, Ando T, Honda M; for Japanese Study Group of Renal Disease in Children: Treatment with microemulsified cyclosporine in children with frequently relapsing nephrotic syndrome. *Nephrol Dial Transplant* 25: 3956–3962, 2010
3. Niaudet P, Reigneau O, Humbert H: A pharmacokinetic study of Neoral in childhood steroid-dependent nephrotic syndrome. *Pediatr Nephrol* 16: 154–155, 2001
4. Gregory MJ, Smoyer WE, Sedman A, Kershaw DB, Valentini RP, Johnson K, Bunchman TE: Long-term cyclosporine therapy for pediatric nephrotic syndrome: A clinical and histologic analysis. *J Am Soc Nephrol* 7: 543–549, 1996
5. Tanaka R, Yoshikawa N, Kitano Y, Ito H, Nakamura H: Long-term ciclosporin treatment in children with steroid-dependent nephrotic syndrome. *Pediatr Nephrol* 7: 249–252, 1993
6. Inoue Y, Iijima K, Nakamura H, Yoshikawa N: Two-year cyclosporin treatment in children with steroid-dependent nephrotic syndrome. *Pediatr Nephrol* 13: 33–38, 1999
7. Kitano Y, Yoshikawa N, Tanaka R, Nakamura H, Ninomiya M, Ito H: Ciclosporin treatment in children with steroid-dependent nephrotic syndrome. *Pediatr Nephrol* 4: 474–477, 1990
8. El-Husseini A, El-Basuony F, Mahmoud I, Sheashaa H, Sabry A, Hassan R, Taha N, Hassan N, Sayed-Ahmad N, Sobh M: Long-term effects of cyclosporine in children with idiopathic nephrotic syndrome: A single-centre experience. *Nephrol Dial Transplant* 20: 2433–2438, 2005
9. Cattran DC, Alexopoulos E, Heering P, Hoyer PF, Johnston A, Meyrier A, Ponticelli C, Saito T, Choukroun G, Nachman P, Praga M, Yoshikawa N: Cyclosporin in idiopathic glomerular disease associated with the nephrotic syndrome: Workshop recommendations. *Kidney Int* 72: 1429–1447, 2007
10. Hulton SA, Neuhaus TJ, Dillon MJ, Barratt TM: Long-term cyclosporin A treatment of minimal-change nephrotic syndrome of childhood. *Pediatr Nephrol* 8: 401–403, 1994
11. Hino S, Takemura T, Okada M, Murakami K, Yagi K, Fukushima K, Yoshioka K: Follow-up study of children with nephrotic syndrome treated with a long-term moderate dose of cyclosporine. *Am J Kidney Dis* 31: 932–939, 1998
12. Kemper MJ, Kuwertz-Broeking E, Bulla M, Mueller-Wiefel DE, Neuhaus TJ: Recurrence of severe steroid dependency in cyclosporin A-treated childhood idiopathic nephrotic syndrome. *Nephrol Dial Transplant* 19: 1136–1141, 2004
13. Fakhouri F, Bocquet N, Taupin P, Presne C, Gagnadoux MF, Landais P, Lesavre P, Chauveau D, Knebelmann B, Broyer M, Grünfeld JP, Niaudet P: Steroid-sensitive nephrotic syndrome: From childhood to adulthood. *Am J Kidney Dis* 41: 550–557, 2003
14. Kabuki N, Okugawa T, Hayakawa H, Tomizawa S, Kasahara T, Uchiyama M: Influence of age at onset on the outcome of steroid-sensitive nephrotic syndrome. *Pediatr Nephrol* 12: 467–470, 1998
15. Rüth EM, Kemper MJ, Leumann EP, Laube GF, Neuhaus TJ: Children with steroid-sensitive nephrotic syndrome come of age: Long-term outcome. *J Pediatr* 147: 202–207, 2005

16. Niaudet P: Long-term outcome of children with steroid-sensitive idiopathic nephrotic syndrome. *Clin J Am Soc Nephrol* 4: 1547–1548, 2009
17. Early identification of frequent relapsers among children with minimal change nephrotic syndrome. A report of the International Study of Kidney Disease in Children. *J Pediatr* 101: 514–518, 1982
18. Schwartz GJ, Muñoz A, Schneider MF, Mak RH, Kaskel F, Warady BA, Furth SL: New equations to estimate GFR in children with CKD. *J Am Soc Nephrol* 20: 629–637, 2009
19. Fujinaga S, Ohtomo Y, Someya T, Shimizu T, Yamashiro Y, Kaneko K: Is single-daily low-dose cyclosporine therapy really effective in children with idiopathic frequent-relapsing nephrotic syndrome? *Clin Nephrol* 69: 84–89, 2008
20. Iijima K, Hamahira K, Tanaka R, Kobayashi A, Nozu K, Nakamura H, Yoshikawa N: Risk factors for cyclosporine-induced tubulointerstitial lesions in children with minimal change nephrotic syndrome. *Kidney Int* 61: 1801–1805, 2002
21. Yoshioka K, Ohashi Y, Sakai T, Ito H, Yoshikawa N, Nakamura H, Tanizawa T, Wada H, Maki S: A multicenter trial of mizoribine compared with placebo in children with frequently relapsing nephrotic syndrome. *Kidney Int* 58: 317–324, 2000
22. Kyrieleis HA, Levtchenko EN, Wetzels JF: Long-term outcome after cyclophosphamide treatment in children with steroid-dependent and frequently relapsing minimal change nephrotic syndrome. *Am J Kidney Dis* 49: 592–597, 2007
23. Esfahani ST, Madani A, Asgharian F, Ataei N, Roohi A, Moghtaderi M, Rahimzadeh P, Moradinejad MH: Clinical course and outcome of children with steroid-sensitive nephrotic syndrome. *Pediatr Nephrol* 26: 1089–1093, 2011
24. van Husen M, Kemper MJ: New therapies in steroid-sensitive and steroid-resistant idiopathic nephrotic syndrome. *Pediatr Nephrol* 26: 881–892, 2011
25. Morgan C, Sis B, Pinsk M, Yiu V: Renal interstitial fibrosis in children treated with FK506 for nephrotic syndrome. *Nephrol Dial Transplant* 26: 2860–2865, 2011
26. Prytuła A, Iijima K, Kamei K, Geary D, Gottlich E, Majeed A, Taylor M, Marks SD, Tuchman S, Camilla R, Ognjanovic M, Filler G, Smith G, Tullus K: Rituximab in refractory nephrotic syndrome. *Pediatr Nephrol* 25: 461–468, 2010

Received: January 5, 2012 **Accepted:** June 25, 2012

Published online ahead of print. Publication date available at www.cjasn.org.

Pediatr Nephrol (2013) 28:765–771
DOI 10.1007/s00467-012-2393-4

ORIGINAL ARTICLE

Prospective 5-year follow-up of cyclosporine treatment in children with steroid-resistant nephrosis

Yuko Hamasaki · Norishige Yoshikawa · Hitoshi Nakazato · Satoshi Sasaki · Kazumoto Iijima · Koichi Nakanishi · Takeshi Matsuyama · Kenji Ishikura · Shuichi Ito · Tetsuji Kaneko · Masataka Honda · for Japanese Study Group of Renal Disease in Children

Received: 20 May 2012 / Revised: 7 December 2012 / Accepted: 7 December 2012 / Published online: 13 January 2013

Abstract
Background Cyclosporine has improved remission rates in children with steroid-resistant nephrotic syndrome (SRNS). However, little prospective long-term follow-up data is available.
Methods We prospectively followed and analyzed 5-year outcomes of all 35 patients enrolled in our previous prospective multicenter trial with cyclosporine and steroids in children with SRNS. At enrollment, 23 cases were classified as minimal change (MC), five as diffuse mesangial proliferation (DMP), and seven as focal segmental glomerulosclerosis (FSGS).
Results Renal survival at 5 years (median 7.7 years) was 94.3 %. Patient status was complete remission (CR) in 31 (88.6 %) (MC/DMP, 25; FSGS, 6); partial remission in one (FSGS); and non-remission in three (MC/DMP), including chronic kidney disease and end-stage kidney disease in one each. Among 31 patients with CR, 22 (71.0 %) were receiving treatment with immunosuppressants at 5 years, including cyclosporine in 19, and seven of these 22 continued to show frequent relapse. Response to cyclosporine at 4 months predicted 5-year outcome in 31 of 35 patients.
Conclusions Although SRNS treatment with cyclosporine provides high renal survival and remission rates, many children require ongoing immunosuppression. Management has advanced from the prevention of end-stage kidney disease to the long-term maintenance of remission and management of relapse after induction therapy.

Keywords Steroid resistance · Nephrotic syndrome · Pediatric nephrology · Cyclosporine · Long-term outcome

Introduction

The use of cyclosporine has revolutionized the treatment of steroid-resistant nephrotic syndrome (SRNS) in children, and outcomes have gradually improved over the last

Y. Hamasaki · K. Ishikura · M. Honda
Department of Nephrology,
Tokyo Metropolitan Children's Medical Center,
Fuchu, Japan

Y. Hamasaki (✉)
Department of Pediatric Nephrology,
Toho University Omori Medical Center,
6-11-1, Omori-Nishi, Ota-ku, Tokyo 143-8541, Japan
e-mail: yuhamasaki@med.toho-u.ac.jp

N. Yoshikawa · K. Nakanishi
Department of Pediatrics, Wakayama Medical University,
Wakayama, Japan

H. Nakazato
Department of Pediatrics, Faculty of Life Sciences,
Kumamoto University, Kumamoto, Japan

S. Sasaki
Department of Pediatrics, Hokkaido University Graduate
School of Medicine, Sapporo, Japan

K. Iijima
Department of Pediatrics,
Kobe University Graduate School of Medicine, Kobe, Japan

T. Matsuyama
Department of Pediatrics, Fussa Hospital, Fussa, Japan

S. Ito
Division of Nephrology and Rheumatology, National Center
for Child Health and Development, Setagaya-ku, Japan

T. Kaneko
Department of Clinical Research, Tokyo Metropolitan
Children's Medical Center, Fuchu, Japan

15 years. In particular, Waldo et al. [1] and Ehrich et al. [2, 3] achieved remission rates of around 80 %, and the recent Cochrane review [4] confirmed the adoption of cyclosporine as the first-line treatment. Most studies of the safety and efficacy of cyclosporine to date have been retrospective, however, and the few prospective studies that have appeared have been short-term ones. No prospective long-term data on the safety and efficacy of cyclosporine for children with SRNS have yet appeared.

Our previous prospective multicenter trial in children with SRNS also obtained high renal survival and remission rates with cyclosporine and steroid therapy. This study was limited to 12-month protocol treatment [5], however, and therefore provided no prospective information on long-term outcome in these patients. The availability of prospective long-term data would confirm these 12-month findings, and validate the fundamental shift in treatment of this disease from the prevention of end-stage kidney disease (ESKD) to the long-term maintenance of remission and management of relapse after induction therapy.

Here, to better understand the long-term safety and efficacy of cyclosporine in children with SRNS, we evaluated outcomes in patients of our previous prospective study at 5 years after enrollment.

Methods

Previous trial

The present study analyzed all 35 patients enrolled in the previous trial, consisting of a consecutive series of 21 boys and 14 girls with a median age of 2.7 years at enrollment between January 2001 and December 2007. Entry criteria are described in the previous report [5]. Nephrotic syndrome was diagnosed based on a urinary protein/creatinine ratio of $\geq$ 1.8 mg/mg and serum albumin level of $\leq$ 2.5 g/dl. SRNS was diagnosed if remission was not achieved (serum albumin level $\leq$ 2.5 g/dl) after treatment with 2 mg/kg prednisolone daily in three divided doses for 4 weeks. Renal biopsy in all patients at enrollment showed minimal change (MC) in 23, diffuse mesangial proliferation (DMP) in five, and focal segmental glomerulosclerosis (FSGS) in seven. All patients received 12-month treatment with cyclosporine at a trough level of 120–150 ng/ml for 3 months, followed by 80–100 ng/ml for 9 months; and prednisolone at 1 mg/kg/day for 4 weeks, followed by 1 mg/kg every second day for months 2–12. Those with FSGS additionally received methylprednisolone pulse therapy (MPT) at 30 mg/kg on 3 consecutive days in weeks 1, 2, 5, 9, and 13. For patients with MC/DMP who did not achieve remission within 4 months, treatment was restarted with the regimen of the FSGS group (cyclosporine, prednisolone, and MPT). Patients with FSGS who did not achieve remission within 4 months were given off-protocol treatment selected at the discretion of the physician. Patients who progressed to SRNS or frequently relapsing nephrotic syndrome (FRNS) after once achieving remission also received off-protocol treatment.

Follow-up study

Treatment of nephrotic syndrome after expiration of the 12-month protocol treatment was at the discretion of the attending physician. However, we recommended that patients who experienced relapse of SRNS receive the previous protocol treatment again, and that while those who had progression to FRNS received cyclosporine therapy [6] or cyclophosphamide. Treatment with cyclosporine was conducted under mainly trough and occasionally C2 control.

Five-year follow-up data were available for all 35 patients, including analysis of outcomes, treatment for nephrotic syndrome, and adverse events. Outcome at year 5 was classified as complete remission (CR), partial remission (PR), or non-remission. CR was defined as negative or trace proteinuria (dipstick method or urinary protein/creatinine ratio $\leq$ 0.20 mg/mg) on urinalysis and a serum albumin level of > 2.5 g/dl. CR was also considered to include cases of steroid-sensitive nephrotic syndrome, including cases with no relapse, infrequent relapse, and frequent relapse, with the latter defined as three relapses within any 6-month period or four or more relapses within any 12-month period. PR was defined as a serum albumin level > 2.5 g/dl but persistent proteinuria (dipstick method +1 or greater; or urinary protein/creatinine ratio > 0.20 mg/mg) on urinalysis. Stage 3 or greater chronic kidney disease (CKD) was defined as estimated GFR (eGFR) < 60 ml/min/1.73 m^2, and ESKD as the requirement for dialysis or kidney transplantation.

Data were obtained from the participating institutions (n=14) for analysis at 1-year intervals, and included body weight and height, blood pressure, blood analysis (complete blood cell count, blood chemistry), urine tests (urinalysis, quantitative proteinuria), and eGFR as determined by the Schwartz equation [7]. Hypertension was defined as the need for antihypertensive medication, including angiotensin-converting enzyme (ACE) inhibitors or angiotensin-receptor blockers (ARB), except when given for renoprotective purposes. Growth was evaluated from height measured at the most recent examination, and expressed as the height standard deviation score. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the primary investigator's institution. Informed consent was obtained from the parents before the start of study procedures. This study has been registered in a public trials registry, the University