Erk 1,2 phosphorylates p27^{Kip1}: Functional evidence for a role in high glucose-induced hypertrophy of mesangial cells

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Abstract

Aims/hypothesis. Mesangial cell hypertrophy is one of the earliest morphological abnormalities of diabetic nephropathy. We have previously shown that high glucose induces p27^{Kip1} by a post-transcriptional mechanism and that mesangial cell hypertrophy depends on G₁-phase arrest mediated by this CDK-inhibitor. However, it remains poorly understood how high glucose stimulates p27^{Kip1} expression in mesangial cells.

Methods. Mesangial cells were isolated from p27^{Kip1} +/+ and –/– mice and characterized by light microscopy and immunohistochemistry. It was tested by Western blotting and autoradiography whether high glucose medium activates Erk 1,2 and whether this activation phosphorylates p27^{Kip1}. The three consensus phosphorylation sites of p27^{Kip1} were mutated and these constructs were expressed in p27^{Kip1} –/– mesangial cells. Hypertrophy was assessed by different methods.

Results. High glucose stimulates phosphorylation of MAP kinases Erk 1,2 in p27^{Kip1} +/+ and –/– mesangial cells. Activation of Erk 1,2 leads to phosphorylation of p27^{Kip1} in vitro and in vivo. Mutations of serine¹⁰ or threonine¹⁸⁷ still supported high glucose-induced hypertrophy. In contrast, a mutation of serine¹⁷⁸ converted the hypertrophic response into a proliferative phenotype. Mutation of serine¹⁷⁸ leads to the attenuated expression of p27^{Kip1} protein in the presence of high glucose.

Conclusions/interpretation. Our study shows that high glucose stimulates Erk 1,2 that phosphorylate p27^{Kip1} at serine¹⁷⁸ increasing its expression. This is an important molecular mechanism of high glucose-induced hypertrophy of mesangial cells. [Diabetologia (2003) 46:1090–1099]

Keywords p27, cyclin dependent kinase, hypertrophy, glucose, mesangial cells.

Glomerular hypertrophy is one of the earliest morphologic changes of Type 1 and 2 diabetic nephropathy, and is principally due to mesangial cell growth [1, 2]. Experimental and clinical studies show that mesangial hypertrophy contributes to the subsequent develop-

ment of glomerulosclerosis and decline in renal function [3, 4, 5]. Thus, delineating the mechanisms underlying mesangial hypertrophy are essential to the design of potential interventions in diabetic nephropathy. We and others have previously shown that cultured mesangial cells exposed to high glucose undergo hypertrophy after 48 h of incubation [6, 7, 9]. Cell cycle analysis showed that cells arrest in the G₁ phase of the cell cycle following prolonged exposure to high glucose-containing medium [6], supporting the idea that the cell cycle plays a critical role in diabetic renal disease.

The cell cycle is positively controlled by cyclins and partner cyclin-dependent kinases (CDK), and is

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negatively governed by CDK inhibitors. We have recently shown that glucose-induced G₁ arrest was associated with an up-regulation of the CDK inhibitor, p27^{Kip1} protein, but not mRNA expression [10]. In vivo studies also showed increased mesangial p27^{Kip1} in models of experimental diabetic nephropathy [11]. A functional role for p27^{Kip1} in mediating mesangial hypertrophy was proven by showing that in contrast to p27^{Kip1} wild-type mesangial cells (+/+), high glucose failed to induce hypertrophy in p27^{Kip1} -/- mesangial cells. Indeed, high glucose facilitated cell cycle progression in p27^{Kip1} -/- cells, but caused cell cycle arrest in p27^{Kip1} +/+ cells [12]. Finally, reconstituting p27^{Kip1} with an inducible vector system restored high glucose-mediated hypertrophic phenotype in p27^{Kip1} -/- cells [12]. These results show that p27^{Kip1} is functionally required for glucose-induced cell cycle arrest and hypertrophy [12].

The mechanisms whereby high glucose increases p27^{Kip1} protein expression by post-transcriptional mechanisms has not been well elucidated. Studies have shown that MAP kinases (Erk 1,2) are activated in diabetic nephropathy, and that inhibition of MAP kinase activation inhibits mesangial hypertrophy [13, 14]. Accordingly, we tested the hypothesis that Erk 1,2 phosphorylates p27^{Kip1} in vitro. In this study we identified specific phosphorylation sites in p27^{Kip1} that are phosphorylated by Erk 1,2, and we utilized mutational analysis to show which of these is required for glucose-induced hypertrophy of mesangial cells.

Material and methods

Cell culture and characterization of mouse mesangial cells. Mesangial cells (MCs) from litter mate p27^{Kip1} +/+ and p27^{Kip1} -/- mice were originally isolated by differential sieving [12]. MCs were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Eggenstein, Germany) containing 100 mg/dl (5.5 mmol/l) D-glucose supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/l glutamine. They were cultured at 37°C in 5% CO₂, and passaged every 4 to 5 days. Experiments were carried out on passage 15 to 30 cells.

Cells of passage 20 were again characterized for this study by light microscopy and immunohistochemistry according to published criteria [13, 14]. Cells were fixed in methanol at -20°C for 20 min and were then washed in phosphate-buffered saline (PBS). Primary antibodies were diluted in PBS containing 1% BSA. All primary antibodies were incubated on the cells at 4°C overnight. The following primary antibodies were used: mouse anti-smooth muscle actin 1:500 (Sigma, Deisenhofen, Germany); mouse anti-desmin 1:100 (Research Diagnostics, Flanders, N.J., USA); mouse anti-vimentin 1:50 (Dako, Carpinteria, Calif., USA); mouse anti-myosin 1:100 (Dako); goat anti-collagen I 1:200 (Southern Biotechnology Associates, Birmingham, Ala., USA); mouse anti-collagen IV 1:50 (Dako); and rabbit anti-AT1 receptor 1:50 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). The unbound primary antibody was washed away and the following secondaries were applied: biotinylated-horse-anti-mouse IgG 1:500 (Vector Labs, Burlingame, Calif., USA), biotinylated-goat-anti-rabbit

IgG 1:200 (Vector Labs), and biotinylated-rabbit-anti-goat IgG 1:200 (Zymed, San Francisco, Calif., USA); and incubated at room temperature for 30 min. Antibodies were visualized using streptavidin-conjugated with Alexa Fluor. 594, 1:200 (Molecular Probes, Eugene, Ore., USA). Images of stained cells were taken on a Leica DMIL inverted microscope (Bartels & Atout, Bellevue, Wash., USA) using a digital CoolSNAPpro camera (Media Cybernetics, Silver Springs, Md., USA).

Leucine Incorporation. The incorporation of ³[H]leucine was used to assess de novo protein synthesis as previously reported [6, 12]. Briefly, cells were plated (10⁵ per well) in 24-well plates, and were made quiescent for 12 h in serum-free media with a normal concentration of glucose (5.5 mmol/l). After an additional 12 h, the medium was replaced with either normal glucose or high glucose (450 mg/dl, 25 mmol/l) for 48 h. 18.5×10⁴ Bq of ³[H]leucine (5.25×10¹² Bq/mmol, Amersham-Pharmacia Biotech, Freiburg, Germany) was added to each well for the last 12 h of the experiment. At the end of the incubation period, MCs were washed twice in ice-cold PBS and proteins were subsequently precipitated with ice-cold 10% trichloroacetic acid. After redissolving the precipitates in 0.5 mol/l NaOH containing 0.1% Triton X-100, 5 ml scintillation cocktail (Roth, Karlsruhe, Germany) was added, and radioactivity was measured by liquid scintillation spectroscopy. ³[H]leucine incorporation experiments were repeated five times with duplicate measurements for each experiment.

Thymidine Incorporation. The incorporation of ³[H]thymidine into DNA was used to measure proliferation [6, 12]. Cells (10⁴ cells per well) were transferred to a 96-well microtitre plate. After incubation for 12 h in normal glucose medium, MCs were subsequently grown for another 48 h in either normal or high glucose. Cells were pulsed with 3.7×10⁴ Bq ³[H]thymidine (18.5×10¹⁰ Bq/mmol, Amersham) during the last 6 h of culture. At the end of the incubation period, MCs were washed in PBS, trypsinized for 10 min at 37°C, and finally collected on glass-fibre paper with an automatic cell harvester. Radioactivity of dry filters was measured by liquid scintillation spectroscopy. ³[H]thymidine experiments were independently carried out four times with triplicate measurements.

Total protein to cell count ratio. The ratio of total protein content to cell number is another well established measure of cellular hypertrophy [12]. To measure this ratio, 10⁵ p27^{Kip1} +/+ and p27^{Kip1} -/- MCs were seeded into each well of a six-well plate and were synchronized into quiescence for 12 h in serum-free medium containing a normal glucose concentration. p27^{Kip1} -/- MCs were transfected with the various constructs and cultured in either normal or high glucose containing medium. In selected experiments, p27^{Kip1} +/+ MCs were also exposed to the specific MAP kinase kinase 1 (MEK1) inhibitor PD98059 [2-(2'-amino-3'-methoxyphenyl)oxanaphthalen-4-one] (10⁻⁵ mol/l, Calbiochem-Novabiochem, Bad Soden, Germany). After incubation for another 48 h, cells were trypsinized, scraped off the plate with a rubber policeman, and washed twice in PBS. A small aliquot of cells were used for cell counting in a Fuchs-Rosenthal chamber after re-suspension in PBS to calculate the cell number/protein ratio. The remaining cells were lysed in 0.5 mol/l NaOH, and total protein content was measured by a modified Lowry method. Total protein content was expressed as µg protein per 10³ cells. These experiments were independently carried out five times.

Cell cycle analysis. In order to carry out cell cycle analysis, cells were trypsinized, washed in ice-cold PBS, and stained for 15 min with 50 µg/ml propidium iodide in staining buffer

(0.1% citrate, 0.1% Triton X100, 50 µg/ml RNase). Cells were analyzed with FACSCalibur (Becton Dickinson, Franklin Lakes, N.J., USA) using CellQuest software (Becton Dickinson). Cell cycle analysis was done with the ModFit LT V 2.0 program (Verity Software House, Topsham, Me., USA). Cell cycle analysis was carried out separately on two occasions. In addition to cell cycle analysis, forward cell scatter was used to measure cell size. Microbead standards (Bangs Laboratories, Fishers, Ind., USA) with defined sizes [4.3, 6.8, 7.9, 8.7, 10.8×10⁻⁶ m diameter) were analyzed in parallel. The peak channel number of each microbead was measured and plotted against bead diameter. A linear relationship was obtained and cell diameters were calculated from this graph. Forward cell scatter experiments were independently done three times (independent stimulation and transfection, if appropriate).

Western blot analysis. MCs were grown in 50 ml flasks in DMEM with 10% FCS to 70 to 80% confluence, at which time the medium was changed to serum-free DMEM for 24 h and cells were subsequently grown in normal or high glucose. At the end of the stimulation period, cells were washed with PBS and lysed with 100 µl lysis buffer (2% SDS, 60 mmol/l Tris-HCl, pH 6.8), and the lysates were cleared by centrifugation. Samples of 80 µg protein (measured by a modification of the Lowry method) were denatured by the addition of 5% glycerol, 5% mercapthoethanol and 0.03% bromophenol blue, and boiled for 10 min. After centrifugation, the supernatants were loaded on a 12% SDS-polyacrylamide gel. Low molecular weight marker (Rainbow-marker, Amersham) served as the molecular weight standard. After electrophoresis, proteins were electroblotted semi-dry for 1 h at 0.8 mA/cm² to a PVDF membrane (Hybond P, Amersham). Selected membranes were stained with Ponceau S (Sigma) to ensure equal loading and transfer of proteins. The blots were blocked in 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 1 h at room temperature. The following antibodies were all used in a 1:1000 dilution: a mouse monoclonal anti-p27^{Kip1} antibody (BD Bioscience, Heidelberg, Germany), a rabbit polyclonal anti-Erk 1,2 antibody, and a monoclonal mouse phospho-specific Erk 1,2 (Thr202/Tyr204) antibody (all from New England Biolabs; Beverly, Mass., USA). Incubation was carried out for 1 h at room temperature. The blots were then washed once for 15 min and twice for 5 min in PBS containing 0.1% Tween 20. After incubation with an appropriate secondary antibody coupled to horseradish peroxidase, blots were washed again for 25 min and the detection was carried out using the ECL system (Amersham). Selected blots were washed and reprobed with an antibody against β-actin (Sigma) to control for small variations in protein loading and transfer. Exposed films were scanned with Fluor-S multi-imager (Bio-Rad Laboratories, Hercules, USA), and data were analyzed with the computer program Multi-Analyst from Bio-Rad. Signal intensities in control lanes were arbitrarily assigned a value of 1.00. Western blots were repeated three times.

Gene construct and transfections. A full length mouse p27^{Kip1} cDNA was cloned into Bluescript (Stratagene, Heidelberg, Germany). The following putative phosphorylation sites for Erk 1,2 were mutated using standard PCR techniques: serine¹⁰ to arginine, serine¹⁷⁸ to alanine, and threonine¹⁸⁷ to alanine. Wild-type and mutated cDNAs were cloned into the pIND vector (Invitrogen, Leek, Netherlands) and were sequenced to confirm correct orientation and the presence or absence of mutations. The expression plasmid pIND contains five modified ecdysone response elements upstream of a minimal heat shock promoter allowing inducible expression. For transient transfections, 10⁵ p27^{Kip1} ^{-/-} MCs were first grown in serum-free

medium for 24 h and then co-transfected with 10 µg of pIND-p27^{Kip1} and 10 µg of the plasmid pVgRXXR (Invitrogen) encoding subunits of the ecdysone receptor using lipofectin (Gibco-BRL) [12]. Cells were then incubated for 48 h in either normal or high glucose in the presence or absence of the inducer muristone (1 µg/ml). Hypertrophy, proliferation, and cell cycle analysis were carried out after 48 h of stimulation.

Expression of recombinant p27^{Kip1} and phosphorylation assays. A full length mouse p27^{Kip1} cDNA with a polyhistidine (6×His) tag cloned into pcDNA 3 was a gift of M. Eilers, University of Marburg, Germany [17]. Recombinant p27^{Kip1} protein was expressed in E.coli and isolated by affinity chromatography using the TALON system (Clontech, Heidelberg, Germany) according to the manufacturer's recommendations. p27^{Kip1} protein was eluted in the second and third fraction. SDS-polyacrylamide electrophoresis and Western blot analysis with a monoclonal anti-p27^{Kip1} antibody revealed the presence of a single band (data not shown). Recombinant p27^{Kip1} (5 µg) was incubated with 2 µg of activated recombinant rat MAP kinase (Erk 2, activity >600 units/µg protein; Calbiochem-Novabiochem, Bad Soden, Germany), 1 µl 10 mmol/l ATP, and 1 µl adenosine 5'-[γ-³²P]triphosphate (>5000 Ci/mmol; Amersham) in kinase buffer (25 mmol/l Tris-HCl, pH 7.5; 2 mmol/l dithiothreitol, 0.1 mmol/l Na₃VO₄, 10 mmol/l MgCl₂) for 30 min at 30°C. The product was separated on a 12% SDS-polyacrylamide gel and autoradiography was carried out for 4 days. Radioactive bands were cut out of the gel, treated with 50 mmol/l ammonium bicarbonate and proteins were precipitated with trichloroacetic acid. After resuspension, proteolyses was done with 6 N HCl for 60 min at 110°C. Electrophoresis was carried out on thin-layer cellulose plates using a Hunter thin layer peptide mapping electrophoresis system [18]. Cold phosphoamino acids (tyrosine, serine, threonine from Sigma) were run together with the sample and served as standards [18]. Bands were visualized with ninhydrin following electrophoresis, and autoradiography of the cellulose plate was then carried out. Phosphoamino acid mapping experiments were done independently on two occasions.

To test whether in vivo activated Erk 1,2 could phosphorylate recombinant p27^{Kip1}, wild-type (p27^{Kip1} ^{+/+}) MCs were exposed for 10 min or 18 h in either normal or high glucose serum-free DMEM. Some cells were also treated with 10⁻⁵ mol/l PD98059. At the end of the incubation, total Erk 1,2 was immunoprecipitated as described above. After extensive washing, immunoprecipitated Erk 1,2 was incubated with recombinant p27^{Kip1} and in vitro phosphorylation was measured.

Statistical analysis. All values are shown as means ± SEM. Statistical significance among multiple groups was tested with non-parametric Kruskal-Wallis test. Individual groups were then tested using the Wilcoxon-Mann-Whitney test. A *p* value of less than 0.05 was considered statistically significant.

Results

Characterization of p27^{Kip1} ^{+/+} and ^{-/-} mouse MCs. Mouse MCs were again characterized for this study. p27^{Kip1} ^{+/+} and ^{-/-} MCs had a "stellate" appearance, overlapped one another and had many processes by light-microscopy (Fig. 1A). They did not grow in the "cobblestone" pattern of glomerular endothelial and epithelial cell types, also they did not form swirls upon confluence as fibroblasts do. p27^{Kip1} ^{-/-} MCs

stained strongly positive for α -smooth muscle actin and, to a lesser extent, positive for the cytoskeletal filaments desmin and vimentin (Fig. 1B). Staining MCs with antibody recognizing collagen type I showed a typical intracellular staining pattern whereas collagen type IV was mainly localized to the basement membrane (Fig. 1B). MCs also expressed angiotensin II receptors of the AT1 type. p27^{Kip1} $\pm/\pm$ MCs showed exactly the same staining pattern (data not shown). Taken together, these findings clearly indicate that our cells were of mesangial origin.

Fig. 1A, B. Characterization of mouse mesangial cells. (A) p27^{Kip1} $\pm/\pm$ and $-/-$ MCs grow in a “stellate” pattern and exhibited many processes. Furthermore, immunohistochemistry exemplarily shown for p27^{Kip1} $-/-$ MCs revealed a strong positive staining for α -smooth muscle (MC) actin, and a lesser positive staining pattern for the cytoskeletal filaments vimentin and desmin (B). Moreover, an antibody against collagen type I stained MCs in a typical intracellular staining pattern whereas collagen type IV was mainly localized to the basement membrane (B). MCs also expressed angiotensin II receptors of the AT1 type whereas negative controls without primary antibody revealed no specific staining. p27^{Kip1} $\pm/\pm$ MCs revealed exactly the same staining pattern (data not shown)

Fig. 2A, B. Western blot for Erk 1,2. Incubation of p27^{Kip1} +/+ (A) and p27^{Kip1} -/- (B) mesangial cells in high glucose (450 mg/dl, 25 mmol/l) containing medium induced after 6 h phosphorylation of Erk 1,2. Phosphorylation was more pronounced for Erk 2 compared to Erk 1. Phosphorylation of Erk 1,2 occurred earlier and was more sustained in p27^{Kip1} -/- cells. The Western blot was reprobed with an antibody against total Erk 1,2 indicating that total amount of Erk 1,2 did not change. This blot is representative of three independent experiments with qualitatively similar results

High glucose mediates Erk 1,2 activation and hypertrophy. To test the hypothesis that the post-transcriptional increase in p27^{Kip1} induced by high glucose requires MAPK activation, we began by measuring ERK1,2 activity. Western blots using a phospho-specific anti-Erk1,2 antibody showed phosphorylation of Erk 1,2 after 6 h exposure of high glucose medium in p27^{Kip1} +/+ and -/- MCs (Fig. 2A,B). Re-probing the Western blot with an antibody to total Erk 1,2 showed equal protein loading.

To test whether inhibition of Erk 1,2 modulates high glucose-induced hypertrophy, p27^{Kip1} +/+ MC were exposed to high glucose serum-free medium in the absence or presence of the MEK1 inhibitor PD98059. Total protein per cell number, a well-established parameter of hypertrophy, was measured. As predicted, exposing mesangial cells to high glucose for 48 h induced hypertrophy. However, co-incubation with 10⁻⁵ mol/l PD98059 prevented high glucose-induced hypertrophy (normal glucose:1.37±0.06, normal glucose + PD98059:1.42±0.14, high glucose:1.74±0.05**, high glucose + PD98059:1.51±0.08 µg protein/10³ cells; ***p*<0.01 vs normal glucose, *n*=6).

MAP kinase phosphorylates p27^{Kip1}. As we showed that an increase in p27^{Kip1} expression is necessary for glucose-induced hypertrophy of mesangial cells [12] we tested whether Erk 2 directly phosphorylates p27^{Kip1}. This isoform was selected because the increase in Erk 2 was more pronounced than Erk 1 in the presence of high glucose (Fig. 2A,B). Exogenous activated Erk 2 enzyme phosphorylated recombinant mouse p27^{Kip1} in vitro (Fig. 3A). Amino acid mapping using two-dimensional thin layer electrophoresis showed that serine and threonine, but not tyrosine residues, are phosphorylated by MAP kinase (Fig. 3B).

In order to investigate whether high glucose mediated activation of Erk 1,2 phosphorylate p27^{Kip1} in vitro, MAP kinases were immunoprecipitated from cells grown in high glucose with an antibody against total Erk 1,2 and immunoprecipitates were used for an in vitro phosphorylation assay of recombinant p27^{Kip1}. Immunoprecipitated complexes containing Erk 1,2 from MCs grown for 18 h, but not for 10 min, in high glucose phosphorylated recombinant p27^{Kip1} (Fig. 3C). In vitro phosphorylation was prevented when Erk 1,2 was precipitated from cells grown in high glucose medium in the presence of PD98059 (Fig. 3C).

Mutation of serine178 prevents the high glucose-induced expression of p27^{Kip1}. The p27^{Kip1} protein contains three consensus sites for MAP kinase-induced phosphorylation (serine¹⁰, serine¹⁷⁸, and threonine¹⁸⁷). These amino acids were individually mutated and cloned into an ecdysone-inducible vector system.

p27^{Kip1} -/- cells were transfected with individual mutant cDNAs and the plasmid pVgRXR encoding subunits of the ecdysone receptor. Transfected cells were exposed to either normal or high glucose for 48 h in the presence of 1 µg/ml muristone, which induced expression. Western blot analysis showed that there was a similar increase in p27^{Kip1} expression in p27^{Kip1} +/+ cells and in p27^{Kip1} -/- MCs transfected with the serine¹⁰ and threonine¹⁸⁷ mutant p27^{Kip1} when exposed to high glucose. In contrast glucose did not increase p27^{Kip1} levels in p27^{Kip1} -/- cells transfected with the serine¹⁷⁸ mutant construct (Fig. 4A; wild-type normal glucose:1.00, wild-type high glucose:3.34±0.7*, serine¹⁰ mutant normal glucose:1.00, serine¹⁰ mutant high glucose:2.5±0.3*, serine¹⁷⁸ mutant normal glucose:1.00, serine¹⁷⁸ mutant high glucose:0.79±0.6, threonine¹⁸⁷ mutant normal glucose:1.00, threonine¹⁸⁷ mutant high glucose:2.06±0.6* relative p27^{Kip1} expression normalized to β-actin, **p*<0.05 versus normal glucose, *n*=3). These results show that the increase in p27^{Kip1} by glucose specifically requires phosphorylation on serine¹⁷⁸.

Functional role of serine 178 mutation of p27^{Kip1} in high glucose-induced hypertrophy. We have previously shown [12] that high glucose inhibits proliferation

Fig. 3A–C. MAP kinase induced phosphorylation of p27^{Kip1}. (A) Recombinant p27^{Kip1} was expressed and incubated with activated phosphorylated Erk 2 in the presence of [γ -³²P]ATP in kinase buffer. Proteins were separated by SDS-polyacrylamide electrophoresis and the autoradiography shows a phosphorylated band at 27 000 Mr. This gel is representative for three independent experiments using different batches of recombinant p27^{Kip1} and Erk 2. (B) Phospho amino acid mapping. Phosphorylated p27^{Kip1} was cut out of the gel and treated as described. Phosphopeptide mapping was done with two-dimensional electrophoresis on thin layer chromatographic plates. Non-radioactive phosphorylated amino acids were run as standards in parallel and were detected with ninhydrin. Erk 2 induces serine and threonine, but not tyrosine phosphoryla-

Fig. 4. Western blot for p27^{Kip1}. p27^{Kip1} cDNAs with mutations encoding serine¹⁰, serine¹⁷⁸, and threonine¹⁸⁷ were transfected into p27^{Kip1} $-/-$ mesangial cells and incubated for 24 h in either normal (NG) or high glucose (HG) with the inducer muristone (1 μ g/ml). p27^{Kip1} $+/+$ wild-type cells, also treated with muristone, served as controls. High glucose stimulates p27^{Kip1} expression in wild-type as well as p27^{Kip1} $-/-$ MCs transfected with serine¹⁰ and threonine¹⁸⁷, but not serine¹⁷⁸ mutants. These blots are representative for three independent experiments

of p27^{Kip1} $+/+$ MCs, but enhances thymidine incorporation in p27^{Kip1} $-/-$ cells (Fig. 5A). In the current study our results show that reconstitution of p27^{Kip1} $-/-$ cells with the serine¹⁰, and threonine¹⁸⁷, but not serine¹⁷⁸ mutants reduced thymidine incorporation in cells grown in high glucose, and thus restored the anti-proliferative effect of high glucose medium (Fig. 5A). Similar results were shown using cell cycle analysis. High glucose arrested p27^{Kip1} $+/+$ cells and p27^{Kip1} $-/-$ reconstituted with serine¹⁰ and threonine¹⁸⁷ mutants in the G₁ phase of the cell cycle, and this was accompanied by significantly fewer cells progressing into the S-phase. In contrast, in p27^{Kip1} $-/-$ cells or MCs p27^{Kip1} $-/-$ transfected with the serine¹⁷⁸ mutant, high glucose-stimulated cell cycle progression (Table 1).

³[H]leucine incorporation, a measure of hypertrophy, was increased by high glucose in p27^{Kip1} $+/+$ and p27^{Kip1} $-/-$ MCs reconstituted with serine¹⁰ and threo-

tion of p27^{Kip1}. Phosphopeptide mapping was carried out twice with similar results. (C) Phosphorylation of recombinant p27^{Kip1} by immunoprecipitated Erk 1,2. MCs were incubated for 10 min or 18 h in normal glucose (NG) or high glucose (HG) in the presence and absence of the MEK1 inhibitor PD98059. Total Erk 1,2 was immunoprecipitated and the precipitates were tested whether they could phosphorylate p27^{Kip1} in vitro. Erk 1,2 immunoprecipitated from mesangial cells grown for 18 h, but not for 10 min in high glucose medium phosphorylate recombinant p27^{Kip1}. This effect was inhibited by the MEK1 kinase inhibitor. This autoradiography is representative for three independent experiments with qualitatively similar results

Fig. 5A–C. Functional consequences of p27^{Kip1} mutations. **(A)** Proliferation measured by ³[H]thymidine incorporation. High glucose medium stimulates proliferation in p27^{Kip1} -/- MCs and cells transfected with the serine¹⁷⁸ mutant. In contrast, high glucose failed to stimulate thymidine incorporation in p27^{Kip1} -/- cells reconstituted with the serine¹⁰ or threonine¹⁸⁷. **p*<0.01 vs normal glucose, *n*=12. **(B)** ³[H]leucine incorporation into de novo synthesized proteins. High glucose stimulates de novo protein synthesis in p27^{Kip1} -/- MCs transfected with the serine¹⁰ or threonine¹⁸⁷ mutant p27^{Kip1} expression vectors, but not with the serine¹⁷⁸ mutant or untransfected p27^{Kip1} -/- cells. **p*<0.01 vs high glucose, *n*=5. **(C)** Measurement of the quotient μg protein/cell number as hypertrophy index. p27^{Kip1} -/- cells reconstituted with p27^{Kip1} serine¹⁰ and threonine¹⁸⁷ mutants develop cellular hypertrophy after exposure to high glucose, but not in p27^{Kip1} -/- cells. The p27^{Kip1} serine¹⁷⁸ mutant failed to reconstitute the hypertrophic phenotype. **p*<0.05 vs high glucose, *n*=5

Table 1. Cell cycle analysis

Group	G ₀ /G ₁	S	G ₂ /M
p27 ^{Kip1} +/+ mesangial cells			
in normal glucose	11.86%	84.78%	3.36%
in high glucose	30.17%	69.83%	0.00%
p27 ^{Kip1} -/- mesangial cells			
in normal glucose	34.39%	65.61%	0.00%
in high glucose	21.56%	78.44%	0.00%
p27 ^{Kip1} -/- mesangial cells transfected with serine ¹⁰ mutant			
in normal glucose	35.27%	64.73%	0.00%
in high glucose	38.00%	62.00%	0.00%
p27 ^{Kip1} -/- mesangial cells transfected with serine ¹⁷⁸ mutant			
in normal glucose	32.38%	67.62%	0.00%
in high glucose	22.08%	75.72%	2.20%
p27 ^{Kip1} -/- mesangial cells transfected with threonine ¹⁸⁷ mutant			
in normal glucose	36.02%	63.98%	0.00%
in high glucose	31.63%	59.75%	8.62%

Mean values from two independent experiments

Table 2. Cell Size (measured by Forward Cell Scatter compared with beads of standard size)

Group	Cell Diameter (10 ⁻⁶ m)
p27 ^{Kip1} +/+ mesangial cells	
in normal glucose	5.71±0.16
in high glucose	6.49±0.10*
p27 ^{Kip1} -/- mesangial cells	
in normal glucose	5.27±0.05
in high glucose	4.88±0.13
p27 ^{Kip1} -/- mesangial cells transfected with serine ¹⁰ mutant	
in normal glucose	5.28±0.14
in high glucose	6.29±0.23*
p27 ^{Kip1} -/- mesangial cells transfected with serine ¹⁷⁸ mutant	
in normal glucose	5.31±0.05
in high glucose	5.17±0.09
p27 ^{Kip1} -/- mesangial cells transfected with threonine ¹⁸⁷ mutant	
in normal glucose	5.60±0.04
in high glucose	6.39±0.19*

Three independent stimulation experiments, **p*<0.05 vs. normal glucose

nine¹⁸⁷ mutants. In contrast, ³[H]leucine incorporation was not increased in p27^{Kip1} -/- cells or p27^{Kip1} -/- cells reconstituted with p27^{Kip1} mutated at serine¹⁷⁸ (Fig. 5B). In addition, calculating μg protein/cell number, a well-established parameter of hypertrophy, also revealed an increase in p27+/+ and p27^{Kip1} -/- cells

reconstituted with serine¹⁰ and threonine¹⁸⁷ mutants after exposure to high glucose, but not in p27^{Kip1} $-/-$ cells transfected with at serine¹⁷⁸. Again, the serine¹⁷⁸ mutant failed to reconstitute the hypertrophic phenotype (Fig. 5C). Finally, we also measured cell size using forward cell scatter. In contrast to p27^{Kip1} $-/-$ cells that slightly reduced their individual size, high glucose increased cell size in p27^{Kip1} $+/+$ MCs. Reconstitution of p27^{Kip1} $(-/-)$ MCs with serine¹⁰ and threonine¹⁸⁷ mutants restored the high glucose-induced increase in cell size in p27^{Kip1} $-/-$ cells. However, the p27^{Kip1} serine¹⁷⁸ mutant was without effect (Table 2). These results show that the hypertrophic and anti-proliferative phenotype induced by high glucose requires phosphorylation of serine¹⁷⁸ of p27^{Kip1}.

Discussion

High glucose induces G₁ phase arrest of cultured mesangial cells with the concomitant development of hypertrophy. We have shown that expression of the CDK-inhibitors p27^{Kip1} and p21^{Cip1} are increased by high glucose in mesangial cells in vivo and in vitro [12, 19, 20, 21]. Studies in p27^{Kip1} $-/-$ and p21^{Cip1} $-/-$ MCs revealed that these CDK-inhibitors are essential for high glucose-mediated hypertrophy of mesangial cells. Furthermore, treatment of BBdp rats, a model of autoimmune diabetes mellitus Type 1, with angiotensin-converting enzyme inhibitors reduced the glomerular expression of p27^{Kip1} and this was accompanied by a decrease in renal hypertrophy [20]. In addition, p21^{Cip1} $-/-$ [21] and p27^{Kip1} $-/-$ [22] mice are protected to some extent of the development of morphological and functional changes of diabetic nephropathy. These studies clearly show a pivotal role for these specific CDK-inhibitors in the development of glomerular hypertrophy of diabetes mellitus.

The observation that high glucose stimulates p27^{Kip1} protein expression in MCs without changes in mRNA abundance suggests a probable post-transcriptional regulation of p27^{Kip1} [10, 12, 19, 23]. Since MAP kinases are activated during diabetic nephropathy, and renal hypertrophy is dependent on MAP kinase activation in other situations [13, 14], in the current study we studied the potential relationship between Erk 1,2 activation and regulation of p27^{Kip1}. Similar to previous studies, we showed Erk 1,2 phosphorylation by high glucose in cultured mesangial cells [13, 14, 24, 25, 26]. The molecular mechanisms underlying high glucose-induced activation of MAP kinases have been recently delineated [27, 28]. Superoxide formation induced by high glucose-mediated activation of the mitochondrial electron-transport chain plays a pivotal role [28]. Excess superoxide inhibits the glycolytic enzyme GAPDH, thereby diverting metabolites from glycolysis into the protein kinase C (PKC) pathway [27, 28]. In turn, stimulation of PKC

activates Erk 1,2, partly by inhibiting expression of MAPK phosphatase-1 (MKP-1; 29).

In this study we showed that inhibition of high glucose-induced Erk 1,2 phosphorylation prevented mesangial cell hypertrophy. Accordingly, we asked if Erk 1,2 directly phosphorylates p27^{Kip1}. Our results showed that active Erk 2 directly phosphorylated recombinant p27^{Kip1} in vitro. Furthermore, Erk 1,2 immunoprecipitated from cells grown in high glucose phosphorylated recombinant p27^{Kip1}, which did not occur when Erk 1,2 was immunoprecipitated from cells grown in medium with normal glucose. Further proof that Erk 1,2 directly phosphorylates p27^{Kip1} was provided by phospho amino acid mapping. We then mutated the three MAP kinase phosphorylation sites of p27^{Kip1}, and showed that changing the serine¹⁷⁸ to alanine prevented glucose-induced p27^{Kip1} expression. Finally, functional studies showed that serine¹⁷⁸ mutation, in contrast to wild-type or serine¹⁰, and threonine¹⁸⁷ failed to promote high glucose-induced hypertrophy of mesangial cells, showing the role for specific protein phosphorylation in mediating the effect of glucose on mesangial cell growth.

p27^{Kip1} expression is thought to be regulated by both translational and post-translational mechanisms, and only under very limited circumstances at the level of transcription [23, 30]. The ubiquitin-proteasome pathway of protein degradation is a major factor in regulating p27^{Kip1} abundance [30]. This is a complex process initiated by CDK2/cyclin E activated phosphorylation of the threonine¹⁸⁷ residue [31, 32]. A multienzyme protein, SCF^{Skp2}, complexes with ubiquitin ligase, then binds to phosphorylated p27^{Kip1} [33]. A critical protein in this complex is Skp2 this assembles the other members of the protein SCF^{Skp2} complex through the F-box motif, while its C-terminal region binds to the phosphorylated threonine¹⁸⁷ of the substrate p27^{Kip1} [33]. It has been recently discovered that the CDK protein Cks1 is an essential accessory factor for the SCF^{Skp2} complex [35]. Cks1 apparently promotes allosteric changes of Skp2 so that SCF^{Skp2} could bind to the phosphorylated threonine¹⁸⁷ of p27^{Kip1}. In addition, Cks1 might also facilitate the recruitment of phosphorylated p27^{Kip1} to the SCF^{Skp2} complex and stabilizes their interaction [35]. Recent studies suggest that p27^{Kip1} degradation is differentially regulated in G₀/G₁ and S/G₂ transitions [37, 38]. One study produced mice expressing p27^{Kip1} in which threonine¹⁸⁷ is mutated to alanine. [38] Although these mice were unable to down-regulate p27^{Kip1} in the S/G₂ phase transition because Skp2 can not bind to p27^{Kip1}, p27^{Kip1} degradation in G₁ was unaffected [38]. Thus, this G₁ phase proteolytic pathway for p27^{Kip1} also required Skp2 binding to p27^{Kip1} but independent of threonine¹⁸⁷ phosphorylation.

The effect of MAP kinase activation on expression of CDK inhibitors varies depending on cell type, stimulus and duration of activation. In fibroblasts, activa-

tion of MAP kinases leads to an increase in p27^{Kip1} degradation that is independent of phosphorylation by CDK2/cyclin E [39, 40, 41]. In contrast, a more delayed and prolonged activation of MAP kinases, for example induced in PC12 cells and fibroblasts by nerve growth factor, leads to cell cycle arrest [42]. This effect is associated by an Erk 1,2-dependent increase in the expression of CDK-inhibitors including p27^{Kip1} [42]. However, the mechanism of this MAP kinase mediated increase in p27^{Kip1} expression has been not investigated.

In agreement with our findings, it has been previously reported that Erk 1,2 phosphorylates p27^{Kip1} in vivo and in vitro [39, 43]. Similar to our experiments, others mutated the three serine/threonine kinase consensus sites of p27^{Kip1} [43]. They found that phosphorylation of serine¹⁰ accounted for 70% of the total p27^{Kip1} phosphorylation [43]. All three p27^{Kip1} mutants inhibited the kinase activity of CDK2/cyclin E or CDK4/cyclin D2 to a similar extent [43]. The same study provides evidence that phosphorylation of serine¹⁰ increased the half-life of p27^{Kip1} about two-fold by promoting binding to CRM1, a carrier protein for nuclear transport, and facilitating cytoplasmic export from the nucleus [44]. In this study, nuclear export controlled by serine¹⁰ phosphorylation was necessary for p27^{Kip1} down-regulation and mitogen-induced G₀/G₁ transition [44]. Others have reported a similar mechanism of serine¹⁰ phosphorylation in nuclear export and cell cycle progression, but found no evidence that this phosphorylation increased p27^{Kip1} expression because serine¹⁰ mutants were efficiently recognized by Skp2 [45]. The effect of serine¹⁷⁸ phosphorylation on p27^{Kip1} stability was not investigated in these studies [44, 45].

However, since high glucose mediates cell cycle arrest with an increase in p27^{Kip1} [10, 12], it is very unlikely that phosphorylation of serine¹⁰ which would induce G₀/G₁-phase progression plays a role in hypertrophy of mesangial cells. Moreover, although one study reported that Erk 1,2 phosphorylates p27^{Kip1} at serine¹⁰ and serine¹⁷⁸ in vitro, serine¹⁰ was not phosphorylated in vivo [43]. This could very well explain our observation that mutation of serine¹⁷⁸ attenuates the high glucose induced increase in p27^{Kip1} and hypertrophy. Assuming that high glucose-induced activation of Erk 1,2 only phosphorylates serine¹⁷⁸ in vivo, it is tempting to assume that phosphorylation of this amino acid might counteract nuclear export transport as mediated by serine¹⁰ phosphorylation. As a consequence, less p27^{Kip1} translocates into the cytoplasm subjected to degradation after binding to Skp2. Alternatively, phosphorylation of serine¹⁷⁸ could directly influence binding of p27^{Kip1} to the SCF^{Skp2} complex by modulating the three dimensional structure of the complex. Further studies are necessary to test these hypothesis. Finally, we have recently observed that under certain conditions cell cycle arrest and hypertrophy are independently regulated by CDK-inhibi-

tors [46]. Transforming growth factor- β still induces G₁ phase arrest in p27^{Kip1} -/- MCs, but not hypertrophy compared to wild-type cells [46]. This observation suggests that p27^{Kip1} can induce hypertrophy by other, currently unknown mechanisms than cell cycle arrest [46]. Perhaps, phosphorylation of serine¹⁷⁸ of p27^{Kip1} could be important for this cell cycle independent pathway of hypertrophy. However, in our study inhibition of proliferation, hypertrophy, and p27^{Kip1} expression was closely associated making this a cell cycle independent mechanism of hypertrophy mediated by p27^{Kip1} less likely.

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