Regulation of nephrin gene by the Ets transcription factor, GA-binding protein

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ABSTRACT

Background. Transcription factor GA-binding protein (GABP) is suggested to be involved in the formation of the neuromuscular junctions by regulating the transcription of synapse genes. Interestingly, neurons and podocytes share molecular and functional similarities that led us to investigate the expression and function of GABP in podocytes and its role in transcriptional regulation of nephrin, the key molecule of the podocyte slit diaphragm that is essential for normal glomerular ultrafiltration.

Methods. The expression and localization of GABP in the rat and human kidney as well as in human embryonic kidney A293 cells and undifferentiated and differentiated human podocytes were analysed by immunoblotting and immunostaining. The role of GABP in activating the nephrin promoter was investigated by reporter gene assay and site-directed mutagenesis of the GABP-binding elements, and the interaction of GABP with the nephrin promoter was analysed by chromatin immunoprecipitation. The function of GABP in podocytes was studied by knocking down GABP\(^{\alpha}\) in differentiated human podocytes using lentiviral shRNA targeting GABP\(^{\alpha}\).

Results. GABP is expressed in the nuclei in rat and human glomeruli. In addition, in A293 cells and undifferentiated and differentiated human podocytes, GABP highly enriches in the nucleus. GABP activates and binds nephrin proximal promoter and Ets sites are essential for this activity. Knock-down of GABP\(^{\alpha}\) stimulates apoptosis in cultured podocytes.

Conclusions. The results show that GABP is expressed in podocytes and is involved in the regulation of nephrin gene expression. Furthermore, GABP may be important in the maintenance of podocyte function by regulating apoptosis.

INTRODUCTION

Podocytes are highly differentiated epithelial cells and essential components of the glomerular filtration barrier in the kidney. They are wrapped around glomerular capillaries and form specialized cell–cell contacts called slit diaphragms (SDs) which function as a sieve to prevent the loss of plasma proteins into urine [1]. Nephrin, a transmembrane Ig superfamily protein, is a key component of the SD functioning as a structural component and a regulator of podocyte signalling. The nephrin gene, \(NPHS1\), is mutated in patients with congenital nephrotic syndrome of the Finnish type (CNF) and lack of functional nephrin leads to loss of SDs and massive proteinuria beginning before birth [2–4].

Despite the importance of nephrin for glomerular function, little is known about the regulation of the nephrin gene in podocytes. Studies have focused on the nephrin-enhancer sequences as well as transcription factors involved in the
regulation of nephrin in man and rodents. A 1.125-kb human nephrin proximal promoter and specifically a highly conserved 186-bp fragment within this region drives its expression in podocytes and is specifically bound by WT1 [5, 6]. However, some of the transgenic lines generated with this 186-bp human promoter fragment fused to the lacZ gene showed mosaic and ectopic lacZ expression, suggesting that WT1 probably requires cooperative action of other elements and factors to ensure podocyte-specific expression of nephrin [6]. We found recently that WT1 and NF-kB function cooperatively in the regulation of nephrin gene expression [7], and further, retinoic acid receptors, peroxisome proliferator-activated receptors and proinflammatory cytokines TNF-α and IL-1β also regulate the human nephrin gene [7–11]. In addition, transcription factors Sp1 and Snail as well as 1,25-dihydroxyvitamin D₃, dexamethasone, retinoic acid receptors and proinflammatory cytokines are involved in the regulation of the rodent nephrin gene [12–16].

GA-binding protein (GABP) (also known as adenovirus E4 transcription factor [E4TF-1] or nuclear respiratory factor 2 [Nrf-2]) is an Ets transcription factor generally acting as a transcriptional activator. It is a heterotetramer composed of GABPα and GABPβ subunits which both are necessary to generate a functional complex [17]. Target genes of GABP encode a functionally diverse range of molecules important for basic cell functions, including cell cycle, apoptosis and differentiation [18]. GABP is widely expressed and has been detected in the adult mouse kidney by northern blotting [19]. However, the expression and function of GABP in podocytes is not known.

Podocytes have several features in common with neuronal cells. Both are highly differentiated and specialized cells and share several expression-restricted proteins including nephrin [20]. Moreover, they both possess cell-type-specific cellular contacts, podocytes, the SDs, and neurons, the synapses. In the brain, GABP is suggested to be involved in the formation of the neuromuscular junctions by regulating the transcription of synapse-specific genes including utrophin [21]. In podocytes, utrophin localizes in the cytoplasm of the foot processes and is involved in linking the podocyte cytoskeleton to the glomerular basement membrane [22]. Thus, GABP may also have an important role in podocytes by participating in the transcriptional regulation of podocyte-specific genes. In this study, we report that the transcription factor GABP is expressed in podocytes and show that GABP is involved in the regulation of nephrin gene expression. We further show that knock-down of GABP stimulates podocyte apoptosis.

**Materials and Methods**

**Rat and human kidney material**

The kidneys of male Sprague–Dawley rats (2 months old) were snap frozen in liquid nitrogen and processed for cryosectioning. Animal work was approved by the national Animal Experiment Board. Human kidney samples were obtained from kidneys removed because of tumours and represent the healthy part of the kidneys. The use of human material was approved by the Hospital District of Helsinki and Uusimaa.

**Cell culture**

A293 (human embryonic kidney) cells [23] and human conditionally immortalized podocytes [24] were cultured as described previously [25]. The 293FT cells (highly transfectable clonal isolate from human embryonic kidney cells) were maintained in 5% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium (glucose 4500 mg/mL) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine. Podocytes proliferated at 33°C (undifferentiated podocytes) and were thermoswitched to 37°C for 14 days for growth arrest and differentiation.

**Preparation of glomerular and tubular lysates, total cell lysates and nuclear extracts**

Glomerular and tubular fractions were isolated from rat kidney cortices using a graded sieving method [26]. Glomeruli, tubules and cells were lysed in lysis buffer containing 1% Nonidet P-40, 20 mM Tris–HCl, pH 7.5, and 150 mM NaCl, supplemented with 1× complete, EDTA-free proteinase inhibitor cocktail (Roche, Mannheim, Germany), 50 mM sodium fluoride and 1 mM sodium orthovanadate by rotation at 4°C for 30 min and insoluble material was removed by centrifugation. Nuclear extracts were prepared according to the protocol of Schreiber et al. [27]. Briefly, cells were resuspended in buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1× complete, EDTA-free proteinase inhibitor cocktail and allowed to swell on ice for 15 min. Cell membranes were disrupted by adding Nonidet P-40 at a final concentration of 0.6% and vortexing vigorously. The homogenate was centrifuged and the nuclear pellet was resuspended in buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA and 1× complete, EDTA-free proteinase inhibitor cocktail and rotated at 4°C for 15 min. The samples were centrifuged and supernatants containing the nuclear proteins were collected. The protein concentrations of glomerular, tubular and total cell lysates and nuclear extracts were measured by using a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

**Immunofluorescence microscopy**

Rat and human kidney frozen sections (6 μm), undifferentiated and differentiated podocytes and A293 cells grown on glass coverslips were fixed with paraformaldehyde (PFA) and immunofluorescence stainings were performed as described previously [7], using mouse anti-GABP-α (G-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-GABP-α (H-180; Santa Cruz Biotechnology), mouse anti-WT1 (clone: 6F-H2; Upstate, Lake Placid, NY, USA) and/or mouse anti-Thy1.1 (AbD Serotec, Kidlington, UK) antibodies followed by Alexa Fluor 488 or 555 (Invitrogen, Carlsbad, CA, USA) anti-mouse or anti-rabbit secondary antibodies and Hoechst 33342 (Sigma) as a nuclear marker.
Immunoblotting
Equivalent amounts of glomerular and tubular lysates (75 μg), total cell lysates and nuclear extracts (50 μg) for the detection of GABPα expression or total cell lysates (100 μg) in the GABPα shRNA studies were resolved on 12–15% SDS–polyacrylamide gels and immunoblotting was performed using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) as described previously using 5% skimmed milk for blocking [28]. Rabbit anti-GABP-α (H-180; Santa Cruz Biotechnology), rabbit anti-cleaved caspase-3 (Asp175; Cell Signaling Technology), mouse anti-actin (Sigma) and rabbit anti-histone H3 (Cell Signaling Technology) were used as primary antibodies and Alexa Fluor 680 (Invitrogen) IRDye 800 (LI-COR Biosciences) antismouse or anti-rabbit as secondary antibodies. The signal was detected using the Odyssey infrared imaging system (LI-COR Biosciences) and quantification was performed using Odyssey software.

Construction of reporter gene plasmids
Creation of the reporter construct Nephrin 5′−837-Luc has been described previously [7]. Reporter constructs Nephrin 5′−392-Luc, Nephrin 5′−48-Luc and Nephrin 5′+25-Luc were produced as previously described by PCR amplification from the TOPO construct [25], using Nephrin 5′−392-FW, Nephrin 5′−48-FW or Nephrin 5′+25-FW as a forward primer and Nephrin 5′+156-RV as a reverse primer (Table 1). The constructs were verified by sequencing.

Site-directed mutagenesis
Mutation of the GABP-binding sites was performed by introducing point mutations into Nephrin 5′−48 plasmid using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Oligonucleotides with site-specific mutations are listed in Table 1. The mutations were confirmed by sequencing.

Transfections and luciferase assay
Transfections of nephrin promoter constructs and expression plasmids for GABP-α and/or GABP-β [29], and luciferase assay were performed as described previously [25].

Chromatin immunoprecipitation
ChIP-IT™ Express Kit (Active Motif, Carlsbad, CA, USA) and differentiated podocytes were used for chromatin immunoprecipitation assay as in [25] with rabbit anti-GABP-α antibody (H-180) and rabbit IgG (Zymed Laboratories, South San Francisco, CA, USA) as a negative control. The fragment spanning nucleotides −48 to +44 from nephrin transcription start site was amplified by real-time PCR with the following PCR profile: pre-incubation at 95°C for 10 min, 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. Primers are shown in Table 1. The relative quantity of DNA was counted by comparing the sample fluorescence to the fluorescence values measured from the total chromatin input dilution series. The specificity of the PCR products was verified by both melting curve analysis and agarose gel electrophoresis.

Knock-down of GABP by lentiviral shRNA
Lentiviruses were produced by co-transfecting the pLKO.1 vector containing a short hairpin sequence targeting GABPα (Functional Genomics Unit, University of Helsinki) together with packaging plasmids pCMV-ΔR8.9 and phCMVg into 293FT cells using Lipofectamine 2000 (Invitrogen). Empty pLKO.1 construct was used as a control. Seventy-two hours after transfection, media were collected, filtered through 0.45 μm filter and viruses were concentrated by ultracentrifugation 85 000 × g for 90 min at 4°C and resuspending the pellet in PBS. The viruses were added to cultured podocytes on Day 12 of differentiation, followed by incubation for 10 min at 37°C and centrifugation 1360 × g for 30 min at 4°C. After 48 h, cells were lysed for immunoblotting analysis.

Bioinformatics and statistics
MatInspector software using Genomatix matrixes (www.genomatix.de) was exploited to study the putative transcription factor binding sites in the nephrin promoter. The core similarity of 1.0 and matrix similarity of 0.9 were selected to scan sequences for matches.

Statistical analyses were performed using independent samples t-test. P-values of 0.05 or less were considered to be statistically significant.

RESULTS

GABP is expressed in the nuclei of glomerular cells
The transcription factor GABP has previously been detected in the adult mouse kidney at the mRNA level by northern blotting [19], but the expression and function of GABP protein in podocytes is not known. Here we found by immunostaining of rat kidney sections with GABPα and WT1 antibodies that GABP is expressed in glomeruli and co-localizes with WT1 in podocyte nuclei (Figure 1A–C). Immunostaining with GABPα and Thy1.1 antibodies revealed that GABP is also expressed in the nuclei of mesangial cells (Figure 1D–F). Immunoblotting of rat glomerular and tubular fractions indicated that GABP is primarily expressed in glomeruli (Figure 1G). Immunostaining of human kidney sections with GABPα antibody showed that GABP is expressed in human kidney glomerulus and localizes in the nuclei as confirmed by Hoechst staining (Figure 1H and I).

Immunoblotting of total cell lysates and nuclear extracts prepared from undifferentiated and differentiated human podocytes and A293 cells indicated that GABP is expressed in all cell types and highly enriches in the nuclear fractions (Figure 2). To confirm the nuclear localization of GABPα, we performed immunofluorescence staining and found that GABPα localizes mainly in the nucleus in cultured podocytes and A293 cells (Figure 3A–F).

GABP activates nephrin promoter in A293 cells
GABP binds to DNA sequences that are rich in nucleotides guanine and adenine (5′-GGAA-3′; Ets motif) [30, 31]. Using MatInspector software, we located several putative binding sites in the nephrin promoter. The core similarity of 1.0 and matrix similarity of 0.9 were selected to scan sequences for matches. Statistical analyses were performed using independent samples t-test. P-values of 0.05 or less were considered to be statistically significant.
binding sites for GABP in human nephrin 5-kb 5′ proximal promoter and in the first exon of the coding region. To map the region where GABP might bind, a series of deletion promoter fragments of nephrin were fused to the luciferase reporter gene in pGL3 vector, and the constructs were transiently transfected together with expression vectors for GABP transiently transfected together with expression vectors for porter gene in pGL3 vector, and the constructs were moter fragments of nephrin were fused to the luciferase re- the region where GABP might bind, a series of deletion pro- the Nephrin 5 GABP fold (P < 0.001) and simultaneous overexpression of both GABP expression by 2.0-fold (P < 0.001) and by simultaneous (Figure 4). The effect of GABP activity of the Nephrin 5 GABP +25 construct was minor, increasing its activity by 1.4-fold (P < 0.001) and simultaneous overexpression of GABPα and GABPβ led to no further activation of this nephrin promoter region (Figure 4). Individual overexpression of GABPβ did not have an effect on any of the promoter constructs. These data suggest that GABP can activate transcription of the nephrin promoter and that the nephrin promoter region between −48 to +25 harbours response element(s) for GABP.

**Ets sites are essential for the activity of the nephrin promoter in A293 cells**

To identify functional elements of GABP crucial for the regulation of nephrin promoter, we did computational analysis on the nephrin proximal promoter region −48 to +25 using MatInspector and found three putative GABP-binding sites named Ets(1) (core element −5/−2), Ets(2) (core element +4/+7) and Ets(3) (core element +10/+13) (Figure 5A). To test whether these Ets motifs are required for the activity of the −48 nephrin promoter, site-directed muta- genesis was performed and Ets motif core sequences were single-, double- and triple-mutated from GGAA to TTAA in the Nephrin 5′ −48 construct. These constructs were transi-ently transfected with GABPα and/or GABPβ expression vectors into A293 cells. The promoter activity of the non-mutated Nephrin 5′ −48-Luc construct was increased by 2.8-fold (P < 0.001) by GABPα overexpression and simultaneous overexpression of GABPα and GABPβ increased promoter activity by 12.8-fold (P < 0.001). Mutation of the Ets(1) site reduced the activity of the Nephrin 5′ −48 promoter construct by 48% (P < 0.01). However, GABP overexpression still highly increased the promoter activity of this mutated

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**Table 1. Oligonucleotides used in the study**

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<th>Name</th>
<th>Position</th>
<th>Sequence 5′−3′</th>
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</thead>
<tbody>
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<td><strong>Primers for reporter constructs</strong></td>
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<tr>
<td>Nephrin 5′ −392-FW</td>
<td>−837 to −820</td>
<td>GACACGCCTGTGCTGTGAGTGTGAGACAAGG</td>
</tr>
<tr>
<td>Nephrin 5′ −48-FW</td>
<td>−48 to −28</td>
<td>GACACGCCTAGAGATTGAGACTGAGACGA</td>
</tr>
<tr>
<td>Nephrin 5′ +45-FW</td>
<td>+28 to +45</td>
<td>GACACGCCTAGAGATTGAGACTGAGACGA</td>
</tr>
<tr>
<td>Nephrin 5′ +156-RV</td>
<td>+139 to +156</td>
<td>GACAGATCTCACAGTCCCCCTATCTGT</td>
</tr>
<tr>
<td><strong>Primers for site-directed mutagenesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nephrin 5′ −48/Ets(1) mut</td>
<td>−22 to +17</td>
<td>GAGAGAGAATCCTACAGTTAAGAGGGGAAGAGAAACG</td>
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<tr>
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<td>−12 to +25</td>
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<tr>
<td>Nephrin 5′ −48/Ets(3) mut</td>
<td>−12 to +25</td>
<td>CTCACAGGGAGAGGGTAAAGGGAACAGGAAAGG</td>
</tr>
<tr>
<td>Nephrin 5′ −48/Ets(1 + 2) mut</td>
<td>−12 to +25</td>
<td>CTCACAGTTAAGAGGGTAAAGGGAACAGGAAAGG</td>
</tr>
<tr>
<td>Nephrin 5′ −48/Ets(1 + 3) mut</td>
<td>−12 to +25</td>
<td>CTCACAGTTAAGAGGGTAAAGGGAACAGGAAAGG</td>
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<tr>
<td>Nephrin 5′ −48/Ets(2 + 3) mut</td>
<td>−12 to +25</td>
<td>CTCACAGTTAAGAGGGTAAAGGGAACAGGAAAGG</td>
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<td><strong>Primers for ChIP</strong></td>
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<tr>
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<td>−48 to −28</td>
<td>CAGAGATTGAGACTGAGACGA</td>
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<tr>
<td>Nephrin 5′ +44-RV ChIP</td>
<td>+24 to +44</td>
<td>CTCTTTCCGTACTCTCTCC</td>
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*aPositions in genomic sequence (GenBank accession no. AC002133) related to nephrin transcription start site [41].

bFor oligonucleotides used in mutagenesis, only the sense primer is shown. The core element is underlined and the mutated oligonucleotides are shown in bold.
overexpression of GABPα alone increased the promoter activity by 2.8-fold (P < 0.001) and simultaneous overexpression of GABPα and GABPβ increased by 12.4-fold (P < 0.001) (Figure 5B). Mutations of the Ets(2) and Ets(3) sites reduced the activity of the Nephrin 5'–48 promoter construct much less than Ets(1) mutation, by 22% (not significant) and 17% (not significant), respectively. Nevertheless, promoter activity of constructs with Ets(2) and Ets(3) mutations was only slightly increased by GABP overexpression. Individual GABPα overexpression increased the activity of Nephrin 5'–48/Ets(2)mut-Luc and Nephrin 5'–48/Ets(3)mut-Luc by 1.6-fold (P < 0.01) and 1.2-fold (not significant) and simultaneous GABPα and GABPβ co-transfection 3.3-fold (P < 0.001) and 1.6-fold (P < 0.01), respectively. Double mutation of the Ets(1) and Ets(2) sites, Ets(1) and Ets(3) sites and Ets(2) and Ets(3) sites reduced the activity of the Nephrin 5'–48 promoter construct by 38% (P < 0.05), 43%

**FIGURE 1:** GABP is expressed in the glomerular cell nuclei. (A–C) GABP and WT1 co-localize in rat glomeruli indicating the expression of GABP in podocyte nuclei. (D–F) Double-staining of GABPα and Thy1.1 indicate the expression of GABP in the nuclei of rat glomerular mesangial cells. The arrows indicate GABP-positive podocytes. (G) GABP is mainly expressed in glomeruli. A total of 75 μg of rat glomerular and tubular lysates were separated by SDS-PAGE and immunoblotted with GABPα antibody (H-180). Actin was used as a loading control. (H and I) GABP localizes to the nuclei of human glomerulus. Rat (A–F) and human (H and I) kidney frozen sections were processed for immunofluorescence staining, labelled with GABPα H-180 (A, D and H), WT1 (B) and Thy1.1 (E) antibodies, and Hoechst (I) to identify nuclei. (C) and (F) show the merged images of (A and B) and (D and E), respectively. Samples were examined by fluorescence microscopy. Scale bar, 50 μm.

**FIGURE 2:** GABP is expressed in cultured human podocytes and A293 kidney cells and enriches in the nucleus. A total of 50 μg of total cell lysates and nuclear extracts from cultured undifferentiated and differentiated podocytes and A293 cells were separated by SDS-PAGE and immunoblotted with GABPα antibody (H-180). Actin was used as a loading control for total cell lysates and histone H3 for nuclear extracts.
Promoter activity of the construct with Ets(1 + 2) mutation was increased by GABP overexpression much less than the promoter activity of the construct with individual mutations of Ets(1) and Ets(2) sites. GABPα overexpression increased the activity of the Ets(1 + 2) mutation construct by 1.4-fold (P < 0.01) and simultaneous GABPα and GABPβ overexpression increased the activity of the construct by 1.8-fold (P < 0.001). Promoter activities of the constructs with Ets(1 + 3) and Ets(2 + 3) mutations stayed at a similar level as the activities of their corresponding single mutation constructs by GABP overexpression. GABPα overexpression increased the activity of the Ets(1 + 3) mutation construct by 1.4-fold (P < 0.01) and the activity of the Ets(2 + 3) mutation construct by 1.3-fold (P < 0.01). Simultaneous GABPα and GABPβ overexpression increased the activity of the Ets(1 + 3) mutation construct by 1.4-fold (P < 0.01) and the activity of the Ets(2 + 3) mutation construct by 1.4-fold (P < 0.01). Triple mutations of all three Ets sites in the Nephrin 5′ −48 promoter construct reduced the nephrin promoter activity by 43% (P < 0.05) and GABP overexpression did not increase the activity of this construct significantly. These results suggest that GABP is able to stimulate nephrin transcription through Ets sites located in the proximal promoter.

GABP binds to the nephrin promoter

In order to study the interaction of GABP with the nephrin promoter, we performed a chromatin immunoprecipitation assay. GABPα antibody (H-180) was used to precipitate DNA–protein complexes from differentiated podocytes that express nephrin mRNA endogenously [24]. Immunoprecipitated DNA fragments including GABP-binding sites were amplified by real-time PCR. GABPα was found to bind the nephrin promoter, whereas no binding was seen when using rabbit IgG as a negative control (Figure 6).

Knock-down of GABPα results in caspase-3 activation

To study the role of GABP in podocytes, GABPα was knocked down in differentiated cultured human podocytes using lentiviral shRNA targeting GABPα. Infection resulted in 58% reduction in the GABPα protein level as quantified by normalizing the amount of GABPα to the amount of actin (Figure 7A and B). GABP controls the expression of genes
necessary for several critical cellular functions and thereby participates, e.g. in the regulation of respiration, differentiation and apoptosis [18]. We measured by quantitative immunoblotting the activation of caspase-3 as an indicator of the induction of apoptosis in podocytes after GABP\(\alpha\) knock-down. The results showed 2-fold increase in the expression of cleaved caspase-3 after GABP\(\alpha\) knock-down (Figure 7A and C), indicating that knock-down of GABP\(\alpha\) stimulates apoptosis in cultured podocytes.

**DISCUSSION**

GABP is an Ets transcription factor whose localization and function in the kidney has thus far been uncharacterized. In the brain, GABP is suggested to be involved in the formation of the neuromuscular junctions by regulating the transcription of synapse-specific genes, of which at least utrophin is also expressed in podocytes [21, 22]. Transcription factor WT1 regulates nephrin in podocytes by binding to nephrin promoter at position −654 to −638 bp upstream from the transcription start site [6], and our recent studies suggest that, in the close proximity of the WT1 binding region locates functional sites for NF-κB, a transcription factor that regulates nephrin co-operatively with WT1 [7]. In this study, we concentrated on the more proximal promoter of nephrin, where putative binding sites for GABP were pinpointed. We found that in glomeruli GABP localizes to the nuclei of podocytes and mesangial cells and that GABP stimulates nephrin transcription through functional Ets sites. Furthermore, knock-down of GABP in podocytes induced apoptosis, indicating that GABP may be important in the maintenance of podocyte function.

GABP is unique among the Ets family of transcription factors because it is an obligate multimeric protein complex composed of GABP\(\alpha\) and GABP\(\beta\) subunits. GABP\(\alpha\) contains the DNA-binding domain and GABP\(\beta\) ankyrin repeats that mediate the highly specific dimerization to the \(\alpha\)-subunit. GABP\(\beta\) also harbours a nuclear localization signal that
translocates GABPα to the nucleus [18] and without GABPβ, GABPα localizes in the cytoplasm [17]. Using GABPα antibody, we found that the GABP complex is expressed in the nuclei of the podocytes. GABPα subunit is unable to stimulate transcription by itself, but the interaction with GABPβ that does not contain DNA-binding activity dramatically enhances the DNA-binding capacity of GABPα and transactivation of transcription [17, 18]. In our experiments, overexpression of GABPα alone increased the activity of the nephrin promoter which is most probably due to endogenous GABP having an effect on the overexpressed subunit. In line with the literature, nephrin promoter activity increased remarkably when GABPβ was overexpressed simultaneously with GABPα.

GABP binds to GA-rich sequences in DNA, which is a characteristic of the Ets transcription factor family and the response elements are called Ets motifs (5’-GGAA-3’) [30, 31]. We analysed the three putative Ets motifs in the nephrin promoter by site-directed mutagenesis. Mutation of Ets(1) reduced the basal activity of the nephrin promoter the most, by 48% (P < 0.01), whereas mutation of Ets(2) and Ets(3) reduced the activity only by 22 and 17%, respectively, and were not statistically significant. The decreasing effect of Ets(1) mutation on the basal level activity of the Nephrin promoter was also seen in the double mutation constructs Ets(1 + 2) and Ets(1 + 3) as well as in the triple mutation construct Ets(1 + 2 + 3), but any of the double mutations or the triple mutation did not strengthen the effect of the single mutations. Even though Ets(1) mutation decreased the basal level activity of the nephrin promoter, Ets(1) mutation did not abolish the activation of the nephrin promoter by GABP overexpression, whereas Ets(2) and Ets(3) mutations did. This may be because GABP affects via the other two Ets-elements since double mutation of Ets(1) and Ets(2) sites strengthened the effect of their individual mutations in GABP overexpression studies. Ets(1 + 3) and Ets(2 + 3) mutations did not strengthen the effect of their individual mutations in GABP overexpression studies, but triple mutation of all three Ets sites abolished the effect of GABP overexpression on nephrin promoter activity completely. The results indicate that all three Ets sites play a role in nephrin regulation by GABP. It has been shown that GABPα and GABPβ form an active dimeric complex on a single GABP consensus motif but a tetrameric complex, composed of two GABPα and two GABPβ subunits, can be formed when two GABP-binding sites are present [30]. This can also be the case for nephrin and various Ets sites can function either separately or together, and also differently under special

**Figure 6:** GABPα binds to the promoter region of nephrin in cultured podocytes. In chromatin immunoprecipitation assay, cross-linked chromatin from differentiated podocytes was extracted and precipitated with GABPα (H-180) antibody (ab). Rabbit IgG was used as a negative control. Real-time PCR was performed with specific primers to amplify extracted DNA fragments. The relative quantity of DNA was counted by comparing the sample fluorescence with the fluorescence values measured from total chromatin input dilution series. Columns indicate the means of two parallel samples. The experiment was repeated three times with similar results.

**Figure 7:** Knock-down of GABPα results in caspase-3 activation in cultured podocytes. Representative immunobots (A), quantification of GABPα (B) and cleaved caspase-3 (C). Differentiated cultured podocytes were infected with lentivirus carrying the empty pLKO.1 vector or pLKO.1 expressing GABPα shRNA. 48 h after infection, cells were lysed and 100 μg of total cell lysates were analysed for GABPα, cleaved caspase-3 and actin expression by immunoblotting. Quantification of the immunobots was performed by normalizing the amount of GABPα and cleaved caspase-3 to the amount of actin. The bars (B–C) represent mean ± SD from three replicas. The experiment was repeated three times. (**P < 0.01, *** 0.001; independent samples t-test.)
circumstances such as during differentiation. The identified GABP-binding sites in the nephrin promoter were located very close to the transcription start site. Interestingly, in addition to being a transcriptional activator, GABP has been shown to function as an initiator factor for genes with a TATA-less promoter [32]. Since the nephrin promoter does not harbour a TATA-box, GABP-directed transcription initiation might be involved in nephrin regulation.

GABP is expressed in a wide variety of tissues, and its target genes represent both lineage-restricted and housekeeping genes [18]. For example, GABP is a key transcriptional regulator of genes that function in the formation of neuromuscular junctions [21]. Furthermore, GABP regulates several key myeloid genes and is suggested to be essential in regulating myeloid cell adhesion to endothelium [33–38]. Since nephrin is a component of the SD junction and has a role in cell adhesion in podocytes [28, 39, 40], it is tempting to speculate that by regulating nephrin and possibly other podocyte genes, GABP may also be involved in junction formation and/or regulation of cell adhesion in podocytes. In general, GABP controls gene expression in several critical cellular settings such as respiration, cell cycle, apoptosis and differentiation [18]. Our finding that knock-down of GABPα in cultured podocytes led to activation of apoptosis suggests that GABP has an important role in the maintenance of podocyte function.

The interactions of GABP with other transcription factors and co-activators are key to its ability to regulate gene expression [18]. Many factors influence nephrin expression, including WT1, NF-κB, Sp1, Snail, retinoic acid receptors and peroxisome proliferator-activated receptors [6–10, 12, 13, 15]. Nephrin transcription is most probably regulated by many of these factors acting together to determine the exact cell and developmental stage-specific expression of nephrin. Understanding how the transcription of the nephrin gene is regulated may shed light on the mechanisms that control gene expression generally in podocytes, and help to better understand the differentiation and function of podocytes.

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CONFLICT OF INTEREST STATEMENT

None declared.

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REFERENCES

Regulation of nephrin gene by the Ets transcription factor