Activation of Cyclin D1-Cdk4 and Cdk4-Directed Phosphorylation of RB Protein in Diabetic Mesangial Hypertrophy

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To determine the role of cell-cycle proteins in regulating pathological renal hypertrophy, diabetes was induced in mice expressing a human retinoblastoma (*RB*) transgene and in wild-type littermates. Whole-kidney and glomerular hypertrophy caused by hyperglycemia was associated with specific G1 phase cell-cycle events: early and sustained increase in expression of cyclin D1 and activation of cyclin D1-cdk4 complexes, but no change in expression of cyclin E or cdk2 activity. Overexpression of RB alone likewise caused hypertrophy and increased only cyclin D1-cdk4 activity; these effects were not further augmented by high glucose. Identical observations were made when isolated mesangial cells conditionally overexpressing RB from a tetracycline-repressible system hypertrophied in response to high glucose. A mitogenic signal in the same cell-culture system, in contrast, transiently and sequentially activated both cyclin D1-cdk4 and cyclin E-cdk2. In vivo and in cultured mesangial cells, high glucose resulted in persistent partial phosphorylation of RB, an event catalyzed specifically by cyclin D1-cdk4. These data indicate that mesangial hypertrophy caused by hyperglycemia in diabetes results in sustained cyclin D1-cdk4–dependent phosphorylation of RB and maintenance of mesangial cells in the early-to-middle G1 phase of the cell cycle.
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Renal hypertrophy is one of the earliest features of diabetic nephropathy, occurring within days of the onset of hyperglycemia. Whole kidneys, glomeruli, and tubules hypertrophy by increase in cell size and by secretion of extracellular matrix (1,2). The increase in kidney size and mesangial mass can be prevented or reversed by maintenance of normal blood glucose concentrations and does not result from osmotic swelling (3). Although proximal tubules comprise the bulk of the renal cortex and contribute to total renal hypertrophy in diabetes, glomerular mesangial cells are just as important in the pathogenesis of diabetic nephropathy. Indeed, the first histologically detectable and relevant features of diabetic nephropathy are thickening of glomerular basement membranes, expansion of the size of the mesangium, and total glomerular enlargement. Moreover, glomerular hypertrophy and expansion of mesangial mass have been correlated with eventual glomerulosclerosis and with detectable albuminuria (4,5).

Control of mesangial hypertrophy at the cellular level is likely to be mediated by specific intracellular signal transduction mechanisms and cell-cycle inhibitory growth factors secreted in response to excess glucose or other components of the diabetic microenvironment (6). Indeed, many studies have suggested that these cell-cycle events are dependent upon the secretion and subsequent effects of transforming growth factor-β (TGF-β) (7–11). Two forms of hypertrophy can be distinguished: cell-cycle dependent and cell-cycle independent (12,13). Cell-cycle independent hypertrophy probably stems from increases in mRNA stability, increases in mRNA translation, or decrements in protein degradation, all of which lead to increases in the overall protein content of the cell. Cell-cycle–dependent hypertrophy is caused by primary or secondary signals that allow cells to enter or remain in the G1 phase of the cell-cycle but not to progress to the S phase (13–16). Synthesis of structural proteins occurs during the G1 phase, without subsequent DNA synthesis or cell division, and causes the cell to hypertrophy.

Hypertrophic signals are believed to activate early G1 cyclin kinase complexes (cyclin D-cdk4/6) but not the late G1/early S phase complex cyclin E-cdk2, which is more directly and proximally responsible for progression from G1 to S phase (13,17,18). Because the activity of retinoblastoma (RB) protein, the crucial gatekeeper during G1 phase, is modulated primarily through phosphorylation by the G1 cdks (19–22), RB is active and able to bind transcription factors, such as E2F, only when partially phosphorylated (hypophosphorylated) by cyclin D-cdk4 (19,23,24). Although RB-independent functions of the E2F family of transcription factors have been suggested, release of free E2F is thought to be required for the G1/S transition, and RB is supposed to keep a cell in the G1/G0 phase as long as it binds E2F. Hypophosphorylated RB also binds to and inhibits the activity of histone deacetylase (HDAC), which normally functions to remodel specific segments of histone-bound chromatin for transcription (25). Activation

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CKI, cyclin kinase inhibitor; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FLS, forward light scatter; HDAC, histone deacetylase; MMC, mouse mesangial cell; MMCtetRB, mouse mesangial cells with tetracycline-controllable expression of human RB transgene; MMCwt, mouse mesangial cells from wild-type littermates; PAS, periodic acid-Schiff; RB, retinoblastoma; STZ, streptozotocin; tet, tetracycline; TGF-β, transforming growth factor-β.

of cyclin E-cdk2 and phosphorylation of RB on additional specific sites (hyperphosphorylation) is required to free HDAC, E2F, and other transcription factors completely and to allow a cell to pass through the G1 restriction point into the S phase. The failure to activate cyclin E-cdk2 may occur through increased expression (i.e., increased transcription or reduced degradation) of the cyclin kinase inhibitors (CKIs), $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, which associate with and inhibit cdk2 (26). It might also occur through release of CKIs from complexes with the G1 cdks. RB thus serves as a focal point for determining whether a cell-cycle is to divide, remain in the G1/G0 phase, or terminally differentiate (27,28).

In G1, RB is sequentially phosphorylated on several serine and threonine residues, first by cyclin D/cdk4 or 6 and then by cyclin E/cdk2 complexes (22,29). One such residue, Ser 780, is a specific target of cdk4 (29–31). Subsequently in the G1 phase, another residue, Ser 795, is phosphorylated. Both cdk4 and cdk2 can phosphorylate GST-$p56^{RB}$ on Ser 795 in vitro (30), and this late G1 event has been shown to be critical for entry into the S phase (30,32,33).

In this report, we show in a mouse model of type 1 diabetes and in cultured cells that mesangial cell hypertrophy in the setting of high glucose is associated with specific and sustained activation of early G1 cyclin D-cdk4 complexes and sustained, cdk4-directed, partial phosphorylation of RB protein.

RESEARCH DESIGN AND METHODS

Induction of diabetes. Streptozotocin (STZ), freshly prepared daily in 10 mmol/l citrate buffer, pH 4.5, was injected intraperitoneally at a dose of 50 μg/100 g body wt for 4 consecutive days (34). The day of the fourth STZ injection, when the animals first developed hyperglycemia, was considered time 0 for induction of type 1 diabetes. Control mice were injected with an equivalent volume of citrate buffer alone. Midmorning blood glucose concentrations were monitored 1, 3, 7, and 9 days after the last dose of STZ to assure that the injected mice became diabetic. Diabetic mice were outwardly normal except for polyuria and slight loss of body mass (Fig. 1*B*); they did not require insulin injections during the 9-day study period.

Mice, kidney sectioning, histological staining, and quantification of glomerular surface area. The RB1 and RB3 lines of mice expressing a human RB transgene have been described (28,35). All mice were fed, housed, and given free access to water in identical cages. The mice were killed by cervical dislocation. Kidneys were harvested immediately and fixed overnight in 10% neutral-buffered formalin. After progressive dehydration and embedding of the kidneys in paraffin, 4-μm sections were stained with Meyer's hematoxylin and eosin or periodic acid-Schiff (PAS) reagents. Stained slides were examined by light microscopy. A digital camera was used to take photomicrographs of several cortical regions. Glomerular surface areas were determined by manual outline of each glomerular cross-section inside Bowmanís capsule, quantification as total number of pixels using Image Pro Plus software (Media Cybernetics), and conversion of pixels to micrometers squared using an internal standard of distance. Total nuclei (including mesangial, epithelial, endothelial, and any infiltrating leukocytes) per glomerular cross-section were counted in the same 4-μm kidney sections.

Cell culture. L9C15 mice expressing the same *RB* transgene from a tetracycline (tet)-repressible promoter system were created by A. Yu. Nikitin and W.-H. Lee (36). Murine mesangial cells (MMCs) were isolated from them and from sex-matched wild-type littermates using standard sieving methods (37). Because murine tubules are difficult to detach completely from glomeruli by sieving and centrifugation alone, individual glomeruli without any visible tubular remnants were picked from plates after 48 h and replated to obtain pure glomerular preparations. MMCs growing from attached glomeruli were carefully removed from the plate with droplets of trypsin-EDTA solution and then replated. MMCs stained positively by indirect immunofluorescence for α-smooth muscle actin and negatively for cytokeratin. Cells were cultured from their inception in Dulbecco's modified Eagle's medium (DMEM) containing 5 mmol/l glucose and supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Mesangial cells (mouse mesangial cells with tet-controllable expression of human RB transgene [MMCtetRB]) grown from mice expressing a human RB transgene under transcriptional control of a tet-repressible promoter were normally maintained in the presence of 1 μg/ml tet to keep transgenic RB protein expression "off." When these cells reached 70% confluence, tet was removed from selected plates to turn RB transgenic protein expression "on." Mesangial cells grown from wild-type littermate mice (MMCwt) were treated identically, i.e., cultured with or without tet. For measurements of kinase activity and phospho-specific RB protein expression as a function of time, cells were made quiescent by serum withdrawal for 24 h before adding 10% FBS or 30 mmol/l glucose at time 0.

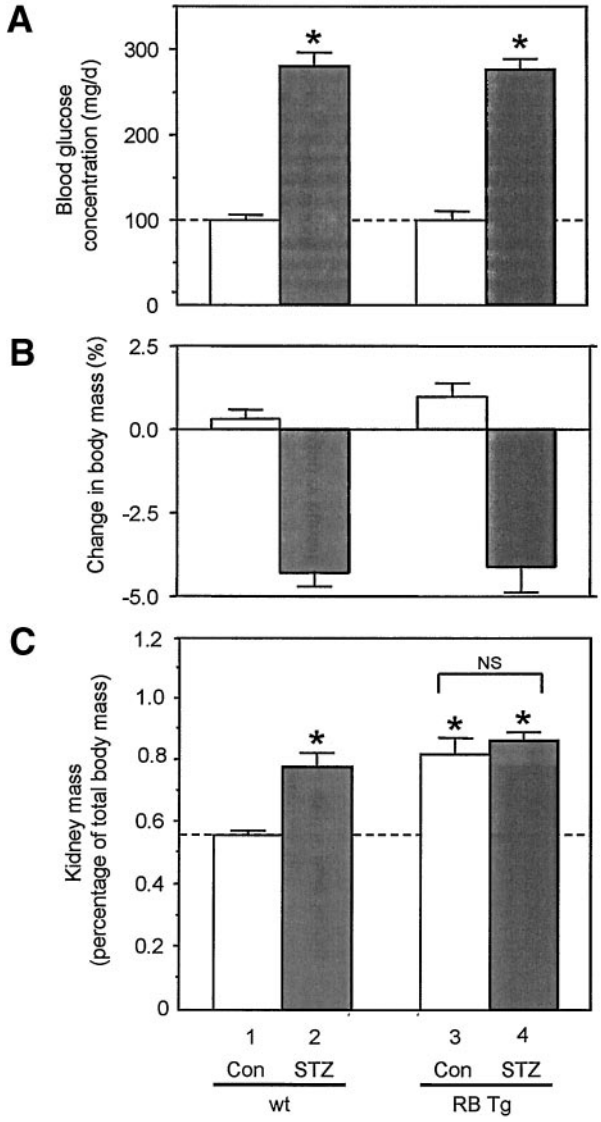

FIG. 1. Effects of diabetes in wild-type mice and in mice overexpressing RB protein. *A*: Hyperglycemia 7–9 days after induction of diabetes by intraperitoneal injection of STZ as described in RESEARCH DESIGN AND METHODS. Histograms represent means ± SD; $n = 4–6$ for each histogram. *B*: Mean change in body mass in the same mice during the study interval of 9 days. *C*: Mean single kidney wet mass at time of death, expressed as a percentage of total body mass. *$P < 0.05$ vs. wild-type control (*lane 1*). □, control mice injected with citrate buffer alone (mean blood glucose concentrations 100 or 103 mg/dl [5.5–5.7 mmol/l]); ■, age and sex-matched mice injected with STZ (mean blood glucose concentrations 280 or 277 mg/dl [15.4–15.6 mmol/l]).

Measures of hypertrophy. Cells were grown with serum to 70–90% confluence in the presence of 10% FBS. The cells were maintained in medium supplemented with FBS for the next 72 h, with or without tet, in the presence of either 5 or 30 mmol/l glucose. Monolayers of adherent cells were removed from the plates with dilute trypsin-EDTA, washed once with DMEM containing 10% FBS, and then washed again in cold PBS. Suspensions of the cells (1×10^6 cells/ml) were fixed in 70% ethanol for 30 min and sorted in a fluorescence-

activated cell sorter. Unitless forward light scatter was measured as a surrogate for cell diameter. Mean values were determined from the peaks of Gaussian distribution curves from 50,000 events counted per experimental condition.

For protein-to-DNA ratios, aliquots of the same cells used for fluorescence-activated cell sorter (FACS) analysis were centrifuged, counted using a hemacytometer, fixed with 70% ethanol, or resuspended in a single detergent protein lysis buffer. Total protein in cell lysates was measured in triplicate using a Bio-Rad colorimetric kit, according to instructions provided by the manufacturer. DNA content was measured by staining the cells for 30 min with propidium iodide (10 μg/ml) and quantifying DNA fluorescence at 260 nm. Protein-to-DNA ratios were then determined, as micrograms protein per micrograms DNA, and compared with those of control cells (MMCwt, 5 mmol/l glucose, no tet).

Antibodies, immunoblots, and kinase assays. Cells were lysed in a buffer containing 50 mmol/l Tris (pH 7.5), 250 mmol/l NaCl, 2 mmol/l EDTA, 1 mmol/l sodium orthovanadate, and 0.1% Nonidet P40. Protein content was measured as above. For immunoblots, 100 μg protein lysate per lane (or amounts otherwise indicated) were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose. After blocking in a 5% nonfat dry milk solution in washing buffer containing 10 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, and 0.05% Tween-20, membranes were incubated overnight at 4°C with primary antibodies, washed, and incubated for 2 h with horseradish peroxidase–coupled secondary antibodies (40 ng/ml) at room temperature. Signals were detected using a chemiluminescent substrate (ECL; Amersham) and recorded on film. Rabbit polyclonal anti-RB (C15), anti-cyclin D1, and anti-cyclin E were obtained from Santa Cruz Biotechnology. The 11D7 mouse monoclonal antibody specific for human RB (28,35) was a gift from W.-H. Lee. Rabbit polyclonal antibodies recognizing phospho-RB-Ser780 and phospho-RB-Ser795 were obtained from Cell Signaling Technology.

For kinase assays, identical aliquots (100 μg) of cellular protein were incubated with molar excess (0.4 μg) of either anti-cdk2 or anti-cdk4 antibody (Santa Cruz) for 2 h at 4°C. Then, 15 μl of protein A/G agarose (Santa Cruz) were added and incubation was carried out for an additional 1 h. Beads were then pelleted by centrifugation, washed three times with lysis buffer and two times with PBS, and resuspended in 20 μl of a kinase buffer containing 50 mmol/l HEPES (pH 7.5), 10 mmol/l $MgCl_2$, 5 mmol/l $MnCl_2$, 2 mmol/l DTT, and 1 mmol/l sodium orthovanadate. The reaction was started by adding a mix of 2 μmol/l ATP and 5 μg of GST-$p56^{RB}$ (27) or 1 μCi of γ-[^{32}P]-ATP and histone H1, continued for 30 min at 30°C and stopped by addition of 6 μl of 6× sample buffer and boiling for 5 min. Phosphorylated substrates were separated by SDS-PAGE and visualized by autoradiography for histone H1 or by immunoblotting with anti-RB Ser 780 for GST-$p56^{RB}$. The intensities of immunoblot signals and phosphorylated substrates on the exposed films were measured by densitometry with National Institutes of Health Image 1.62 software.

Statistical analyses. For analysis of a single variable, Student's *t* tests were performed. When more than one condition was changed in the same experiment, ANOVA was performed instead. In either case, InStat and Prism software (GraphPad) was used to compare mean values and to compute standard deviations and standard errors. Two-tailed $P < 0.05$ was considered significant.

RESULTS

Hyperglycemia associated with STZ-induced diabetes was equivalent in both RB-transgenic mice and sex-matched, wild-type littermates. Diabetic mice in both groups maintained blood glucose concentrations of ~280 mg/dl (15 mmol/l) (Fig. 1*A*). Diabetic mice lost ~4% of their body weight 9 days after induction of hyperglycemia but did not look ill; transgenic and wild-type mice were equivalently affected (Fig. 1*B*). As expected, whole kidney mass was significantly greater in wild-type diabetic mice than in wild-type control mice (Fig. 1*B*, *lane 2* vs. *lane 1*). The kidneys of mice overexpressing RB were also hypertrophied relative to the dwarf body size (Fig. 1*B*, *lane 3* vs. *lane 1*) to an extent similar to that observed in diabetic wild-type animals versus nondiabetic wild-type controls, even if the mice were not diabetic. Induction of diabetes and hyperglycemia in RB transgenic mice did not cause any further renal hypertrophy (Fig. 1*B*, *lane 4* vs. *lane 3*). These data indicate that RB protein may be involved in renal hypertrophy and that RB lies distal to hyperglycemia in the pathway of renal hypertrophy in diabetes.

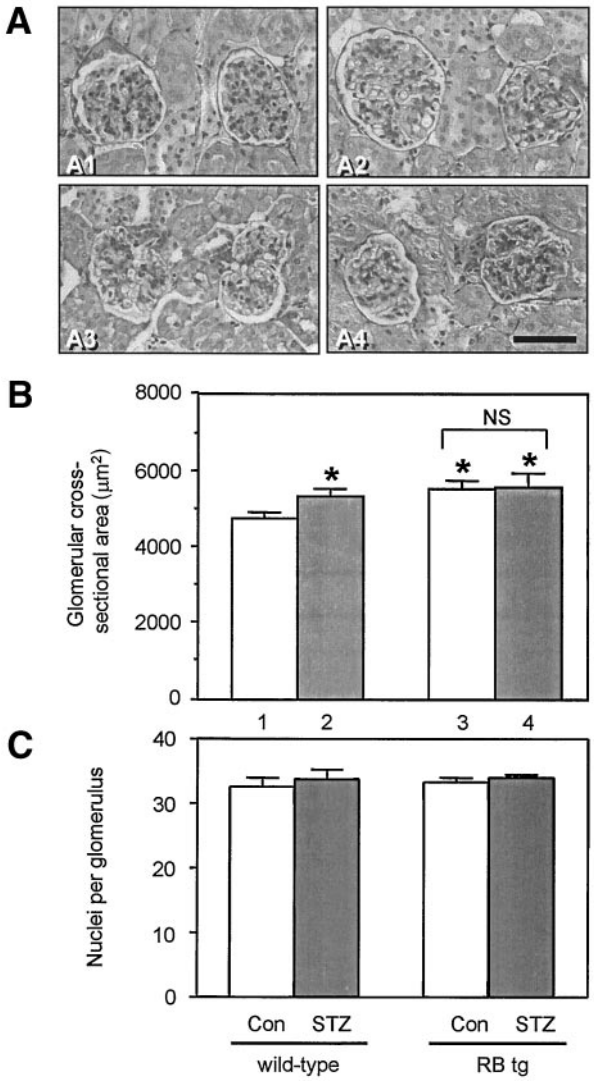

FIG. 2. Glomerular hypertrophy accompanies whole-kidney hypertrophy in diabetic mice and in mice overexpressing RB. *A*: Representative photomicrographs of kidney sections 9 days after four consecutive daily injections with citrate buffer (control) or STZ in citrate buffer. PAS stain; bar = 100 μm. *A1*: Wild-type mouse, citrate buffer alone. *A2*: Wild-type mouse made diabetic with STZ. *A3*: RB transgenic mouse, citrate buffer. *A4*: RB transgenic mouse made diabetic with STZ. *B*: Quantification and comparison of glomerular size. Glomerular cross-sectional areas were measured from kidney sections stained with hematoxylin and eosin or PAS. The histograms represent means ± SE from examining 25–60 individual glomeruli in sections from four individual mice in each group. *$P < 0.01$ vs. wild-type control (*lane 1*). *C*: Quantification and comparison of glomerular cellularity. Total numbers of nuclei per glomerular cross-section were counted in histological sections from the same mice. Neither STZ-induced diabetes nor RB overexpression resulted in any significant hypercellularity at 9 days. tg, transgenic.

We next sought to determine whether glomerular and mesangial hypertrophy paralleled whole-kidney hypertrophy. Glomerular surface area was examined and quantified in histological sections of kidneys removed from control and diabetic wild-type and RB-transgenic mice (Fig. 2*A* and *B*). The mean glomerular area of wild-type diabetic mice was ~15% greater than that of wild-type control mice (Fig. 2*B*, *lane 2* vs. *lane 1*). Glomeruli from RB-transgenic animals were significantly larger than those from sex-

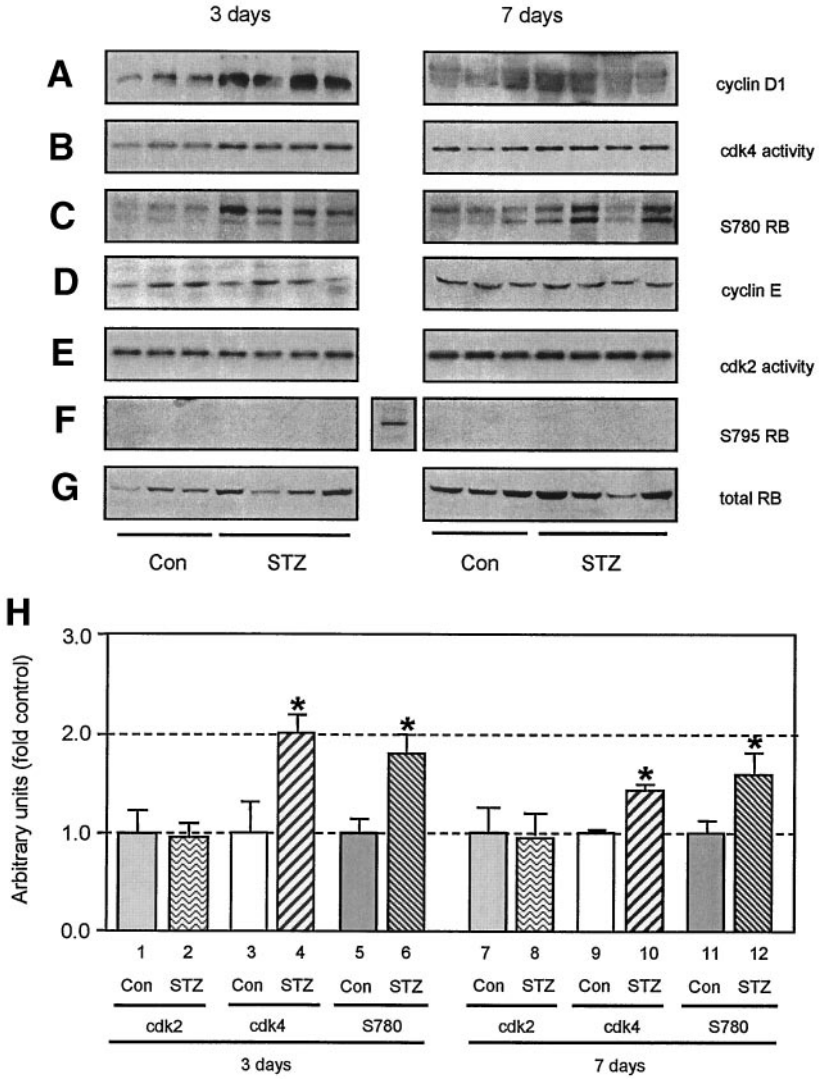

FIG. 3. Increased cyclin D1 expression, cdk4 activity, and cdk4-directed RB phosphorylation in renal cortices from diabetic mice and from mice overexpressing RB from a transgene. Mice were killed 3 or 7 days after induction of diabetes by STZ. *A*: Cyclin D1 expression by immunoblotting. *B*: Cdk4 activity, using GST-p56^{RB} as a substrate, was measured as described in RESEARCH DESIGN AND METHODS. *C*: RB phosphorylated on Ser 780 was measured by immunoblotting with a specific anti–Ser780-phospho-RB monoclonal antibody. *D* and *E*: No significant change in cyclin E expression or cdk2 activity. *F*: RB phosphorylated on Ser 795 was detected in the same lysates using another specific anti–phospho-RB antibody. A positive control for expected RB phosphorylation on Ser 795, lysate from rapidly cycling 786-O renal cell carcinoma cells, was included in the middle panel. *G*: Total RB expression was measured by immunoblot using rabbit polyclonal antibody C15, which recognizes both endogenous murine RB and human transgenic RB proteins (28). *H*: Histograms represent means ± SE from quantification of blots from the renal cortices of six control and seven diabetic mice, only some of which are shown in panels *B*, *C*, and *F*. *$P < 0.05$ vs. control mice.

matched wild-type littermates (Fig. 2*A*, *lane 3* vs. *lane 1*), but did not enlarge further after induction of diabetes (Fig. 2*A*, *lane 4* vs. *lane 3*). No significant change in glomerular cellularity was observed in hypertrophic kidneys (Fig. 2*C*). These results indicate that glomerular hypertrophy accompanies whole kidney hypertrophy caused by diabetes or by overexpression of RB protein and that neither hypertrophic stimulus is associated with mesangial proliferation. Because kidneys and glomeruli in mice overexpressing RB do not hypertrophy further under diabetic conditions, the data suggest that RB lies distal to hyperglycemia in the pathway that leads to both whole kidney and glomerular hypertrophy.

G1 cyclin expression, cdk activity, and site-specific cdk-dependent phosphorylation of RB in the renal cortices of control and diabetic mice were measured 3 and 7 days after STZ-induced diabetes (Fig. 3). At 3 days, cyclin D1 was more abundant in the renal cortices from hypertrophic kidneys in diabetic mice compared with that in control cortices (Fig. 3*A*). Cdk4 activity was also increased approximately twofold in diabetic kidney cortices (Fig. 3*B* and *H*) and was accompanied by a comparable increase in phosphorylation of RB on Ser 780 (Fig. 3*C* and *H*). The amount of total RB, measured by immune blotting, was not significantly different between control and diabetic mice (Fig. 3*H*). Cyclin D1 abundance (Fig. 3*A*), cdk4 activity (Fig. 3*B* and *H*), and phosphorylation of RB on Ser 780 (Fig. 3*C* and *H*) were still higher in diabetic kidney cortices 7 days after the induction of hyperglycemia.

Unlike cyclin D1 expression and cdk4 activity, cyclin E expression (Fig. 3*D*) and cdk2 activity (Fig. 3*E* and *H*) were not increased in diabetic kidney cortices after 3 or 7 days of diabetes and hyperglycemia. This lack of cdk2 activation was paralleled in diabetic kidneys by the lack of detectable phosphorylation of RB on Ser 795 (Fig. 3*D* and *H*). The increase in cdk4 activity and phosphorylation of RB on Ser 780, but lack of activation of cdk2 and phosphorylation of RB on Ser 795 in diabetic hypertrophic kidney cortices, is consistent with block of the cell-cycle in early or mid-G1 during diabetic renal hypertrophy.

Because of the heterogeneous population of cells comprising the renal cortex and glomeruli, definitive conclusions about mesangial hypertrophy could not be drawn from examination of whole-kidneys alone. To investigate whether mesangial cells undergo hypertrophy in high-glucose conditions, cells were isolated from RB transgenic mice and from wild-type mice. Primary cells from mice expressing human RB protein abundantly from a constitu-

tively active promoter (35) could not, however, be propagated, owing to the antiproliferative effects of excess RB (38). To overcome this impediment, mesangial cells were instead grown from mice expressing the same *RB* minitransgene via a tet-regulatable (tet "off") system (36). Mesangial cells from the transgenic mice (murine mesangial cells with tet-regulatable RB expression or MMCtetRB) express the cDNA for human *RB* under control of its own 1.6-kb mini-promoter, as well as eight copies of a tet-repressible promoter upstream (39). When the MMCtetRB were cultured in the absence of tet, they expressed endogenous murine RB in small amounts and human RB from the transgene in great abundance (Fig. 4*A*). The expression of small amounts of human transgenic RB in the absence of tet was expected, due to some "leakiness" in the tet-repressible promoter (39). Preliminary dose-response experiments showed that tet in the culture medium at a concentration of 1 μg/ml maximally repressed the ability of RB to inhibit DNA synthesis and cellular proliferation, and that maximum RB protein expression after withdrawal of tet from MMCtetRB required 72 h (data not shown). MMCwts were isolated from nontransgenic, sex-matched littermate mice and grown in conditions identical to those of MMCtetRB in the various experiments.

Cellular hypertrophy was measured in MMCtetRB and MMCwt after incubation in high glucose concentrations. Hypertrophy was assayed in two ways: by sorting according to cell diameter and forward light scatter (FLS) and by using protein-to-DNA ratios. FLS analysis showed that overexpression of RB caused an increase in cell diameter (Fig. 4*B*), consistent with direct induction of cellular hypertrophy by RB overexpression. Tet itself, as expected, had no effect on cell size in MMCwt. High glucose also caused mesangial cells to hypertrophy. MMCs were cultured in 5 or 30 mmol/l glucose for 3 days before their sizes were measured. Culture in the higher concentration of glucose caused a small increase in the mean diameter of MMCwt cultured with or without tet. MMCtetRB cultured with tet (RB transgene "off") responded exactly like wild-type cells (RB transgene "on"). Overexpression of RB in MMCtetRB caused an increase in cell diameter of 15–40%, corresponding to an increase in cell volume of 150–250%. The hypertrophy caused by RB overexpression was not further increased by high glucose.

Cellular hypertrophy was also measured by protein-to-DNA ratio (Fig. 4*C*). Using this method, high glucose consistently caused hypertrophy in MMCwt (Fig. 4*A*, *lane 2* vs. *lane 1*) and in MMCtetRB cultured with tet (Fig. 4*A*, *lane 4* vs. *lane 3*). Overexpression of RB in MMCtetRB caused hypertrophy to a similar degree, even in 5 mmol/l glucose (Fig. 4*A*, *lane 5* vs. *lane 3*). The hypertrophy caused by RB overexpression was not further increased by high glucose (Fig. 4*C*, *lane 6* vs. *lane 5*). Both methods of measuring hypertrophy in mesangial cells thus confirmed the observations first made in whole-kidneys from diabetic mice. They also showed that cultured mesangial cells could be used to examine mechanisms of hypertrophy in response to high concentrations of glucose.

Next, the activities of cyclin-dependent kinases important for G1 phase progression were determined in MMCs cultured in high glucose concentration for periods of time preceding the development of the hypertrophic phenotype. In MMCtetRB expressing normal amounts of RB

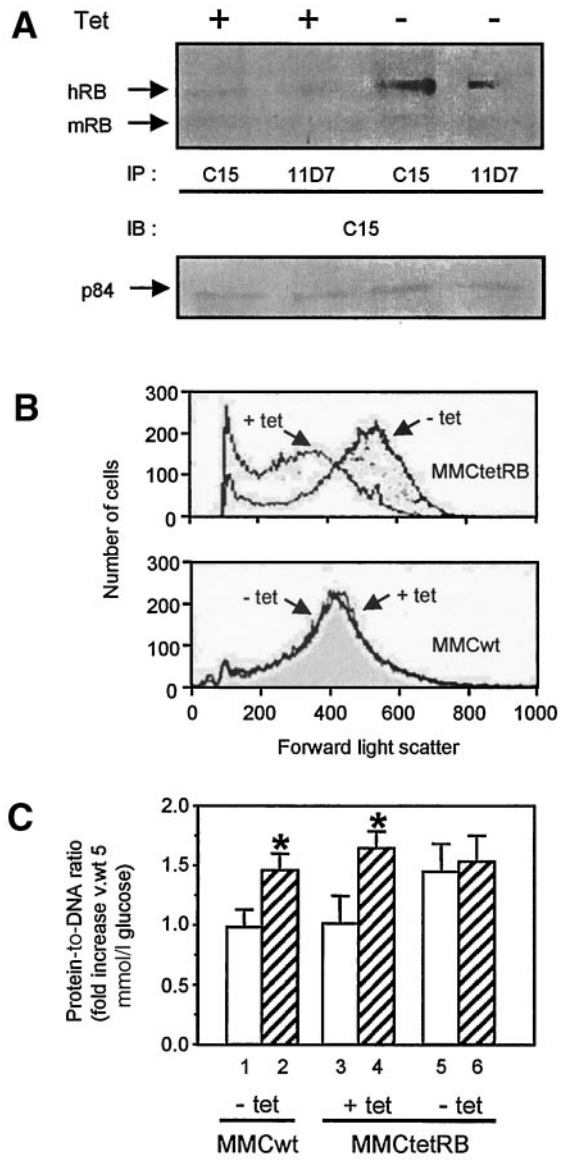

FIG. 4. MMCs hypertrophy in response to overexpression of RB or high glucose concentrations. *A*: Tet-regulatable overexpression of RB protein in mesangial cells. MMCtetRB were cultured in medium containing serum, in the presence or absence of 1 μg/ml tet, for 3 days. RB was immunoprecipitated with either an antibody that recognizes both murine and human RB (C15) or an antibody that recognizes only human RB (mouse monoclonal antibody 11D7 [28,35]). The hypophosphorylated form of murine RB migrates with an apparent molecular mass of 105 kDa and human RB with an apparent mass of 110 kDa. The protein expressed by the human RB transgene is conditionally expressed in large amounts in the absence of tet, in minimal amounts in the presence of tet. Equal loading in each lane was verified by Western blotting of the first immunoprecipitate wash from each sample for p84, a constitutively expressed nuclear matrix protein (45). *B*: Example of increased cell size measured by FACS in MMCtetRB cultured in the absence tet (–tet, RB transgene "on"), compared with identical cells cultured in the presence of tet (+tet, RB transgene "off"). Tet had no effect on the size of wild-type cells (MMCwt). Number of cells (*y*-axis) is plotted against cell size (*x*-axis, arbitrary units) measured as forward light scatter. *C*: Quantification of cellular hypertrophy by protein-to-DNA ratio. The same cells were cultured in the same conditions, but hypertrophy was measured as micrograms protein–to-micrograms DNA ratio, expressed as percent of control (MMCwt, 5 mmol/l glucose, −tet). Histograms represent means ± SE from three independent experiments, each performed on cells from three separate plates. *$P < 0.05$ vs. MMCwt, 5 mmol/l glucose, −tet.

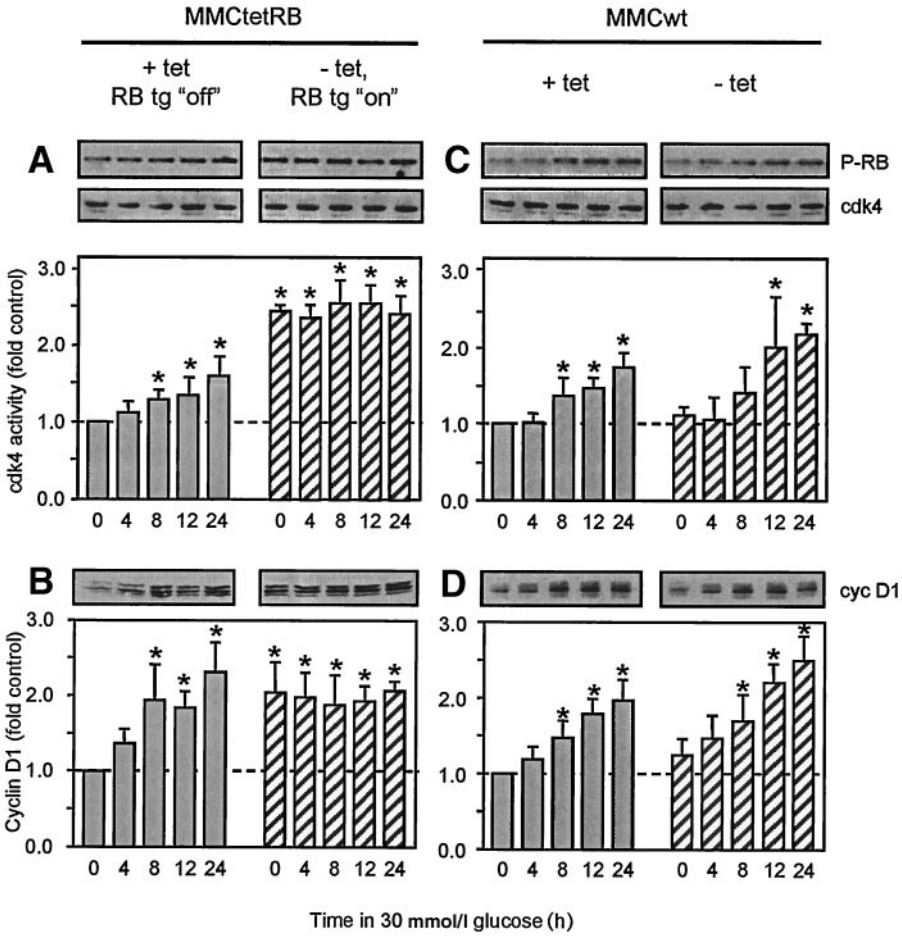

FIG. 5. High glucose or RB overexpression causes time-dependent sustained increases in cdk4 activity and cyclin D1 expression. *A*: cdk4 activity in MMCtetRB. *B*: cyclin D1 expression in MMCtetRB. *C*: cdk4 activity in MMCwt. *D*: cyclin D1 expression in MMCwt. **P* < 0.05 vs. untreated, +tet. MMCs were made quiescent by culture in serum-free medium for 48 h before the addition of 25 mmol/l glucose (30 mmol/l final concentration) for 0–24 h, in the presence (■) or absence (▨) of 1 μg/ml tet, for a total of 3 days before addition of glucose. For each panel, a representative immunoblot or kinase assay (using glutathione S transferase p56RB as substrate) and quantification of three to five independent experiments are shown. In panels *A* and *C*, equivalent aliquots of the same cellular lysates were separated by SDS-PAGE and immunoblotted with an anti-cdk4 antibody to assure equivalent loading of each lane in the kinase assay. Histograms represent means ± SE.

(plus tet, *RB* transgene expression "off"), high glucose activated cdk4 in a sustained manner, starting at 8 h and lasting for >24 h (Fig. 5*A*, 72-h data not shown). Overexpression of RB by itself also significantly activated cdk4, even in the absence of high glucose. In these conditions, cdk4 was not further activated by high glucose. The activity of the cyclin D-cdk4 complex is determined primarily by the relative abundance of its regulatory cyclin subunit (40). As expected, therefore, cdk4 protein amounts in cells cultured with high glucose or in cells overexpressing RB protein did not change significantly. Cyclin D1 abundance, however, was increased by high glucose concentrations, with a time course that precisely matched cdk4 activity (Fig. 5*B*) When RB was overexpressed, cyclin D1 was abundantly expressed compared with control cells but did not increase further after incubation with high glucose. Tet by itself had no effect on cdk4 activity or cyclin D1 expression in MMCwt (Fig. 5*C* and *D*). In MMCwt, high glucose increased cdk4 activity and cyclin D1 abundance in a manner very similar to that seen for MMCtetRB cultured with tet (plus tet, RB transgene "off"). These results show that high glucose and overexpression of RB activate cdk4 in mesangial cells by increasing the expression of cyclin D1, its binding partner.

Unlike cdk4, the cyclin-dependent kinase that functions later in G1, cdk2, was not activated in MMCs cultured in high glucose (Fig. 6), regardless of whether RB was overexpressed. The pattern of G1 cyclin and cdk activation in MMCs cultured in high glucose was the same as that observed in kidney cortices early after the induction of diabetes and hyperglycemia (Fig. 3). These findings suggest that high glucose concentrations in cultured mesangial cells mimic hyperglycemia in vivo, and that hyperglycemia is responsible for the changes in cyclin D1/cdk4 observed in diabetic kidneys, glomeruli, and mesangial cells.

The pattern of cdk4 and cdk2 activation with a mitogenic stimulus was very different from that observed for hypertrophic stimuli in the same MMCs (Fig. 7). Serum was used as the mitogen because of its predictable potency in murine mesangial cells. In contrast to high glucose or RB overexpression, i.e., hypertrophic stimuli, serum caused rapid, sequential, and transient activation of both cdk4 and cdk2. Activation of cdk2 occurs later in G1 and is required for cells to pass the restriction point and to enter the S phase (18,22).

Finally, to show the connection between activation of cyclin-cdk complexes and the resultant kinase activity on the most pertinent substrate, the consequence of increased cdk4 activity on RB protein was examined using specific anti–phospho-RB antibodies in MMCtetRB. Phosphorylation of RB on Ser 780, a target for specific cdk4-dependent phosphorylation in vitro (30,31), closely paralleled cdk4 activation. Increased phosphorylation of RB on Ser 780 started at 8 h after the addition of high glucose and was sustained for >24 h (Fig. 8*A*, *left panels*, 72-h data not shown). When RB was overexpressed, with or without high glucose, Ser 780 was phosphorylated

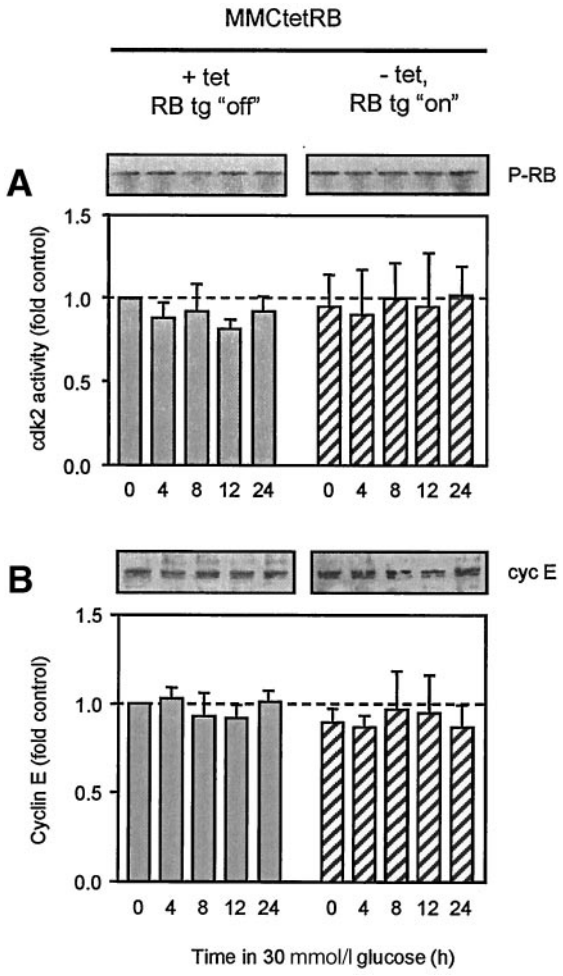

FIG. 6. Neither high glucose nor RB overexpression change cdk2 activity or cyclin E expression. *A*: Cdk2 activity in MMCtetRB. *B*: cyclin E expression in MMCtetRB. None of the mean activities or amounts of protein was significantly different from control values (MMCtetRB +tet or MMCwt −tet). Experiments were performed with aliquots of the same cells used to generate the data in Fig. 5. Very similar results were obtained with MMCwt (data not shown).

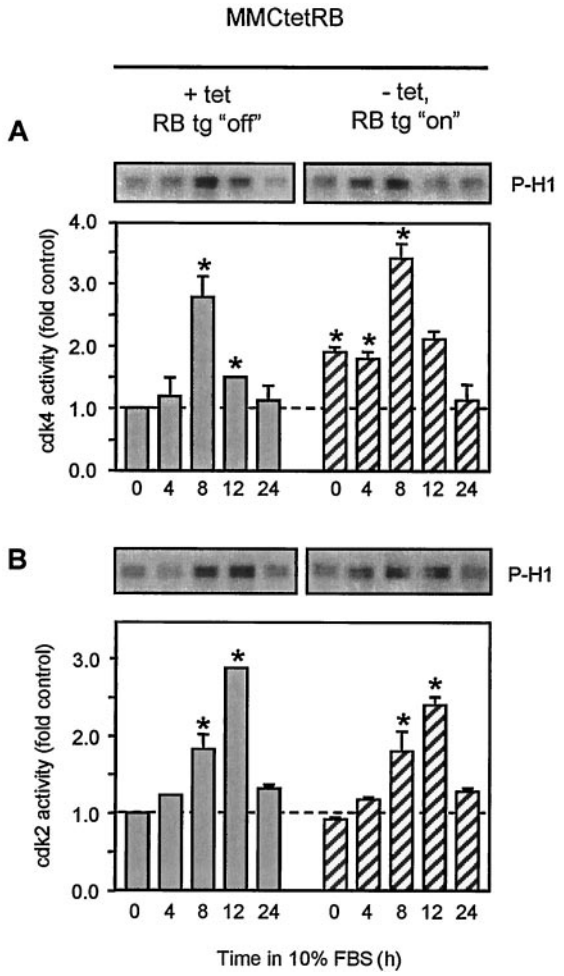

FIG. 7. Rapid but transient activation of both cdk4 and cdk2 by a mitogen in MMCtetRB. *A*: Cdk4 activity. *B*: cdk2 activity (*$P < 0.05$ vs. untreated, +tet). Cells were made quiescent by culture in serum-free medium for 24 h before addition of 10% FBS for 0–24 h in the presence (■) or absence (▨) of 1 μg/ml tet. Tet was added to the medium in appropriate culture plates for 3 days before addition of FBS. For each panel, a representative kinase assay (using histone H1 as substrate) and quantification of at least three independent experiments are shown. Histograms again represent means ± SE.

maximally (Fig. 8*A*, *right panels*). In contrast, phosphorylation of RB on Ser 795 failed to increase with either high glucose or RB overexpression (Fig. 8*B*). The activation of cyclin D1-cdk4 complexes that precedes and accompanies mesangial cell hypertrophy in the setting of high glucose concentrations therefore resulted in specific, cdk4-directed RB phosphorylation early in G1 phase. Neither cyclin D1-cdk4 nor cyclin E-cdk2 resulted in significantly phosphorylation of RB on the Ser 795 residue crucial for passage into the S phase (30).

DISCUSSION

Cdk4 and RB are involved early in the pathway to diabetic mesangial hypertrophy. We have shown that high glucose results in patterns of G1 cyclin expression and cyclin-dependent kinase activation that fit with the current paradigm explaining how cyclin D-cdk4 and cyclin E-cdk2 sequentially activate or inactivate RB during the G1 phase of the cell-cycle (22). To our knowledge, our report is the first to show that hyperglycemia regulates cyclin D1 expression, activates cdk4, and specifically phosphorylates RB on a cdk4-directed serine residue (Ser 780) before mesangial cell hypertrophy. This sequence of events places RB directly in the pathway of glucose-induced renal and mesangial cell hypertrophy. Furthermore, our data provide the first evidence that high glucose results in activation of cdk4 very early in the pathway, leading to mesangial hypertrophy. Because the G1 cell-cycle events occur within hours of incubation of cultured mesangial cells with high concentrations of glucose, they are unlikely to be the indirect result of cell-cycle inhibitory properties of growth factors. Cdk6 and cdk4 are highly homologous (40), but similar activities for cdk4 and cd6 in phosphorylating RB in vivo have never been demonstrated. Indeed, recent studies (41,42) and preliminary experiments in our lab suggest that cdk6 binds preferentially to cyclin D3, not to cyclin D1, and that cdk6-cyclin D3 complexes are more directly involved in progression to the S phase and cellular proliferation than in earlier G1 events associated with hypertrophy (data not shown).

According to data presented here, the cellular pathway to whole kidney, glomerular, and mesangial cell hypertrophy involves early and sustained expression of cyclin D1,

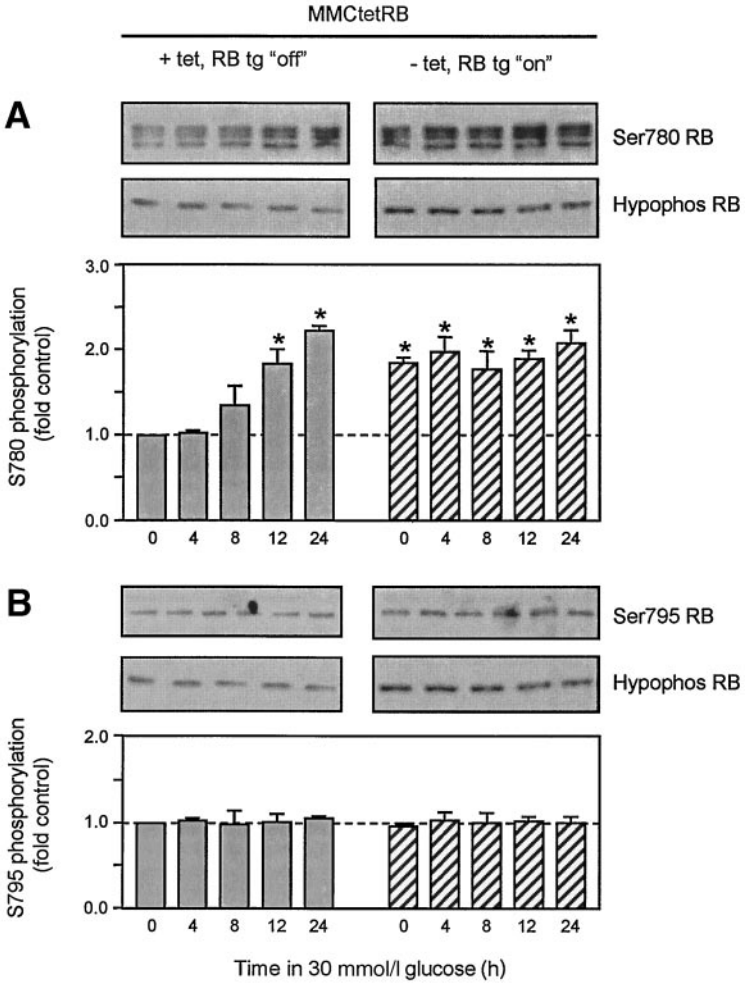

FIG. 8. High glucose induces cdk4-dependent RB phosphorylation in MMCs. Cells were made quiescent by serum deprivation for 24 h before incubation with 30 mmol/l glucose for 0–24 h in the presence (■) or absence (▨) of 1 μg/ml tet. Conditions were identical to those used in Fig. 5. The abundance of RB phosphorylated on Ser 780 or Ser 795 was detected with the same specific anti–phospho-RB antibodies used in Fig. 3. Total hypophosphorylated RB expression was measured by immunoblotting using the C15 antibody (*P < 0.05 vs. 0 h, 5 mmol/l glucose, +tet).

activation of cyclin D1-cdk4 complexes, and phosphorylation of RB by cdk4 on specific residues, including Ser 780. Such specific phosphorylation may cause conformational change, allowing RB to bind and positively regulate factors responsible for transcription of structural proteins during early or mid-G1 phase, but at the same time still negatively regulate E2Fs and HDAC (22,25). This early pathway may be reversible if stopped quickly enough, before secreted growth-inhibitory factors or their receptors could be upregulated, and before the extracellular matrices for sclerosis could accumulate.

Role of RB and RB phosphorylation in the pathway of high-glucose–induced hypertrophy. The same patterns of G1 cyclins and cyclin-dependent kinase activation are associated with renal and glomerular hypertrophy in vivo and with mesangial cell hypertrophy in culture. The effects of high glucose in both systems are associated with cyclin D1-cdk4–dependent partial phosphorylation of RB protein. Forced expression of hypophosphorylated RB resulted in nearly the same pattern of G1 cyclin expression, cdk4 activation, and specific cdk4-dependent RB phosphorylation as high glucose concentrations. Whereas the weaker activation of cdk4 by high glucose could be augmented further by overexpression of RB, high glucose did not further augment cyclin D1 expression (data not shown), cdk4 activity, or mesangial hypertrophy when hypophosphorylated RB was already overexpressed. Precisely how high glucose and overexpression of RB mediate hypertrophy remains to be explored.

Proximal tubular hypertrophy versus mesangial hypertrophy. Whether our findings in mesangial cells are applicable to other kidney cells and to the early cell-cycle–dependent events resulting in tubular hypertrophy also remains to be determined. In investigating proximal tubular cell hypertrophy in experimental diabetes, Preisig et al. (9,13) have suggested that the response of proximal tubules to diabetes and high glucose concentrations is initially proliferative. They argue convincingly that this initial response is converted by autocrine and paracrine secretion of TFG-β and by upregulation of TFG-β receptors to a hypertrophic response that predominates and persists for days (7,43). In cultured proximal tubular epithelial cells, these same investigators implicated RB in the analogous conversion of an epidermal growth factor–induced hyperplastic response to a hypertrophic response by the addition of an antiproliferative factor, TGF-β (44). The importance of functional RB in this process was demonstrated by prevention of the TGF-β–induced hypertrophy by treatment with DNA tumor virus oncoproteins, which functionally inactivate RB.

Diabetic nephropathy is characterized predominantly not by hyperplasia but by hypertrophy of both mesangial and tubular epithelial cell types. Eventually this results in a nonproliferative, sclerotic response in mesangium, glo-

meruli, and tubulo-interstitium. It seems likely that the findings presented in this present study are applicable to diabetic renal hypertrophy in general as well as to mesangial cell hypertrophy. Activation of cyclin D1-cdk4 and partial modification of RB protein by specific cdk4-directed phosphorylation occurs very early in response to high glucose. These events delay progression through the cell-division cycle by preventing cells from passing through the G1 restriction point characterized by cyclin E-cdk2 activation.

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