Table 1 Annual incidence and primary renal disease of ESKD in Japan in 2009

Primary kidney diseases	Dialysis patients		Pre-emptive transplantation		Total	
	Number	%	Number	%	Number	%
Chronic glomerulonephritis	8,228	22.0	41	40.6	8,269	22.1
Diabetes	16,827	45.0	11	10.9	16,838	44.9
Nephrosclerosis	4,055	10.9	1	1.0	4,056	10.8
Polycystic kidney	873	2.3	7	6.9	880	2.3
Rapidly progressive glomerulonephritis	466	1.2	0	0.0	466	1.2
Others	6,917	18.5	41	40.6	6,958	18.6
Total	37,365	100.0	101	100.0	37,466	100.0

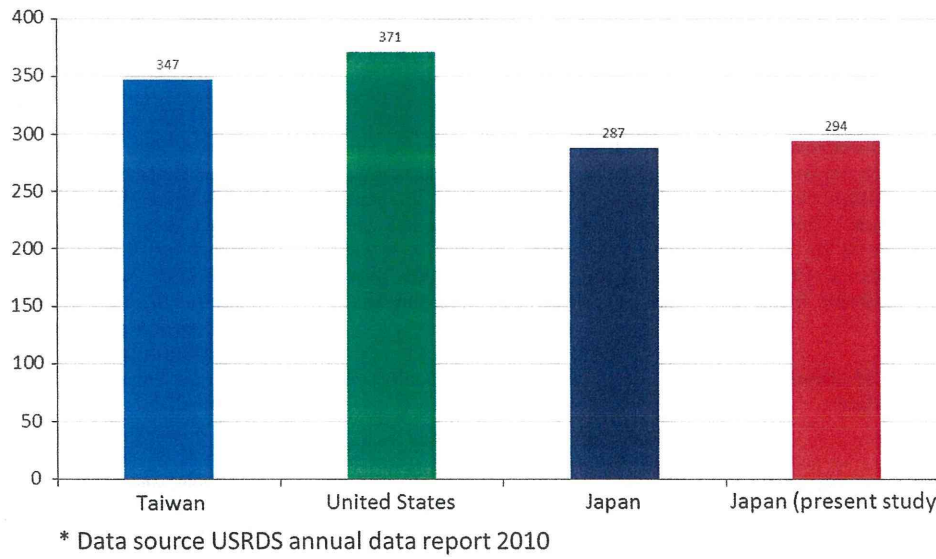

Fig. 9 Comparison of annual incidence of ESKD in 2009 among USA, Taiwan, and Japan. From the USRDS international comparison data, annual incidence of ESKD in Japan was 287 per million population, after our estimation, it was 294 per million population. The order of these countries was the same

Table 2 Prevalence and primary kidney disease of ESKD in Japan at Dec 31, 2007

Primary kidney diseases	Dialysis patients		Transplanted patients		Total ESKD	
	Estimated number	%	Number	%	Estimated number	%
Chronic glomerulonephritis	111,098	40.40	5,879	58.71	116,977	41.01
Diabetes	91,892	33.40	505	5.04	92,397	32.39
Nephrosclerosis	17,850	6.50	105	1.05	17,955	6.29
Polycystic kidney	9,287	3.40	252	2.52	9,539	3.34
Rapidly progressive glomerulonephritis	1,814	0.70	0	0.00	1,814	0.64
Others	43,301	15.70	3,272	32.68	46,573	16.33
Total	275,242		10,013	100.00	285,255	100.00

kidney transplantation in this year. In total, there were 37,466 patients who newly required RRT in 2009. Not only the average ages, but also the primary kidney diseases of the new ESRD patients in each RRT modality were quite different.

It is important to show the incidence and prevalence of the total ESKD population, including dialysis, KT, and predialysis ESKD patients, to determine an effective treatment and care strategy.

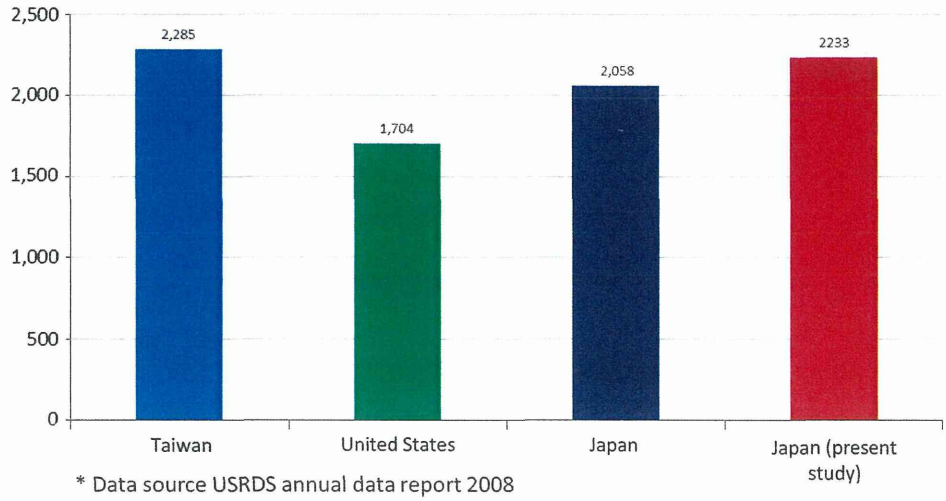

Fig. 10 Comparison of prevalence of ESKD at Dec 31, 2007, among USA, Taiwan, and Japan. Prevalence of ESKD at Dec 31, 2007 in Japan was 2,058 per million population. However, adding KT subjects, our final estimated total ESKD population in Japan as of Dec 31, 2007 was 2,233 per million population

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Conflict of interest The authors have declared that no Conflict of interest exists.

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ORIGINAL ARTICLE

Decreased glomerular filtration as the primary factor of elevated circulating suPAR levels in focal segmental glomerulosclerosis

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Abstract
Background Circulating factor(s) has been thought to be the underlying cause of focal segmental glomerulosclerosis (FSGS), and recent studies foster this idea by demonstrating increased soluble urokinase receptor (suPAR) levels in the serum of FSGS patients.
Methods To explore the possible contribution of suPAR in FSGS pathogenesis, we analyzed serum suPAR levels in 17 patients with FSGS and compared them with those in patients with steroid-sensitive nephrotic syndrome, chronic glomerulonephritis, or non-glomerular kidney diseases.
Results Serum suPAR levels in patients with FSGS were higher than those in patients with steroid-sensitive nephrotic syndrome or chronic glomerulonephritis, but not higher than those in patients with non-glomerular kidney diseases. suPAR levels negatively correlate with estimated glomerular filtration rate and were decreased after renal transplantation in patients with FSGS as well as in those with non-glomerular kidney diseases. Furthermore, 6 FSGS patients with post-transplant recurrence demonstrated that suPAR levels were not high during the recurrence.
Conclusions Based on our results, elevated suPAR levels in FSGS patients were attributed mainly to decreased glomerular filtration. These data warrant further analysis for involvement of possible circulating factor(s) in FSGS pathogenesis.

Keywords Soluble urokinase receptor · Focal segmental glomerulosclerosis · Post-transplant recurrence

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Introduction

Focal segmental glomerulosclerosis (FSGS) is a common pattern of histopathological findings seen in children with nephrotic syndrome, and it is one of the leading causes of end-stage renal disease (ESRD) [1]. The pathogenesis of primary FSGS remains largely unclear. Intrinsic defects, which affect the structure and function of glomerular epithelial cells called podocytes, have been found in some FSGS patients [2]. In most patients with sporadic FSGS, however, serological dysregulation has been thought to be the cause because FSGS recurs in 40 % patients after transplantation and can often be treated with therapeutic post-transplant plasmapheresis [1, 3]. Post-transplant recurrence is of great clinical importance because the recurrence of proteinuria after renal transplantation has a negative impact on graft survival [4]. Recently, a case of recovery from FSGS after retransplantation of an allograft that was failing in the first recipient because of recurrent primary FSGS was reported [5], further emphasizing clinical evidence of specific disease-causing circulating factor(s) in FSGS patients.

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A recent study demonstrated increased soluble urokinase receptor (suPAR) levels in the serum of FSGS patients [6, 7]. uPAR is a glycosylphosphatidylinositol (GPI)-anchored three-domain protein, which has been identified as being a cellular receptor for urokinase [8]. suPAR, its soluble form, is released into the circulation by cleavage of the GPI anchor [9]. suPAR is present under physiological conditions in the human blood, and elevated suPAR levels have also been reported in some malignant neoplasms or infectious diseases [10].

However, subsequent studies have provided conflicting results with regard to the association of serum suPAR levels with proteinuria and specific elevation of suPAR levels in FSGS patients [11–13]. Serum suPAR levels can also be affected by kidney function, complicating the issue [7, 12]. While suPAR levels at the time of post-transplant recurrence of FSGS are crucial for elucidating the role of suPAR in the pathogenesis of FSGS, serial suPAR levels across kidney transplantation have not been reported. Further, changes in suPAR levels by kidney transplantation in FSGS and other kidney diseases have also been unclear.

To determine the correlation of suPAR levels with FSGS activity, we measured suPAR levels in patients with primary FSGS and other kidney diseases across renal transplantation. In some cases, we analyzed circulating suPAR levels in the recurrent state after transplantation. The results demonstrated that circulating suPAR levels depend largely on renal function, and there was no disease-specific elevation of suPAR in FSGS compared with those in other kidney diseases with comparable estimated glomerular filtration rate (eGFR). Notably, suPAR levels were not elevated in the recurrent state after renal transplantation in the 6 FSGS cases analyzed. These data warrant further analysis for possible circulating factor(s) in FSGS pathogenesis.

Materials and methods

Samples

The study was approved by the ethics committee of the Tokyo Women's Medical University School of Medicine, the Tokyo University School of Medicine, and the Japanese Red Cross Nagoya Daini Hospital. Samples were retrieved from blood collected for tests performed for other medical purposes, but in which remaining blood volume was sufficient for our purposes. The samples were assigned codes to render them anonymous.

The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul, as outlined in the "Declaration of Istanbul on Organ Trafficking and Transplant Tourism."

Patient characteristics

We analyzed serum suPAR in 20 samples from 17 biopsy-proven FSGS patients (Table 1). Four patients were analyzed for NPHS2 mutations, and none had a causative mutation [14]. These 20 samples were obtained at the time when patients had not received kidney transplantation. Paired samples before and after kidney transplantation from 4 patients were also obtained. Paired samples before transplantation and during post-transplant recurrence were obtained from 2 FSGS patients (Table 2). From 4 other FSGS patients with post-transplant recurrence, a total of 6 heparinized samples obtained during the post-transplant recurrence were also analyzed (Table 2). Plasma suPAR levels or serum samples obtained after renal transplantation were not included in the statistical analysis in Tables 1 and 3 and Fig. 1, and are only shown in Fig. 2 and Table 2.

To compare suPAR levels of FSGS with those of other kidney diseases, suPAR levels in 26 serum samples from 20 patients with steroid-sensitive nephrotic syndrome (SSNS), 24 samples from 24 chronic glomerulonephritis (CGN) patients, and 24 samples from 24 patients with non-glomerular kidney diseases were also examined (Table 1).

Patients with steroid-sensitive nephrotic syndrome (SSNS group) exhibited normal kidney function, and none of them underwent kidney transplantation. Among 20 patients with SSNS, 7 had steroid-dependent NS and 5 had frequently relapsing NS. Seven samples were obtained when the patients had massive proteinuria.

Diagnoses of 24 patients with chronic glomerulonephritis (CGN group) included IgA nephropathy (n=12), Henoch–Schönlein purpura nephritis (n=3), membranoproliferative glomerulonephritis (n=3), and other diseases (n=6). None of this group underwent kidney transplantation.

In the non-glomerular kidney disease group, 11 patients exhibited hypo-/dysplastic kidney disease, and 2 patients were diagnosed with autosomal recessive polycystic kidney disease. The remainder of the patients had other diseases (autosomal dominant polycystic kidney disease, Joubert syndrome, Bardet–Biedel syndrome, interstitial nephritis, or another). Among them, 3 underwent analysis of paired samples before and after kidney transplantation (Fig. 2). Samples obtained after renal transplantation were not included in statistical analysis in the Tables and Fig. 1, and are only shown in Fig. 2.

Demographic data of the study population are shown in Table 1. The mean age was 13.9±8.8 years. Age, sex distributions, and serum C-reactive protein (CRP) levels were not statistically different among the four groups.

Repeat samples from patients were analyzed only when the clinical status of patients, including degree of proteinuria and renal function (change in eGFR by 25 %), changed.

Table 1 Characteristics of the study cohort. Data are presented as mean ± standard deviation

	FSGS	SSNS	CGN	Non-glomerular kidney disease
n (sample)	20	26	24	24
Sex (male/female)	8/12	15/11	12/12	16/8
Age (years)	13.1±5.2	13.7±10.2	16.4±9.1	12.4±9.3
Urinary protein (g/gCre)	5.5±6.4	2.5±4.5	1.3±3.8	0.6±1.5
CRP (mg/dL)	0.02±0.05	0.04±0.13	0.12±0.39	0.06±0.17
eGFR (mL/min/1.73 m^2)	66.3±60.1	123.7±21.5	104.7±42.3	42.4±36.8
(95%CI)	(38.2–94.5)	(115.0–132.4)	(86.9–122.6)	(26.9–58.0)
Serum suPAR (pg/mL)	2,837±1,266	1,824±859	1,686±950	3,483±1,777
(95%CI)	(2,245–3,430)	(1,477–2,171)	(1,284–2,087)	(2,732–4,233)

CRP C-reactive protein, *eGFR* estimated glomerular filtration rate, *CI* confidence interval, *suPAR* soluble urokinase receptor, *FSGS* focal segmental glomerular sclerosis, *SSNS* steroid-sensitive nephrotic syndrome, *CGN* chronic glomerulonephritis

Definition and clinical assessment

A diagnosis of post-transplant recurrence of FSGS was based on the presence of clinical recurrence of NS (massive proteinuria [more than 1 g protein in a 24-h collected urine]) after renal transplantation. Renal biopsy of recurrent cases showed diffuse effacement of podocyte foot processes by electron microscopy or FSGS region by light microscopy in the absence of transplant glomerulopathy.

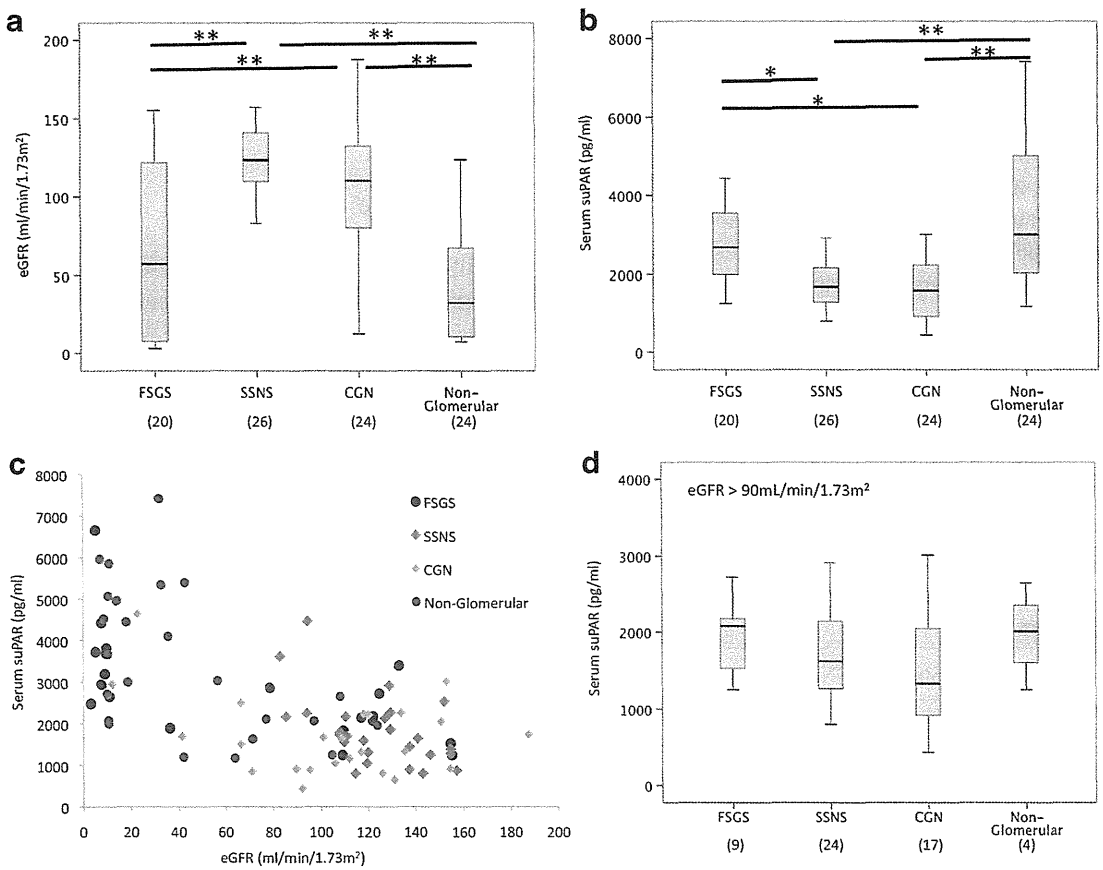

Fig. 1 **a** Estimated glomerular filtration rate (eGFR) and **b** serum suPAR levels in the four groups with numbers of the samples in each group. *Single and double asterisk* denote statistical significance $p<0.05$ and $p<0.01$ respectively. **c** Serum soluble urokinase receptor (suPAR) levels and eGFR in patients with focal segmental glomerular sclerosis (FSGS; *red*), steroid-sensitive nephrotic syndrome (SSNS; *green*), CGN (*grey*), and non-glomerular kidney disease (*blue*). **d** Serum suPAR levels in patients with normal kidney function (eGFR>90 mL/min/1.73 m^2). The differences between groups did not reach statistical significance

Table 2 Circulating suPAR levels before and during post-transplant recurrence of FSGS

Patient	Age	Sex	POD	Sample	suPAR (pg/mL)	eGFR	Urinary protein	Response to PEX
1	17.5	Female	−7	Plasma	3,010	4.7	N.D.	CR
	17.5		7	Plasma	*1,560*	39.6	27.2	
2	8.1	Male	24	Plasma	*1,854*	68.4	5.2	CR
3	6.6	Male	43	Plasma	*2,223*	23.3	5.7	CR
4	17.7	Female	−2	Serum	4,430	7.6	N.D.	PR
	17.8		37	Serum	*2,497*	42.4	282 mg/dL*	
5	17.2	Female	−7	Plasma	3,698	7.7	N.D.	Failure
	17.3		21	Plasma	*1,941*	83.6	9.5	
6	12.9	Male	−5	Serum	2,652	10.9	N.D.	Failure
	13		3	Serum	*1,568*	150	49.1	

These samples were obtained as plasma or serum

suPAR levels during post-transplant recurrence are marked in *italics*

Urinary protein levels were indicated by concentration (*asterisk*) or urinary protein creatinine ratio (g/gCre)

N.D. not determined, *POD* post-operative day, *PEX* plasma exchange, *CR* complete response, *PR* partial response, *eGFR* estimated glomerular filtration rate

Recorded clinical parameters included clinical diagnosis, sex, age, height, kidney transplant status, immunosuppressant therapy, spot urine protein/creatinine ratio, serum creatinine (milligram per deciliter) and serum CRP corresponding in time to when the suPAR specimen was retrieved. Patients with apparent infection were excluded. FSGS was diagnosed by means of renal biopsy. The eGFR in children was estimated using the Schwartz formula [15] (younger than 2 and older than 11 years of age) and the formula of the Japanese Society of Pediatric Nephrology (JSPN) [16] (2–11 years old). In patients older than 22 years, the Japanese equation for eGFR [17] was used.

Measurement of suPAR

Serum suPAR levels were measured using a Human uPAR Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). Whole blood samples were collected in blood-collecting tubes without adding ethylenediaminetetraacetic acid (EDTA) or heparin. After clot removal by centrifugation, the supernatant was designated as serum. Heparinized plasma samples were obtained from four FSGS patients (Table 2). Serum or plasma samples were thawed once or twice to eliminate the effects of multiple freeze/thaw cycles.

Table 3 Correlation coefficients between serum suPAR levels and the indicated parameters

	FSGS	SSNS	CGN	Non-glomerular	Total
eGFR	−0.626*	−0.530*	–	−0.535*	−0.636*
Age	–	–	–	−0.545*	−0.269*
Urinary protein	–	–	–	–	–

eGFR estimated glomerular filtration rate, *FSGS* focal segmental glomerular sclerosis, *SSNS* steroid sensitive nephrotic syndrome, *CGN* chronic glomerulonephritis

Correlation coefficients are filled in the table if they are statistically significant ($p<0.05$)

*Statistical significance ($p<0.01$)

Statistical analyses

Normally distributed variables were expressed as mean ± standard deviation and compared using Student's t test or one-way analysis of variance (ANOVA). Further analysis between cohorts was performed using Bonferroni's test. The Pearson product–moment correlation coefficient was measured to evaluate the association between circulating suPAR and the variables of interest, while controlling for age, proteinuria, and eGFR. p values of less than 0.05 were considered to be statistically significant. Analyses were performed using SPSS software (version 20; SPSS, Chicago, IL, USA).

Results

Serum suPAR in FSGS and other kidney diseases

Characteristics of the patients in the four groups were shown in Table 1. Among the four groups, eGFR in the FSGS and non-glomerular kidney disease groups was lower than that in the SSNS and CGN groups (Fig. 1a). On the contrary, serum suPAR levels were higher in the FSGS and non-glomerular kidney disease groups than those in the SSNS and CGN groups (Fig. 1b). The difference in suPAR between the FSGS

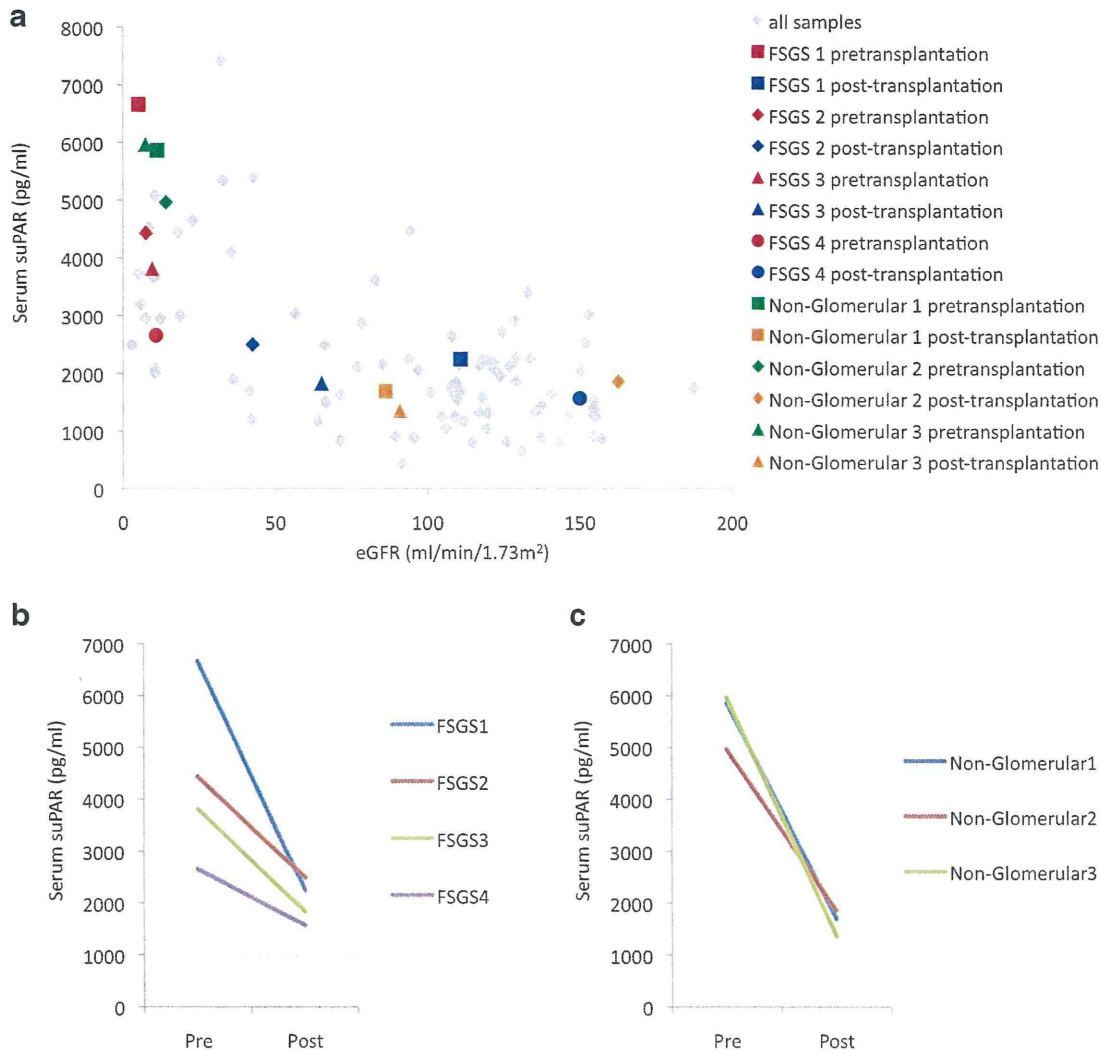

Fig. 2 Serum suPAR levels and eGFR of 7 patients who underwent kidney transplantation. Results of the analysis of paired samples obtained before and after transplantation in 4 patients with FSGS **a**, **b** and 3 patients with non-glomerular kidney disease (1 patient with Joubert syndrome and 2 patients with hypoplastic kidney) (**a**, **c**) are shown. suPAR soluble urokinase receptor, eGFR estimated glomerular filtration rate

group and non-glomerular kidney group did not reach statistical significance (p=0.56).

Using a data set of 94 serum samples from patients with FSGS and other diseases, we analyzed the association of serum suPAR levels with proteinuria, age, and kidney function (Table 3). Serum suPAR inversely correlated with eGFR in the overall patient cohort and in the FSGS, SSNS, and non-glomerular kidney group (Fig. 1c, Table 3). Serum suPAR levels showed a weak inverse correlation with age in the overall patient cohort and in the non-glomerular kidney disease group (Table 3). Of note, serum suPAR levels did not correlate with levels of proteinuria in the overall patient cohort or in any of the four groups (Table 3).

Because suPAR correlates negatively with eGFR, suPAR with normal eGFR (>90 mL/min/1.73 m^2) in each group was compared (Fig. 1d). Serum suPAR from the patients with normal kidney function was 2,123±703 pg/mL, and the difference among the four groups did not reach statistical significance (Fig. 1d). These results suggest that higher levels of suPAR in the FSGS and non-glomerular kidney disease groups might be attributed to lower glomerular filtration in these two groups.

Effects of renal transplantation on circulating suPAR levels

To analyze the effect of glomerular filtration on serum suPAR in FSGS patients, suPAR levels in paired serum samples obtained before and after transplantation were analyzed (Fig. 2). Four FSGS patients underwent living donor kidney transplantation because of end-stage renal failure. In all 4 of these FSGS patients, eGFR was

successfully increased after transplantation (8.4±2.5 to 92.1±48.0 ml/min/1.73 m^2, Fig. 2). Compared with suPAR levels before transplantation (4,389±1,685 pg/ml), suPAR after transplantation was significantly decreased in these FSGS patients (2,035±415 pg/ml, Fig. 2).

Paired samples from 3 patients in the non-glomerular kidney disease group who had hypo-/dysplastic kidney disease or Joubert syndrome and underwent living donor kidney transplantation were also analyzed. Before transplantation, all 3 patients exhibited elevated suPAR levels (5,594±550 pg/ml). After successful transplantation with recovery of kidney function, suPAR levels significantly decreased (1,638±255 pg/ml, Fig. 2). These results clearly indicate that high suPAR levels before transplantation in FSGS are not disease-specific, and are due to decreased glomerular filtration.

suPAR levels during post-transplant recurrence of FSGS

In 6 FSGS patients, serum or plasma samples were obtained during early post-transplant recurrence. Circulating suPAR levels before or during post-transplant recurrence in these patients are shown in Table 2. Some of these patients responded well to plasma exchange therapy, potentially supporting the pathogenic role of circulating factor(s). However, even in these patients, serum or plasma suPAR levels at recurrence in the acute phase were not significantly high (less than 2,500 pg/ml). These results indicate that post-transplant recurrence was not caused by elevated suPAR levels in these patients.

Discussion

In the present study, we analyzed circulating suPAR levels in patients with FSGS and compared them with those in patients with other kidney diseases. suPAR levels of FSGS patients were higher than those of SSNS or CGN patients, but not higher than those in patients with non-glomerular kidney diseases. Serum suPAR levels correlated inversely with eGFR, and suPAR levels were decreased after renal transplantation in ESRD patients with FSGS and hypo-/dysplastic kidney diseases. Furthermore, suPAR levels during post-transplantation recurrence were not high compared with those of patients with other kidney diseases.

Inverse correlation of glomerular filtration rate and serum suPAR levels has been verified in several previous reports [11–13, 18–20]. Our results demonstrated that suPAR levels in FSGS patients were not higher than those of other kidney diseases with comparable renal function, and that suPAR levels of patients with various kinds of diseases with decreased glomerular filtration often exceed 4,000 pg/mL (Fig. 1c). While the estimated molecular mass of uPAR is 55–60 kDa [21], circulating suPAR is produced and modified by intramolecular cleavage and variable glycosylation [21], resulting in the 22-kDa fragment in serum [6]. As proteins of this size are known to be sieved through the glomerular filter, decreased glomerular filtration can result in increased serum suPAR levels. Our results suggest that loss of functional nephrons, whether caused by FSGS or by other kidney diseases, results in high circulating suPAR levels, and recovery of kidney function by renal transplantation restores normal suPAR levels. Because overexpression of suPAR in podocytes or the administration of suPAR in mice results in proteinuria by activating podocyte β-integrin [6], elevated suPAR levels in patients with decreased glomerular filtration can lead to a vicious cycle of renal damage. Renal transplantation and recovery of glomerular filtration could contribute to amelioration of the damage caused by uremic toxins and suPAR. Removal or neutralization strategies for circulating suPAR levels [22, 23] will provide evidence for the role of suPAR in patients with kidney disease.

The correlativity of suPAR levels with proteinuria has been controversial. In some FSGS patients, the decrease in proteinuria after immunosuppressant therapy accompanies the relative decline in suPAR levels [6]. In another cohort, a weak correlation was reported between serum suPAR levels and proteinuria [13]. In the event of post-transplant FSGS recurrence, resolution of effacement of podocyte foot processes after plasmapheresis or immunosuppression has been reportedly linked to the decline in serum suPAR levels [24]. However, other groups demonstrated that the degree of proteinuria did not correlate with suPAR levels in the kidney disease patient cohort, or in the FSGS patient cohort [12, 20]. In the present study, serum suPAR levels did not correlate with proteinuria in all four groups. Moreover, increased suPAR levels during post-transplant recurrence were not observed in FSGS patients with relapsed massive proteinuria within several weeks of transplantation. Some of these patients were at least partially responsive to plasmapheresis, indicating that circulating factor(s) underlie the pathogenesis of proteinuria in these patients. These results suggest that circulating factor(s) other than suPAR, or a specific suPAR fragment that is not detected by ELISA analysis, can cause proteinuria and post-transplant recurrence in these patients.

This study has some limitations. First, our results are based on samples from small and mono-racial patients from a few institutions. Owing to the small number of patients in this study, it is still unclear whether the negative data for the specific role of suPAR in FSGS are generally applicable to FSGS with a broad range of clinical scenarios. Regarding this point, the results of a cohort study of Asian [25, 26] and European [27] FSGS patients were published during the revision process of this manuscript. These data have also shown that suPAR levels are significantly affected by renal function and have little diagnostic value. Together with our results that

suPAR levels in post-transplant recurrence were not elevated, these results question the validity of the pathogenic role of suPAR in FSGS. Second, although FSGS was confirmed by renal biopsy in all patients, secondary FSGS may not be excluded in the FSGS cohort, as genetic analysis of mutations was not performed in the majority of FSGS patients. This could have resulted in the inclusion of FSGS patients with podocyte dysfunction rather than circulating factor(s) in our study, although *NPHS2* mutations are not a major cause of chronic renal insufficiency caused by sporadic steroid-resistant nephrotic syndrome or heavy proteinuria in Japanese children [14, 28]. Third, all samples were obtained during on-treatment visits and the timing of sampling during the disease course varies greatly. Therefore, the effects of therapy or other factors may have interfered with intrinsic serum factor levels [7]. Prospective studies on the measurement of suPAR levels before any treatment or at specific time points in the disease course may be required to define the genuine association between serum factors and FSGS pathogenesis.

This study examined suPAR levels before and after renal transplantation in FSGS as well as other kidney diseases. Our study revealed that elevated suPAR levels in FSGS patients are primarily attributed to low glomerular filtration. We also demonstrated that, although the number of patients is small, elevated suPAR levels are not common in the post-transplant recurrence of FSGS, even in those in whom circulating factor(s) seem to play a role in FSGS pathogenesis. These data warrant further analysis of the possible humoral mediators of FSGS pathogenesis.

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aPKCλ maintains the integrity of the glomerular slit diaphragm through trafficking of nephrin to the cell surface

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The slit diaphragm (SD), the specialized intercellular junction between renal glomerular epithelial cells (podocytes), provides a selective-filtration barrier in renal glomeruli. Dysfunction of the SD results in glomerular diseases that are characterized by disappearance of SD components, such as nephrin, from the cell surface. Although the importance of endocytosis and degradation of SD components for the maintenance of SD integrity has been suggested, the dynamic nature of the turnover of intact cell-surface SD components remained unclear. Using isolated rat glomeruli we show that the turnover rates of cell-surface SD components are relatively high; they almost completely disappear from the cell surface within minutes. The exocytosis, but not endocytosis, of heterologously expressed nephrin requires the kinase activity of the cell polarity regulator atypical protein kinase C (aPKC). Consistently, we demonstrate that podocyte-specific deletion of aPKCλ resulted in a decrease of cell-surface localization of SD components, causing massive proteinuria. In conclusion, the regulation of SD turnover by aPKC is crucial for the maintenance of SD integrity and defects in aPKC signalling can lead to proteinuria. These findings not only reveal the pivotal importance of the dynamic turnover of cell-surface SD components but also suggest a novel pathophysiological basis in glomerular disease.

Keywords: aPKC/cell-surface localization/glomerular disease/nephrin/slit diaphragm.

Abbreviations: aPKC, atypical protein kinase C; aPKC KN, kinase negative form of aPKC; aPKC-Par, aPKC (partitioning defective); aPKC-PS, aPKC pseudosubstrate inhibitor; BSD, blastcidin-*S*-deaminase; cKO, conditional knockout; EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FRT, Flp recombination target; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBM, glomerular basement membrane; MβCD, methyl-β-cyclodextrin; MESNA, β-mercaptoethane sulfonate; PI3K, phosphatidylinositol 3-kinase; SC, scramble peptide; SD, slit diaphragm; STED, stimulation emission depletion; TfR, transferrin receptor; TRE, tetracycline response element; ZO-1, zonula occludens-1.

The glomerular filtration barrier is composed of fenestrated capillary endothelial cells, the glomerular basement membrane (GBM) and glomerular visceral epithelial cells (podocytes). These components work together to form a size- and charge-selective filtration barrier that prevents the leakage of cells or macromolecules into the urine. Podocytes extend primary processes with branched protrusions called foot processes to cover the outer surface of glomerular capillaries. These foot processes from neighbouring podocytes are interdigitated to form a specialized intercellular junction, called the slit diaphragm (SD), that plays a critical role in glomerular filtration (*1*). Most glomerular disease states are characterized by dramatic morphological alteration of podocyte foot processes, called foot process effacement, which involves loss of typical SD structure, resulting in proteinuria, progressive renal damage and eventually the loss of renal function (*1*).

The framework of the SD is composed largely of nephrin, a transmembrane protein of the immunoglobulin superfamily. Nephrin molecules interact with one another in a homophilic manner, or with other SD components, such as neph1 and podocin, to form the zipper-like structure of the SD (*2*, *3*). Nephrin is encoded by the *NPHS1* gene, and patients who harbour mutations in the *NPHS1* gene develop heavy proteinuria before birth and rapidly progress to end stage renal failure, this is known as congenital nephrotic syndrome of the Finnish type (*4*, *5*). Mutated forms of nephrin or podocin found in congenital nephrotic syndrome show defects in cell-surface localization when expressed in HEK293 cells; most localized to the endoplasmic reticulum (ER), whereas wild-type proteins localized to the cell surface (*6*, *7*). In addition, staining of nephrin in patients with various nephrotic syndromes shows defects in the continuous linear pattern, a decrease in the staining at foot processes and an increase in intracellular compartments (*8–12*). Furthermore, administration of a monoclonal antibody against the extracellular domain of nephrin into rats causes proteinuria,

which is also associated with defects in the cell-surface localization of nephrin (*13*, *14*). Change of the staining pattern of nephrin from a linear pattern to a granular pattern is also seen in an animal model, puromycin aminonucleoside nephrosis (*15–18*). Previously, nephrin has been shown to be rapidly endocytosed through clathrin and raft-dependent pathways in cultured COS-7 cells (*19*). Furthermore, the endocytosis of nephrin is facilitated by disease-causing conditions through the molecular interaction with β-arrestin2, PKCα or CIN85 (*20–23*). Recent reports also revealed the importance of protein trafficking, autophagy and protein degradation in the maintenance of the SD (*24–27*). These observations all support the importance of membrane trafficking of SD components, especially the cell-surface localization of nephrin and other SD components, in the physiology and pathology of the SD. However, the molecular mechanisms leading to cell-surface localization of SD components, as well as those regulating the turnover of SD, are largely unknown.

The atypical protein kinase C-partitioning defective (aPKC-Par) complex is an evolutionally conserved ternary complex composed of the serine-threonine kinase aPKC and two scaffold proteins, Par3 and Par6 (*28*). This complex plays a critical role in the formation and maintenance of the cell–cell junction in epithelial cells (*28*). The importance of the aPKC-Par complex is highlighted by the finding that it associates with nephrin, neph1 and podocin through the direct interaction between nephrin and Par3 (*29*, *30*). Furthermore, prevention of the formation of the aPKC-Par complex by podocyte-specific depletion of the aPKC isoform aPKCλ in mice (aPKCλ conditional knockout (cKO)) leads to massive proteinuria, the disassembly of the SD with effacement of podocyte foot processes, and finally develops into severe glomerulosclerosis (*30*, *31*). However, the precise role of the aPKC-Par complex in the maintenance of the SD is largely unknown. Several recent studies have shown that aPKC regulates the turnover of adherence junction proteins and cell-surface receptors through the suppression of their endocytosis (*32–34*). aPKC is also suggested to regulate polarized exocytosis in epithelial cells (*35*, *36*). These observations raise the possibility that aPKC regulates the cell-surface localization of SD components to maintain SD integrity.

In this study, we employed isolated intact glomeruli in a cell-surface biotinylation assay system to evaluate the turnover of cell-surface SD components. We found that SD components were constantly and rapidly exocytosed, endocytosed and degraded in steady-state glomeruli. We also revealed that aPKC is crucial for the turnover of cell-surface SD components to maintain SD integrity through the regulation of exocytosis in cellular models, isolated glomeruli and in aPKCλ cKO mice. These findings not only reveal a novel aspect of the mechanism regulating the integrity of the SD but also imply that the impairment of SD turnover might contribute to the pathogenesis of proteinuria.

Materials and Methods

Antibodies and reagents

The antibodies used in this study were: rabbit anti-nephrin and rabbit anti-podocin (IBL; Immuno-Biological Laboratories, Fujioka, Gunma, Japan), rabbit anti-aPKC and mouse anti-GFP (B-2; Santa Cruz Biotechnology, Dallas, TX, USA), HRP-conjugated rat anti-HA (Roche Diagnostics, Basel, Switzerland), rabbit anti-Par3 (Merck Millipore, Billerica, MA, USA), mouse anti-E-cadherin (36/E-Cadherin) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (BD Biosciences, San Jose, CA, USA), mouse anti-transferrin receptor (TfR) (H68.4), mouse anti-zonula occludens-1 (ZO1-1A12), Alexa488 and Alexa555-conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA), HRP-conjugated secondary antibodies (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and rabbit anti-neph1 (as previously described (*37*)). The reagents used in this study were chlorpromazine (LKT Laboratories Inc., St Paul, MN, USA), methyl-β-cyclodextrin (MβCD) (Sigma, St Louis, MO, USA) and doxycycline-HCl (ICN Biochemicals Inc., Irvine, CA, USA). Myristoylated pseudosubstrate inhibitor specific for atypical PKC subtypes (myr-SIYRRGARRWRKL) or scramble peptide (SC) (myr-RLYRKRIWRSAGR) was purchased from Peptide Institute Inc. (Osaka, Japan).

Animals

Podocyte-specific aPKCλ cKO mice (*aPkc*$^{\Delta E5,floxE5}$; *Nphs1-Cre*^{Tg} mice) were described previously (*30*). Male Wistar rats (200–400 g, 5–9 weeks old) were purchased from Oriental Yeast Co. (Tokyo, Japan). Animal care and handling in experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at Yokohama City University.

Cell lines and transfections

We generated HCT116-nephrin cells using the Flp-In System (Life Technologies). First, we made the lentivirus-based Flp-In target site vector, pLenti6/FRT/LacZeo2. pLenti6/FRT/LacZeo2 was constructed by replacing the segment containing pCMV to Zeo^{R} of pLenti6/V5-DEST with the segment of SV40Δ to FRT/LacZ-Zeocin of pFRTZeo2. Then, we constructed the pLenti6/FRT/enhanced green fluorescent protein (eGFP)-blasticidin-*S*-deaminase (BSD), which replaced the LacZeo2 coding region of pLenti6/FRT/LacZeo2 to enhanced green fluorescent protein (eGFP)–blasticidin-*S*-deaminase (BSD) fusion protein coding sequence for efficient selection of HCT116_FRT cells. The detailed sequence will be provided upon request. Lentivirus was produced according to the instructions for the pLenti6 system (Life Technologies). HCT116 cells were infected with pLent6/FRT/eGFP-BSD lentivirus, selected with 400 μg/ml of blasticidin, single clones isolated and a single integration of Flp recombination target (FRT) site were confirmed by Southern blotting. HCT116-nephrin cells were prepared using clone #19, by Flp-In System (Life Technologies) and maintained in McCOY's 5A medium (Life Technologies) containing 10% foetal bovine serum. HeLa Tet-On Advanced cells (Clontech Laboratories Inc., Mountain View, CA, USA) were maintained in Dulbecco's modified Eagle medium containing 10% foetal bovine serum. Plasmid transfections were performed using lipofectamine LTX reagent (Invitrogen) or linear polyethyleneimine (MW 25,000 Da; Polyscience Inc., Warrington, PA, USA), and siRNA transfections were performed using lipofectamine RNAiMAX (Life Technologies), following the manufacturer's instructions.

Expression constructs and siRNA

Human nephrin and podocin cDNA was described previously (*30*). cDNA fragments of human nephrin were amplified by PCR, and subcloned into pcDNA5-FRT-TO (Life Technologies) or pTRET-FRT-Hyg-TetOn vectors. HA-tagged wild-type mouse aPKCλ and its kinase-deficient mutant (kinase negative form of aPKC (aPKC KN): K273E) were described previously (*38*).

pTRE-tight(TRET)/FRT vector was constructed by linking the fragment of SV40pA to AmpR of pcDNA5/FRT/TO (Life Technologies) and the PCR amplified tetracycline response element (TRE)-tight promoter, containing six TRE, from pTRE-tight (Clontech). The fragment of CMV promoter to SV40pA from the pTet-On Advanced vector (Clontech Laboratories Inc.) was then inserted at the blunted PciI site of pTRET/FRT vector to generate

pTERT/FRT/TetOn vector. Detailed sequence information is available upon request.

siRNA duplexes were purchased from Sigma-Genosys, and nucleotide sequences were as follows: aPKCλ knockdown, sense, 5′-CAAGUGUUCUGAAGAGUUUdTdT-3′ and antisense, 5′-AAACUCUUCAGAACACUUGdTdT-3′; aPKCζ knockdown, sense, 5′-GGAAGCAUAUGGAUUCUGUdTdT-3′ and antisense, 5′-ACAGAAUCCAUAUGCUUCCdTdG-3′; Par3 knockdown #3, sense, 5′-UAGCUGUUUAGAAUACUAUUU-3′ and antisense, 5′-AAAUAGUAUUCUAAACAGCUA-3′; #6, sense, 5′-AUCCAUAUCGACUGCUCUGAU-3′ and antisense, 5′-AUCAGAGCAGUCGAUAUGGAU-3′; #8, sense, 5′-GUCACUUAACCUAAAGCAAUU-3′ and antisense, 5′-AAUUGCUUUAGGUUAACUGAC-3′. For a negative control, we used the All-Stars negative control siRNA (Qiagen, Hilden, Germany, catalogue number 1027281).

Isolation of intact glomeruli from rat kidneys

Glomerular isolation was performed as described previously (*30*, *39*). Deeply anaesthetized rats (50 mg/kg nembutal and 5 mg/kg ketoprofen, i.p.) were transcardially perfused with ice-cold phosphate-buffered saline (PBS) containing protease/phosphatase inhibitors and the sliced cortices from the dissected kidneys were minced into small pieces at 4°C. The minced tissues suspended in the same buffer were passed through successive stainless steel sieves (pore size: 250, 125 and 63 μm, respectively; AS ONE, Osaka, Japan) at 4°C. The glomeruli were collected with ice-cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ by centrifugation at 2,000 × g for 10 min at 4°C, and then processed for the surface biotinylation assay.

Surface biotinylation assay

The details of the surface biotinylation assay have been described previously (*40*). Briefly, the isolated glomeruli, HCT116-nephrin cells or HeLa Tet-On Advanced cells were washed with PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBSCM), and cell-surface proteins biotinylated with 0.5 mg/ml sulfo-NHS-SS-biotin (Thermo Scientific, Waltham, MA, USA) in PBSCM at 4°C for 30 min. Then, the glomeruli or cells were washed with 20 mM glycine in PBSCM at 4°C for 15 min to quench free sulfo-NSH-SS-biotin. In this protocol, we can specifically biotinylate the cell-surface proteins. Cell proteins were extracted with lysis buffer (20 mM Hepes–NaOH (pH7.5), 150 mM NaCl, 5 mM EGTA, 15 mM $MgCl_2$, 0.1% SDS, 0.2% sodium deoxycholate, 1% TritonX-100, protease inhibitor cocktail (Sigma–Aldrich) and PhosSTOP (Roche Diagnostics)). The biotinylated proteins were isolated with streptavidin sepharose (GE Healthcare Bio-Sciences). The samples were then prepared for immunoblot analysis. The total protein level and the cell-surface localization of SD components were normalized to those at the start of labelling.

Endocytosis assay

The details of the endocytosis assay were described previously (*40*). The isolated glomeruli or HCT116-nephrin cells were cell-surface biotinylated as described above, and incubate at 37°C in HBSS (Life Technologies) for the indicated times to allow endocytosis. The remaining sulfo-NHS-SS-biotin on the cell surface was stripped for 1 h at 4°C with 200 mM (isolated glomeruli) or 50 mM β-mercaptoethane sulfonate (MESNA) (Sigma–Aldrich) in 100 mM Tris–HCl (pH 8.6) containing 100 mM NaCl and 2.5 mM $CaCl_2$ and washed with 5 mg/ml iodoacetamide in PBSCM for 15 min at 4°C to quench remaining MESNA. Cell proteins were extracted with cell lysis buffer and the biotinylated proteins were isolated and affinity purified with streptavidin sepharose (GE Healthcare Bio-Sciences). The samples were then prepared for immunoblot analysis. The percentages of internalized proteins were calculated using the following formula: internalized proteins = [(biotinylated protein after incubation at 37°C) − (biotinylated protein at 0 min)]/(biotinylated protein at the start of labelling) × 100 (*40*).

Biotinylation degradation assay

The details of the biotinylation degradation assay have been described previously (*41*). The isolated glomeruli were cell-surface biotinylated as described above, and incubated at 37°C in HBSS to allow endocytosis and degradation. At the indicated time points, glomeruli were lysed and analysed as per the endocytosis assay. The total protein level and the cell-surface localization of SD components were normalized to those at the start of labelling. The percentages of degraded proteins were calculated using the following formula: degraded proteins = [(biotinylated protein at the start of labelling) − (biotinylated protein after incubation at 37°C)]/(biotinylated protein at the start of labelling) × 100 (*41*).

Doxycycline-inducible expression of nephrin

HeLa Tet-On Advanced cells were transiently transfected with the pTRET-FRT-Hyg-Teton-nphs1 vector. After incubation for 48 h, cells were incubated in growth medium with 100 ng/ml doxycycline (ICN Biomedicals Inc.) for the indicate times. After induction, cells were subjected to surface biotinylation and affinity purification with streptavidin sepharose. The samples were then prepared for immunoblot analysis.

Immunofluorescence

Isolated rat glomeruli or HCT116-nephrin cells were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.1% TritonX-100 for 10 min. The glomeruli or the cells were then incubated with the indicated primary antibodies in TBST-containing 0.1% BSA for 1 h, followed by incubation with Alexa Fluor-conjugated secondary antibodies for 1 h. The glomeruli or the cells were mounted on glass slides using Prolong Gold antifade reagent (Invitrogen). The samples were examined and photographed with a fluorescent microscope (Axioimager Z1, Carl Zeiss, Oberkochen, Germany), equipped with a confocal unit (CSU10, Yokogawa Electric Corporation, Tokyo, Japan) and a cooled CCD camera (ORCA-R2, Hamamatsu Photonics K.K., Hamamatsu, Shizuoka, Japan), and objective lens (Plan-Apochromat NA 1.4 63×, Carl Zeiss). Images were processed using ImageJ (NIH, Bethesda, MD, USA) and Photoshop CS4 (Adobe, San Jose, CA, USA).

For stimulation emission depletion (STED) microscopic analysis, 5-μm thick cryosections were cut using a Jung Frigocut 2800E (Leica, Wetzlar, Germany), mounted on silane-coated glass slides and processed for staining with several antibodies. Sections were incubated for 4 h at room temperature with primary antibodies. Next, the sections were incubated with Alexa 488-labelled anti-rabbit IgG (Life Technologies) and V500-labelled streptavidin (BD Biosciences) for 1 h at room temperature. In control experiments, incubation with the primary antibody was omitted. All sections were examined with a TCS STED CW (Stimulated Emission Depletion) super-resolution microscope from Leica Microsystems GmbH (Mannheim, Germany). Samples were imaged using with a 1.4 NA 100× objective lens (HCX PL APO, Leica). Confocal images were acquired using the 488 nm argon laser line for the excitation of Alexa 488 and 458 nm to excite V500. Alexa 488 was detected using PMT2 of the spectral detection unit with the detection range set to 519–602 nm and V500 was detected with the detection range of 463–502 nm. Imaging speed was at 400 Hz using 4× line averaging and the pinhole was set to 1.0 Airy units. For STED microscopy, all conditions were identical. For the stimulated emission depletion the pulsed Ti:Sapphire laser (MaiTai laser, Spectra-Physics, Santa Clara, CA, USA) was tuned to 592 nm and the power set to 100%. Signal was detected by use of a GaAsP hybrid detection system (Leica). We performed deconvolution by applying Huygens STED deconvolution option software (Leica). Colocalization analysis of double-stained samples was performed with the LAS-AF software provided by Leica.

In vivo *biotinylation of mouse kidney*

aPKC cKO mice at P10 or P11, or control mice were transcardially perfused sequentially for 2 min with 1 ml/g body weight of PBSCM, 2 mg/ml sulfo-NHS-SS-biotin/PBSCM and 20 mM glycine/PBSCM. All solutions were prepared at room temperature. After perfusion, the kidneys were homogenized with a Potter homogenizer in 1 ml of lysis buffer on ice. The lysates were diluted to a protein concentration of 0.3 mg/ml and subjected to affinity purification using streptavidin sepharose. The samples were then prepared for immunoblot analysis.

Statistical analysis

Two-tailed Student's *t*-test (Microsoft Excel 2004) and two-tailed Mann–Whitney *U*-test (VassarStats, http://faculty.vassar.edu/lowry/VassarStats.html) were used to analyse the differences between the pairs of groups. Values were regarded statistically significant at $P < 0.05$.

Results

SD components are persistently and rapidly exocytosed and endocytosed in isolated glomeruli

To evaluate the turnover rate of cell-surface SD components in intact glomeruli, we employed rat glomeruli isolated by sieving techniques (*39*) and examined whether total expression and cell-surface localization of SD components were maintained during culture conditions. In isolated glomeruli, the immunofluorescence signals for nephrin and podocin were detected as a linear pattern along the glomerular capillary wall. This staining pattern was preserved in the 60 min incubation at 37°C, suggesting that SD integrity is maintained during this period (Supplementary Fig. S1). Then, we measured the total protein and cell-surface localization level of SD components using a cell-surface biotinylation system (Fig. 1A). Not only the total protein level of nephrin, neph1 and podocin, but also their cell-surface localization levels remained stable for 45 min during incubation (Fig. 1B and C). It has been reported that SD components are localized both in detergent soluble and detergent-resistant membrane domains called lipid-rafts (*42*). In our experiments, the majority of the nephrin was detected in the detergent soluble fraction and only part of the nephrin was detected in the detergent-resistant fraction. However, we found that both fractions of nephrin were biotinylated with a similar efficacy (Supplementary Fig. S2). This result eliminates the possibility that only a subset of SD components is preferentially biotinylated. These results also indicate that SD integrity is maintained in isolated glomeruli *ex vivo* and suggests that they faithfully recapitulate steady-state glomeruli *in vivo*.

Next, we evaluated the amount of protein internalized from the cell surface by an endocytosis assay (*40*) (Fig. 1D). Cell surface proteins were biotinylated and incubated at 37°C for the indicated times to allow endocytosis. By stripping of the remaining biotin from the cell surface with MESNA, we can specifically maintain the biotinylation of the endocytosed proteins and can quantitatively evaluate the endocytosis rate of SD components. As shown in Fig. 1E and F, all three SD components examined were rapidly internalized within 5 min after the induction of protein trafficking (nephrin, $72.1 \pm 8.9\%$; neph1, $61.0 \pm 4.4\%$; podocin, $52.7 \pm 10.0\%$; mean $\pm$ SD ($n = 3$)). This suggests that the remaining fraction consists of SD components that were either degraded after the induction of protein trafficking or retained at the cell surface that was not endocytosed or both. Based on the data taken at 45 min after the induction of protein trafficking, we assume that ~30–40% of SD components were either degraded and/or retained at the cell surface. To evaluate these possibilities, we chased the amount of SD components after biotinylation (*41*) (Fig. 1G). To do this, cell-surface proteins were first biotinylated and then incubated at 37°C for various durations to allow endocytosis and degradation. By chasing the remaining amount of biotinylated proteins, we can determine the degradation rate of SD components that localized to the cell surface from the start of labelling. Our quantitative analysis demonstrates a $31.4 \pm 11.0\%$ decrease in the amount of biotinylated nephrin and $43.6 \pm 9.6\%$ decrease in neph1 (mean $\pm$ SD ($n = 3$)) 30 min after the induction of protein trafficking (Fig. 1H and I), suggesting that most of the internalized SD components were rapidly degraded rather than recycled back to the cell surface. In addition, the amount of podocin, the affinity-purified intracellular binding partner of nephrin and neph1, was also decreased in a time-dependent manner (Fig. 1H and I), suggesting that podocin is also rapidly degraded with these proteins or dissociates from nephrin and neph1. Therefore, although cell-surface localized SD components are rapidly endocytosed and degraded, total expression and cell-surface levels of SD components were constantly maintained, probably by compensating *de novo* synthesis and exocytosis.

aPKC plays a critical role in the surface localization of SD components

As described above, aPKC has an essential role in SD integrity (*30*, *31*) and is also involved in both endocytosis and exocytosis in a variety of other systems (*32–36*, *43–45*). These observations prompted us to investigate the involvement of aPKC in the turnover of cell-surface SD components. First, we evaluated the cell-surface localization of SD components in isolated glomeruli with or without treatment with an aPKC pseudosubstrate inhibitor (aPKC-PS). The amount of SD components at the cell surface was dramatically decreased by aPKC-PS treatment for 30 min compared with control or SC-treated glomeruli, whereas the total glomerular expression level of these proteins was unaffected (Fig. 2A and B). The result implies that aPKC is involved in the turnover of cell-surface SD components in glomeruli. To further examine the involvement of aPKC, we established epithelial cells stably expressing human nephrin using the colon cancer cell-line HCT116 (HCT116-nephrin cells). In these cells, nephrin localized to both apical and intercellular junction regions, and co-localized with the adherens junction protein, E-cadherin (Supplementary Fig. S3 and data not shown). Not only aPKC-PS treatment (Fig. 2C and D), but also the overexpression of a aPKC KN (Fig. 2E and F), as well as the siRNA knockdown of *aPKC* (Fig. 2G and H) all resulted in a decrease of the cell-surface localization of nephrin, without affecting the total expression level. The cell-surface localization, as well as the total amount, of the TfR was not affected by the suppression of aPKC. These data indicate that aPKC plays a crucial role in the cell-surface localization of SD components, especially nephrin. We also examined the role of Par3, a component of aPKC-Par complex, in the maintenance of cell-surface localization of nephrin. Knockdown of *Par3* in HCT116-nephrin cells also resulted in a significant decrease of the cell-surface localization of nephrin (Supplementary Fig. S4), suggesting that the aPKC-Par3 complex regulates the cell-surface localization of nephrin.

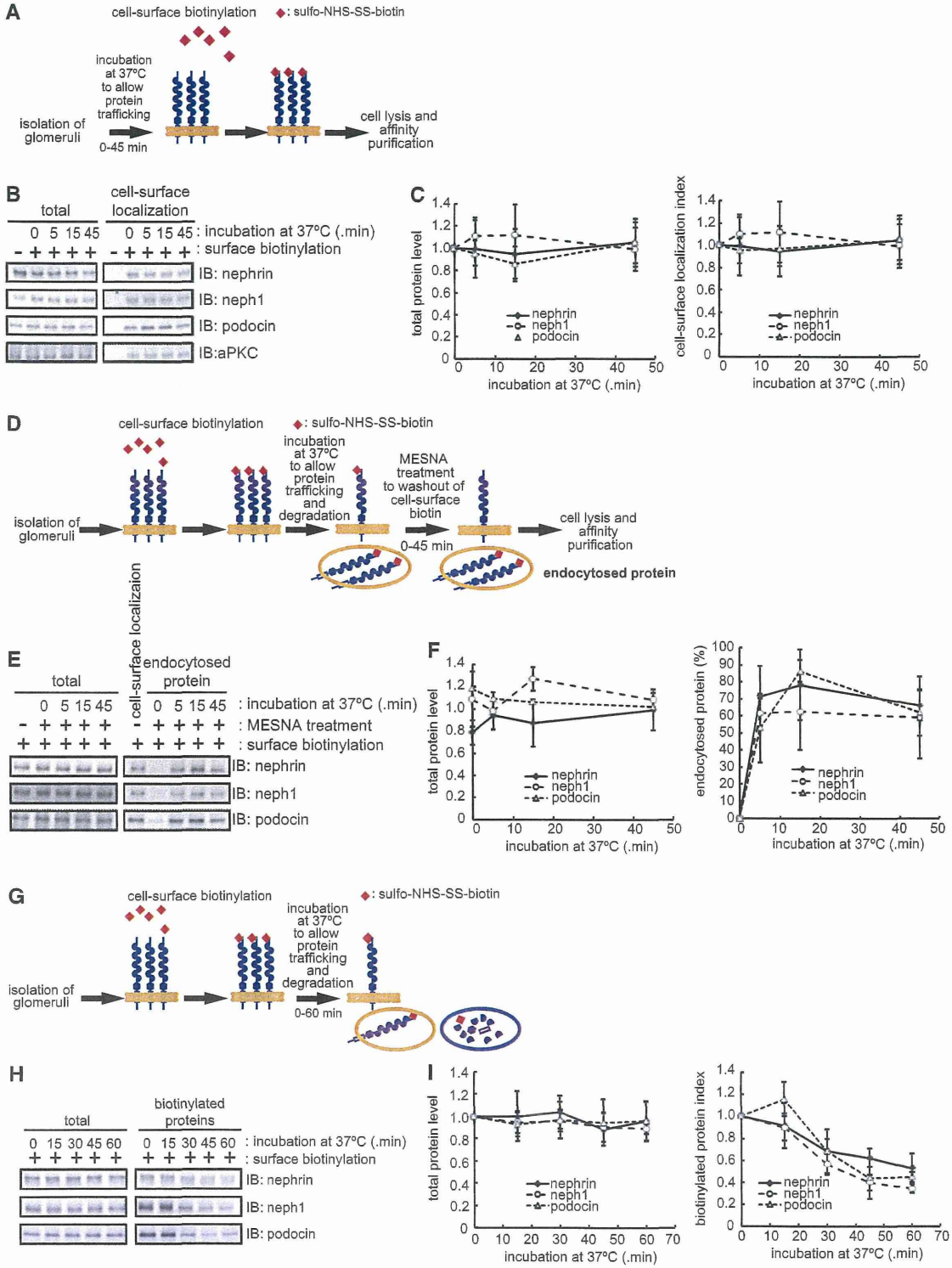

Fig. 1 SD integrity is maintained by rapid turnover of cell-surface SD components. (**A**) Schematic representation of the cell-surface biotinylation assay. (**B**) Isolated glomeruli were subjected to the cell-surface biotinylation assay as in (A). Biotinylated SD components were isolated with streptavidin sepharose and isolated proteins detected by immunoblot. (**C**) Quantification of the results in (B). The total protein level and cell-surface localization of SD components were normalized to those at the start of labelling. (**D**) Schematic representation of the endocytosis assay. (**E**) Isolated glomeruli were subjected to the endocytosis assay and analysed by immunoblot. (**F**) Quantification of the results in (E). The endocytosed proteins were expressed as the percentage of to those at the start of labelling (see 'Materials and Methods' section). (**G**) Schematic representation of the biotinylation degradation assay. (**H**) Isolated glomeruli were subjected to biotinylation degradation and analysed by immunoblot. (**I**) Quantification of the results in (H). The biotinylated SD components were normalized to those at the start of labelling. The data shown in C, F and I are the mean ± SD of three independent experiments.

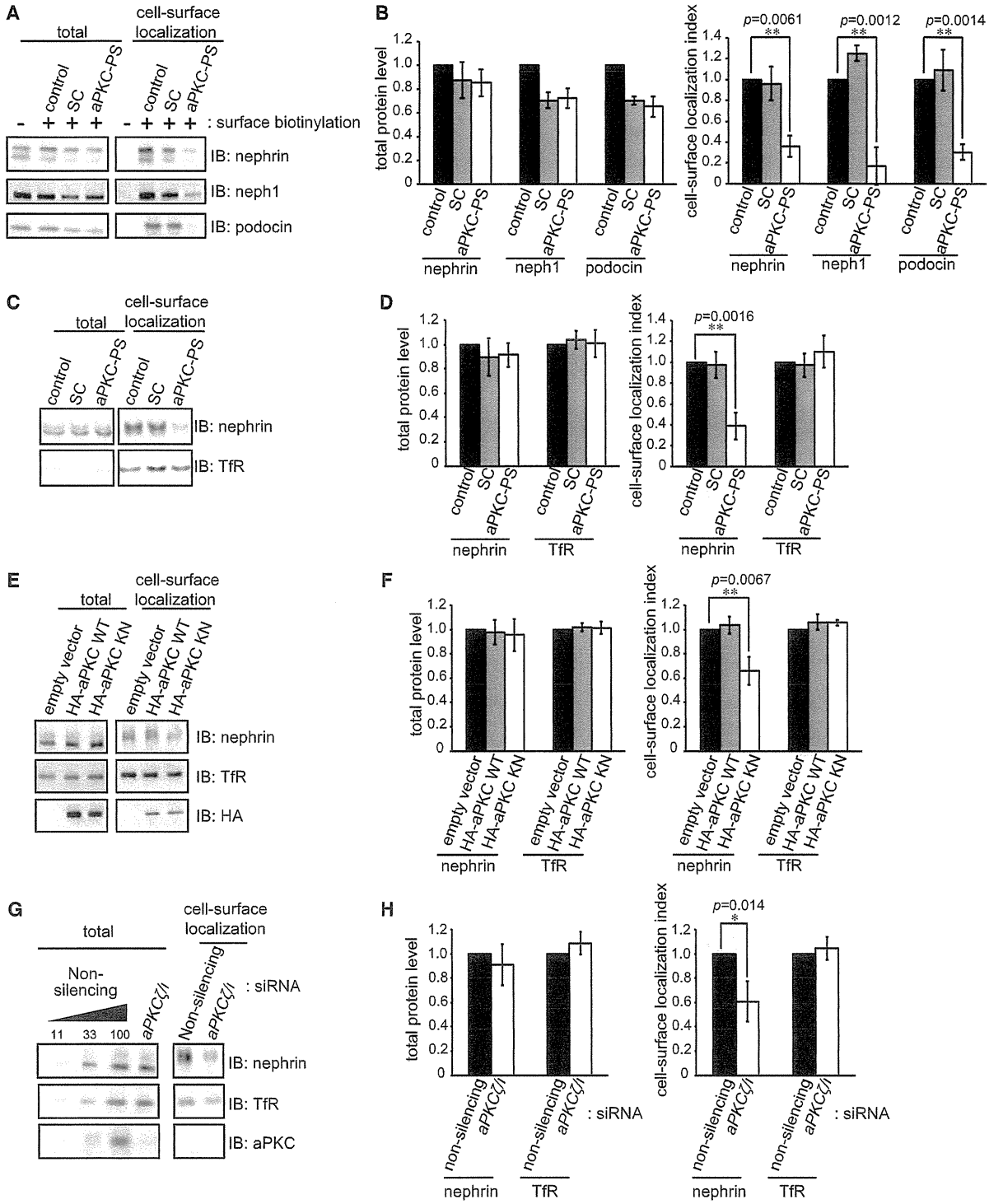

Fig. 2 aPKC is required for the cell-surface localization of SD components, including nephrin. (**A**) Isolated rat glomeruli were treated with 10 μM aPKC pseudosubstrate (PS) or SC for 30 min at 37°C in HBSS(+), then subjected to the cell-surface biotinylation assay. (**B**) Quantification of the results in (A). (**C**) HCT116-nephrin cells were treated with 20 μM of aPKC-PS or SC for 2 h at 37°C and subjected to the cell-surface biotinylation assay. (**D**) Quantification of the results in (C). (**E**) HCT116-nephrin cells were transiently transfected with aPKC WT or KN cDNA and incubated for 48 h and then subjected to the cell-surface biotinylation assay. (**F**) Quantification of the results in (E). (**G**) HCT116-nephrin cells were transiently transfected with *aPKC/ζ* siRNA and incubated for 70 h. Both isotypes of aPKC are expressed in HCT116 cells (data not shown). After incubation, the cells were subjected to the cell-surface biotinylation assay. (**H**) Quantification of the results in (G). The values shown in B, D, F and H were normalized to the appropriate control and are the mean ± SD of three independent experiments. The *P* values were determined by two-tailed Student's *t*-test.

The kinase activity of aPKC is required for exocytosis of newly synthesized nephrin, but not for the suppression of endocytosis

The decrease of cell-surface localization of nephrin by aPKC inhibition is caused by either the facilitation of endocytosis or the suppression of exocytosis, or both, because the total amount of nephrin was not altered by aPKC inhibition (Fig. 2). To evaluate these possibilities, we first examined whether the inhibition of aPKC activity affects the rate of endocytosis of nephrin in HCT116-nephrin cells using the endocytosis assay (Fig. 1D). aPKC-PS treatment (Fig. 3A and B) or overexpression of aPKC KN (Fig. 3C and D) did not alter the rate of nephrin endocytosis. The total expression level of nephrin was also unaffected by these manipulations. We further investigated whether endocytosis inhibitors can compensate for the decrease in cell-surface localization of nephrin by aPKC-PS treatment. Neither chlorpromazine (clathrin-dependent endocytosis inhibitor (*46*)) nor MβCD (raft-mediated endocytosis inhibitor (*47*)) could compensate for the decreased cell-surface localization of nephrin induced by aPKC-PS treatment (Fig. 3E). These data suggest that aPKC does not play a significant role in the endocytosis of nephrin in our system.

To directly evaluate exocytosis of nephrin, we employed a tetracycline-inducible (Tet-On) expression system in the HeLa Tet-On Advanced cell line. In this cell line, the total expression level of nephrin accumulated and reached a constant at 6 h after induction (Fig. 4A–D). The amount of nephrin targeted to the cell surface continued to increase during the 8 h following induction of nephrin expression (Fig. 4A–D). Suppression of aPKC by aPKC-PS treatment (Fig. 4A and B) or overexpression of aPKC KN (Fig. 4C and D) significantly decreased cell-surface localization of newly synthesized nephrin, while the total amount of nephrin was not affected (Fig. 4A–D). The cell-surface localization of TfR was not affected by the inhibition of aPKC in this cell line (Fig. 4A and C). Taken together, these results indicate that aPKC is required for exocytosis of newly synthesized nephrin, but not for the suppression of endocytosis.

aPKC is required for the surface localization of SD components in vivo

The above experiments using isolated glomeruli and nephrin-expressing epithelial cells strongly support the role of aPKC in maintaining the cell-surface localization of nephrin by stimulating the exocytosis of newly synthesized nephrin. To evaluate this possibility *in vivo*, we next analysed the cell-surface localization of SD components in the aPKC-deficient kidney by cell-surface biotinylation *in vivo*. aPKCλ cKO mice at P10 or P11, which developed heavy proteinuria (*30*), and control mice were transcardially perfused with sulfo-NHS-SS-biotin and the biotinylated cell-surface proteins were isolated with streptavidin sepharose from the kidney lysate. The total expression level of SD components was not different between control and mutant mice. However, the ratio between total and cell-surface localization of SD components significantly differed between control and aPKCλ cKO mice (nephrin, 0.447 ± 0.116; neph1, 0.480 ± 0.238; podocin, 0.533 ± 0.234; mean ± SD ($n = 3$), Fig. 5A and B). Exocytosis of nephrin is associated with specific glycosylation of its extracellular domain. Thus by western blot analysis, the upper band of nephrin (Fig. 5A, white arrowhead) represents the mature-glycosylated cell-surface form, and the lower band (Fig. 5A, black arrowhead) represents the *N*-glycosylated ER-form (*48*). Notably, the proportion of the ER-form of nephrin was higher in aPKCλ cKO mice compared with that of control mice; however, these mice also had a reduction in the cell-surface fraction (Fig. 5A). To our knowledge, there is no evidence that aPKC regulates the glycosylation of cell-surface proteins. Therefore, this suggests that the exocytic process of nephrin is disturbed by podocyte-specific deletion of aPKCλ.

To confirm the disturbance of cell-surface localization of nephrin in aPKCλ-deficient glomeruli, we assessed the localization of SD components using super-resolution confocal microscopy based on the STED system (*49*). In control glomeruli, the signals of nephrin, neph1 and podocin were detected as a linear pattern along the glomerular capillary and colocalized with cell-surface biotin labels. However, in glomeruli from aPKCλ cKO mice, the colocalization with cell-surface biotin was disrupted and the signals of nephrin, neph1 and podocin were mainly seen at the cell body of podocytes (Fig. 5C and Supplementary Fig. S5). Finally, we examined the localization of nephrin and podocin using immunoelectron microscopy. In control podocytes, most of the immunolabelled gold particles for nephrin and podocin were detected at the plasma membrane of foot processes. However, in aPKCλ-deficient podocytes, the gold particles for nephrin were dissociated from the plasma membrane and predominantly detected in the intracellular region (Fig. 5D and E). Conversely, the particles for podocin were detected near the plasma membrane, similar to control podocytes. Taken together, these findings demonstrate that aPKC is required for the cell-surface localization of SD components *in vivo* through the regulation of their exocytosis.

Discussion

Our previous observations in aPKCλ cKO mice revealed that aPKCλ depletion gradually causes severe glomerular dysfunction with a drastic change in the ultrastructure of the SD resulting in apically dislocated SDs and foot process effacement, suggesting the involvement of aPKCλ in the turnover of cell-surface SD components (*30*). In this study, we provide direct evidence demonstrating the rapid turnover of SD components at the cell surface using both isolated glomeruli and aPKCλ cKO mice. Furthermore, we provide evidence for the involvement of aPKCλ in the trafficking of SD components to the cell surface.

The physiological and pathological importance of the rapid turnover of SD components at the cell surface

Previous studies have revealed the critical importance of the specialized intercellular junctions formed between podocyte foot processes, the SD, in the

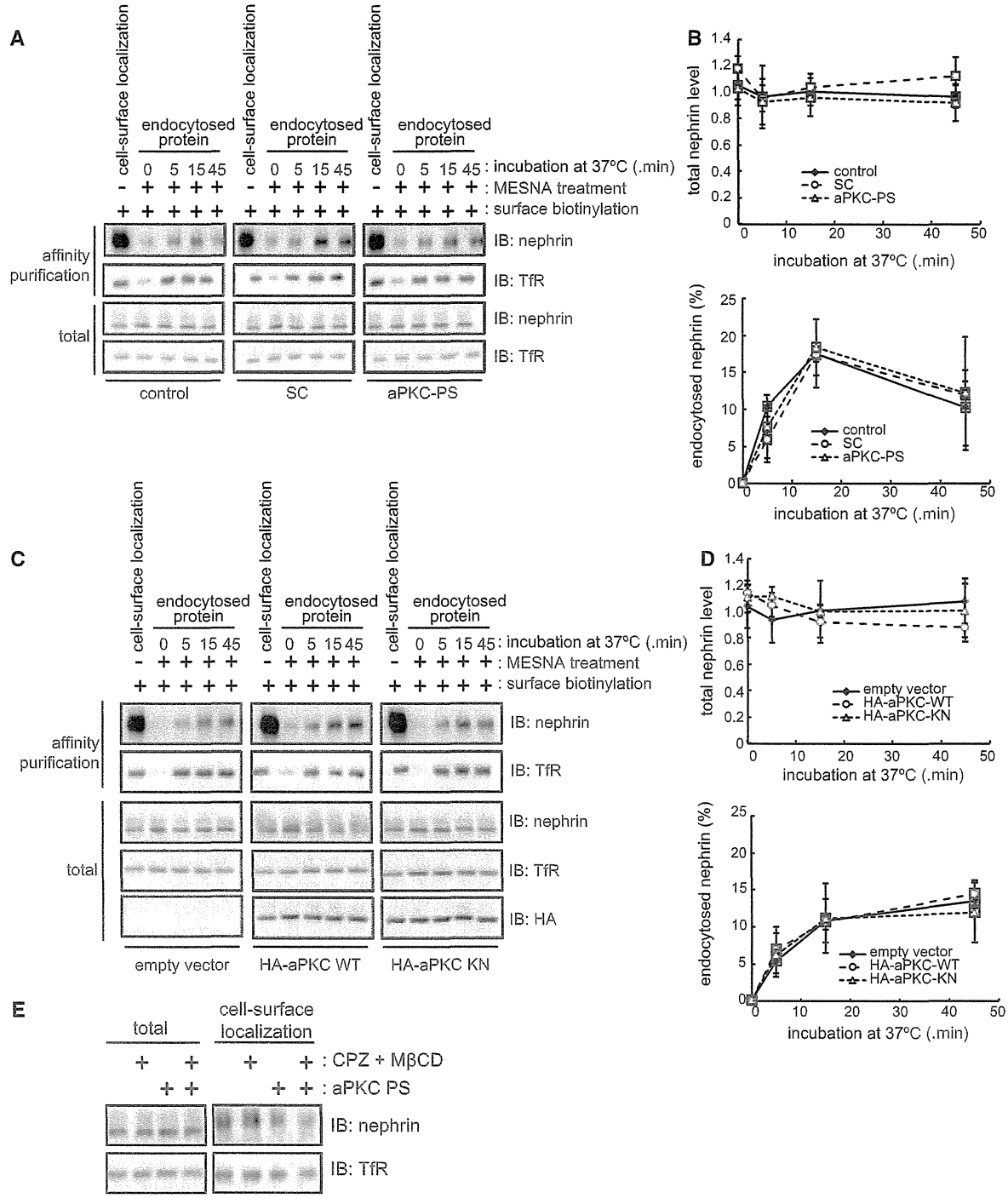

Fig. 3 aPKC does not suppress the endocytosis of nephrin. (**A**) HCT116-nephrin cells were treated with 20 μM aPKC-PS or SC for 2 h at 37°C and then subjected to the endocytosis assay as in Fig. 1D. (**B**) Quantification of the results in (A). (**C**) HCT116-nephrin cells were transiently transfected with aPKC WT or KN cDNA and incubate for 48 h and then subjected to the endocytosis assay. (**D**) Quantification of the results in (C). The amount of endocytosed nephrin shown in B and D was expressed as the percentage of those at the start of labelling and are the mean ± SD of three independent experiments. (**E**) HCT116-nephrin cells were treated with 20 μM aPKC PS with or without 10 μM chlorpromazine or 10 mM MβCD for 30 min at 37°C and then subjected to the cell-surface biotinylation assay.

maintenance of the glomerular filtration barrier and that the disassembly of the SD leads directly to the pathogenesis of proteinuria (*1*, *50*). These studies also revealed the importance of SD components and associated proteins in the development and maintenance of SD structures and function. Among the mechanisms implicated in SD integrity, endocytosis of SD components and regulation of actin cytoskeleton have

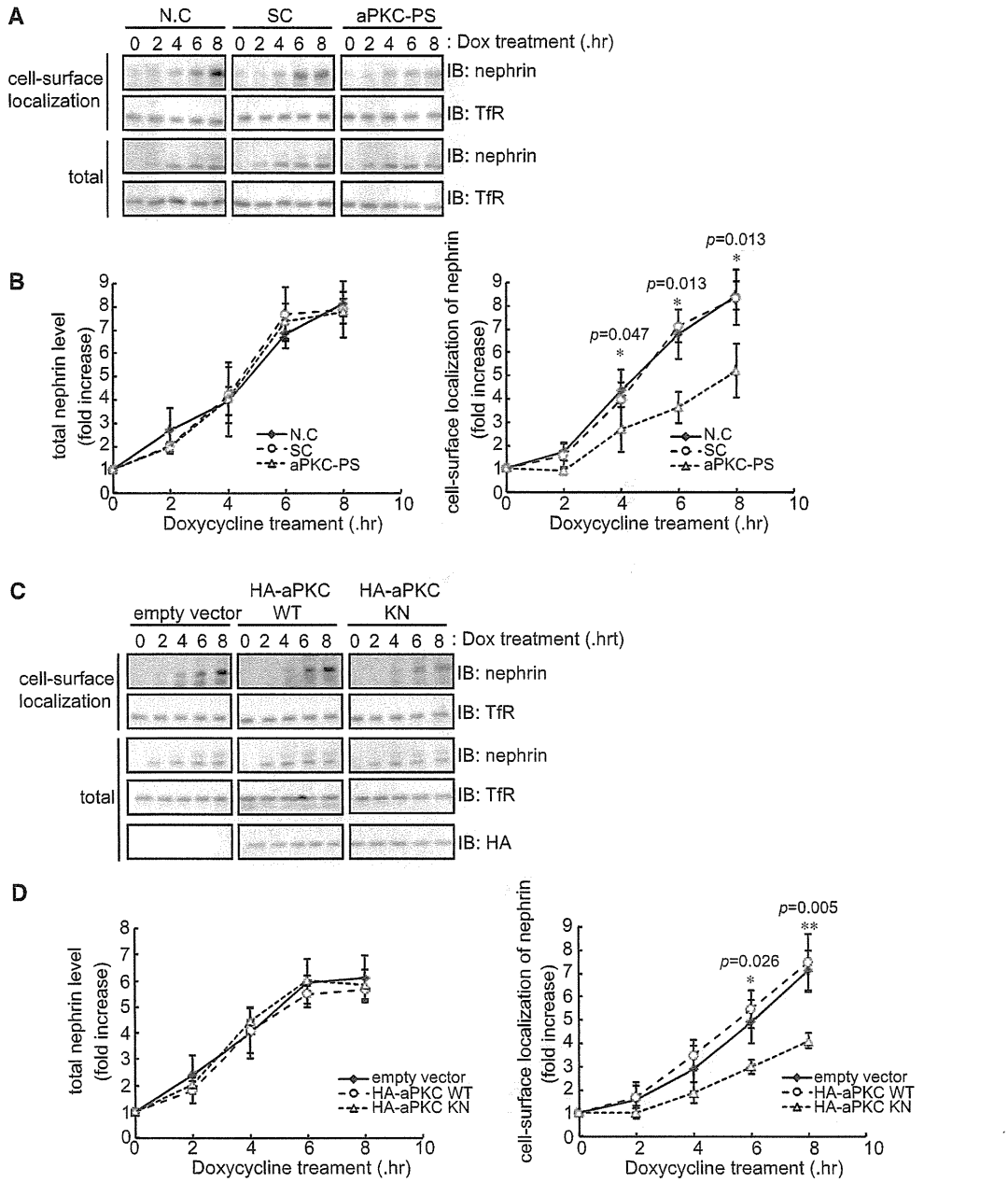

Fig. 4 aPKC is required for exocytosis of newly synthesized nephrin. (**A**) HeLa Tet-On Advanced cells were transiently transfected with nephrin cDNA and incubated for 48 h. After incubation, cells were treated with 20 μM of aPKC-PS or SC for 2 h, and then incubated with 100 ng/ml doxycycline for the indicated times to induce the expression of nephrin. After doxycycline treatment, the cells were subjected to the cell-surface biotinylation assay. (**B**) Quantification of the results in (A). (**C**) HeLa Tet-On Advanced cells were transiently transfected with nephrin and aPKC WT or KN cDNA, and incubated for 48 h. After incubation, the cells were incubated with 100 ng/ml doxycycline for the indicated times to induce the expression of nephrin. After doxycycline treatment, the cells were subjected to the cell-surface biotinylation assay. (**D**) Quantification of the results in (C). The values shown in B and D were normalized to those at the start of doxycycline treatment and are the mean ± SD of three independent experiments. The *P* values were determined by two-tailed Student's *t*-test.

been extensively investigated using cultured podocytes or epithelial cell lines (*50*, *51*). However, these *in vitro* culture systems do not recapitulate the dynamic nature of the SD, thus they are insufficient to directly evaluate the turnover of cell-surface SD components.

By using cell-surface biotinylation and endocytosis assays on isolated glomeruli, we revealed an unexpectedly high turnover rate of cell-surface-localized SD components such as nephrin; they almost completely undergo exocytosis within minutes. We further show that suppression of aPKC kinase activity greatly affected cell-surface localization of SD components, while it did not affect endocytosis, implying the critical role of aPKC on exocytosis of SD components and the critical importance of exocytosis of SD components in maintaining SD integrity. This notion is supported by