High Glucose Stimulates Synthesis of Fibronectin via a Novel Protein Kinase C, Rap1b, and B-Raf Signaling Pathway*

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The molecular mechanism(s) by which high glucose induces fibronectin expression via G-protein activation in the kidney are largely unknown. This investigation describes the effect of high glucose (HG) on a small GTP-binding protein, Rap1b, expression and activation, and the relevance of protein kinase C (PKC) and Raf pathways in fibronectin synthesis in cultured renal glomerular mesangial cells (MCs). In vivo experiments revealed a dose-dependent increase in Rap1b expression in glomeruli of diabetic rat kidneys. Similarly, in vitro exposure of MCs to HG led to an up-regulation of Rap1b with concomitant increase in fibronectin (FN) mRNA and protein expression. The up-regulation of Rap1b mRNA was PKC- and B-Raf-dependent and PDGF-independent, but involves B-Raf, which was largely unaffected by anti-HG. These findings indicate that hyperglycemia and HG cause an activation and up-regulation of Rap1b in renal glomeruli and in cultured MCs, which then stimulates FN synthesis. This effect appears to be PKC-dependent and PDGF-independent, but involves B-Raf, suggesting a novel PKC-Rap1b-B-Raf pathway responsible for HG-induced increased mesangial matrix synthesis, a hallmark of diabetic nephropathy.

Diabetic nephropathy is a common complication of both type-I and -II diabetes mellitus, and it is characterized by excessive accumulation of extracellular matrix (ECM) proteins in the renal glomerulus (1). The major ECM proteins include various types of collagens, laminin, fibronectin, and proteoglycans, and they are an integral part of the capillary basement membrane and mesangial matrix, the latter being situated in the intercapillary region of the glomerulus (2). Several animal and human studies have revealed an altered synthesis or expression of various mesangial ECM proteins, especially of fibronectin, type-I, -III, and -IV collagens, and proteoglycans in diabetic nephropathy (1, 3). Similarly, in vitro cell culture studies indicate that high glucose induces an increased synthesis of various ECM proteins expressed in glomerular mesangial and epithelial cells (4). The initial events include increased proliferation and activation of specific ECM receptors (RAGE) leads to an activation of the PKC pathway followed by that of transcription factors, which then affect the target genes and cause increased expression of fibronectin (9, 15).

Fibronectin, a large glycoprotein consisting of two similar polypeptide chains, is a key component of the mesangial matrix (16). It may exist in a soluble dimeric form or as oligomers of fibronectin or a highly insoluble fibrillar form in the extracellular matrix. The latter form has been shown to modulate various biological processes such as cell adhesion, migration, and differentiation (17). Fibronectin matrix assembly is a highly ordered stepwise process, and many domains along its monomer have been shown to be important for its formation diacylglycerol; GEF, guanine nucleotide exchange factor; PKC, protein kinase C; Rap1b, Ras-proximate GTP-binding protein 1b; PFA, protein kinase A; TGF-β, transforming growth factor β; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; CREB, cAMP-responsive element; CREB, CAMP-responsive element-binding protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; MC, mesangial cell; HG, high glucose; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcriptase; PBS, phosphate-buffered saline; GST, glutathione S-transferase; RBD, Rap binding domain; WT, wild type; FN, fibronectin.

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‡ The abbreviations used are: ECM, extracellular matrix; AGE, advanced glycation end products; ROS, reactive oxygen species; DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; PKC, protein kinase C; Rap1b, Ras-proximate GTP-binding protein 1b; PFA, protein kinase A; TGF-β, transforming growth factor β; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; CREB, cAMP-responsive element; CREB, CAMP-responsive element-binding protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; MC, mesangial cell; HG, high glucose; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcriptase; PBS, phosphate-buffered saline; GST, glutathione S-transferase; RBD, Rap binding domain; WT, wild type; FN, fibronectin.

WITHDRAWN

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This article has been withdrawn by the authors. Fig. 1F was flipped horizontally and reused in Fig. 9D. Fig. 1G was rotated 180 degrees and reused in Fig. 3D. Fig. 2B was flipped horizontally and reused in Fig. 2F. A portion of Fig. 6C was reused in Fig. 6D. Fig. 7A was inappropriately manipulated.
and ultimate proper incorporation into the mesangial ECM (16, 17). Conceivably, excessive production and a disordered incorporation or assembly of fibronectin or of other matrix proteins, secondary to the phenotypic change in the mesangial cell or due to glycoxidative- or ROS-induced damage to the proteins, is expected to result in an abnormal accumulation of ECM in diabetic nephropathy with the evolution of Kimmelstiel-Wilson lesions that progress to glomerulosclerosis and end stage renal disease with kidney failure (1-3). The information regarding the mechanisms of excessive synthesis of ECM proteins is available in the literature; further insights into the mechanisms(s) involved in the evolution of diabetic glomerulosclerosis still remains to be explored, which may be helpful in developing future therapies in the amelioration of this disease process.

Previously, by suppressive subtractive hybridization procedures a small GTP-binding protein, Rap1b, was found to be up-regulated in the kidneys of diabetic newborn mice (18). Similarly, up-regulation of Rap1b was seen in diabetic rats as well (18). The up-regulation was observed in several other organs, but Rap1b expression in the kidney was increased in parallel to the alterations in the blood levels of glucose, suggesting that glucose by itself, rather than any cytokine, e.g., insulin-like growth factor, may be responsible for the increased Rap1b expression. Indeed, an increased Rap1b expression was seen in the embryonic metanephric explants exposed to high glucose ambiences (18). Based on these observations the present study was undertaken to determine the effect of hyperglycemia on the expression of Rap1b in the glomerular compartment of diabetic rats and to assess the effect of glucose on the expression and activation of Rap1b in the glomerular mesangial cells (MCs) and the in vitro investigations in the stimulation of fibronectin synthesis.

**EXPERIMENTAL**

**Animals and Induction of Diabetes—** A diabetic state was induced in 4-week-old Spargue-Dawley rats by the injection of streptozotocin by the differential sieving method (19). Briefly, cortices were dissected, and then the cortex was minced in citrate buffer, pH 4.6 (18). A week later the rats with blood glucose level >200 mg/dl were regarded to have a diabetic state, and their kidneys were processed for mRNA expression and immunohistochemical studies. Rats were injected with citrate buffer only.

**Immunohistochemical Staining—** The glomeruli from both diabetic and control rats were snap-frozen in liquid nitrogen and embedded in OCT compound (Sakura, Torrance, CA). Four-micrometer-thick cryostat sections were prepared. The sections were overlaid with primary monoclonal antibody directed against Rap1b antibody (Santa Cruz Biotechnology, Inc.) with gentle agitation at 4°C for 2 h, followed by addition of 50 μl of 50% protein A-Sepharose 4B™ (Amersham Biosciences) and incubation extended for another 1 h at 4°C. The protein A-Sepharose™ beads were then washed with IP buffer: 20 mM Tris-HCl, pH 7.5, 150 mM CaCl₂, 1 mM MgCl₂, 10 mM benzamidine, 2 mM p-nitrophenylphosphoric acid (Sigma), 2 mM ethylmercurithiolate (Sigma, Los Angeles, CA), and 0.1% SDS-PAGE under reducing conditions. The gel proteins were then electrophoresed onto a nitrocellulose membrane. The membrane was then immersed in a blocking solution containing 5% nonfat dry milk in TBS-T (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% Tween 20) for 1 h before being applied to a nitrocellulose membrane and being dried at 4°C. The membranes were then stained with avidin-biotin peroxidase by following the instructions provided by the vendor (Ultra Streptavidin Detection System, Signet Laboratories Inc.).

**Preparation of Isolated Glomeruli—** The glomeruli were isolated from kidneys of diabetic rats with different blood glucose level 5 days following the injection of streptozotocin by the differential sieving method with minor modifications (19). Briefly, cortices were dissected, and then they were then gently minced with a razor blade and pressed through 100-μm pore size mesh, and finally the glomeruli were collected on the surface of a third mesh with a 75-μm pore size. The purity of the glomerular preparation was examined by light microscopy.

**Cell Culture Studies—** Glomerular MCs were isolated from kidneys of Sprague-Dawley rats (Harlan Laboratories), and they were used to establish primary cell cultures as described previously (20). The cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Gibco, Carlsbad, CA) with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin and streptomycin, and 0.3 unit/ml insulin. They were maintained in 75-cm² flasks in a humidified atmosphere of 5% CO₂ and 95% air at 37°C, and at ~80% confluence they were trypsinized, propagated, and utilized between passages 5 and 7. At a given passage, the cell cultures were replaced with fresh serum-free Opti-MEM medium (Invitrogen). After 48 h, the medium was supplemented with varying concentrations of t-glucose (10-30 mM) and the quiescent mesangial cell cultures were maintained for 24 h. The t-glucose (30 mM) and 5 mM t-glucose-treated cells served as controls.

**Northern Blot Analysis—** Total RNAs were prepared from both the isolated glomeruli and MCs by the acid guanidium isothiocyanate-phenol-chloroform extraction method (21). About 10 μg of RNA from various experiments was subjected to 1.5% agarose gel electrophoresis containing 2.2 μM formaldehyde and capillary-transferred to the Hybond N⁺ nylon membrane (Amershams Biosciences). After cross-linking of RNA to the membrane, prehybridization and hybridization of different blots was carried out with various [32P]dCTP-labeled (1 × 10⁶ cpm/ml) cDNA probes (18, 19). The cDNA probes for Rap1a, Rap2a, and Rap2b were generated from plasmid constructs provided by Martina Schmidt (Institut fur Pharmakologie, Universitatsklinikum, Essen, Germany). The Rap1b cDNA probe was generated as described previously in our laboratory (18). The fibronectin (type-III) 590-bp cDNA probe was generated by PCR using a sense (5'-GCC CCT CCT GTC ACG GAG GAC-3') and an antisense (5'-GGC ACT GAC GAA GAG CCC TTA GAG GCC-3') primers and single-stranded cDNA prepared from rat kidney mRNA. Following the preparation of autoradiograms, the membrane blots were stripped and re-hybridized with radiolabeled β-actin cDNA probe. The integrity of RNA was monitored by visualization of the intact 18 and 28 S bands on the membrane blots stained with 0.05% methylene blue.

**Immunoprecipitation—** Glomerular immunoprecipitation, 12.6% polyacrylamide gels and 4 control normoglycemic MCs were subjected to immunoprecipitation (IP) in the acid guanidinium isothiocyanate-phenol-chloroform extraction method (21). About 10 μg of RNA from various experiments was subjected to 1.5% agarose gel electrophoresis containing 2.2 μM formaldehyde and capillary-transferred to the Hybond N⁺ nylon membrane (Amershams Biosciences). After cross-linking of RNA to the membrane, prehybridization and hybridization of different blots was carried out with various [32P]dCTP-labeled (1 × 10⁶ cpm/ml) cDNA probes (18, 19). The cDNA probes for Rap1a, Rap2a, and Rap2b were generated from plasmid constructs provided by Martina Schmidt (Institut fur Pharmakologie, Universitatsklinikum, Essen, Germany). The Rap1b cDNA probe was generated as described previously in our laboratory (18). The fibronectin (type-III) 590-bp cDNA probe was generated by PCR using a sense (5'-GCC CCT CCT GTC ACG GAG GAC-3') and an antisense (5'-GGC ACT GAC GAA GAG CCC TTA GAG GCC-3') primers and single-stranded cDNA prepared from rat kidney mRNA. Following the preparation of autoradiograms, the membrane blots were stripped and re-hybridized with radiolabeled β-actin cDNA probe. The integrity of RNA was monitored by visualization of the intact 18 and 28 S bands on the membrane blots stained with 0.05% methylene blue.

**Western Blot Analysis—** Glomerular immunoprecipitation, 12.6% polyacrylamide gels and 4 control normoglycemic MCs were subjected to immunoprecipitation (IP) in the acid guanidinium isothiocyanate-phenol-chloroform extraction method (21). About 10 μg of RNA from various experiments was subjected to 1.5% agarose gel electrophoresis containing 2.2 μM formaldehyde and capillary-transferred to the Hybond N⁺ nylon membrane (Amershams Biosciences). After cross-linking of RNA to the membrane, prehybridization and hybridization of different blots was carried out with various [32P]dCTP-labeled (1 × 10⁶ cpm/ml) cDNA probes (18, 19). The cDNA probes for Rap1a, Rap2a, and Rap2b were generated from plasmid constructs provided by Martina Schmidt (Institut fur Pharmakologie, Universitatsklinikum, Essen, Germany). The Rap1b cDNA probe was generated as described previously in our laboratory (18). The fibronectin (type-III) 590-bp cDNA probe was generated by PCR using a sense (5'-GCC CCT CCT GTC ACG GAG GAC-3') and an antisense (5'-GGC ACT GAC GAA GAG CCC TTA GAG GCC-3') primers and single-stranded cDNA prepared from rat kidney mRNA. Following the preparation of autoradiograms, the membrane blots were stripped and re-hybridized with radiolabeled β-actin cDNA probe. The integrity of RNA was monitored by visualization of the intact 18 and 28 S bands on the membrane blots stained with 0.05% methylene blue.

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Na, VO₃, and 1 mM dithiothreitol). After freezing and thawing on ice for 30 min, the cells were scrapped from the flask by using a Dounce homogenizer for 5 min at 4 °C. The homogenates were centrifuged at 1,000 × g for 10 min. The supernatant was saved and centrifuged at 100,000 × g for 90 min at 4 °C. The pellet (membrane proteins) was solubilized with 1% Triton X-100 and protein concentration adjusted to 1 mg/ml. Equal amounts of protein (20 µg) from various experiments were loaded onto the gel well and subjected to 10% SDS-PAGE, followed by Western blot analyses using anti-B-Raf and anti-Raf-1 antibodies (Santa Cruz Biotechnology).

**Generation of Rap1b Eukaryotic Expression Construct and Stable Transfectants**—A full-length Rap1b cDNA in PCR II vector (18) and sense (5'-GGGGGGGGGGATCCATGGCGCGCGGCGAGCCCGCATGCGTGAACGCGCGCTTTG-3') and antisense (5'-GGGGGGGGGGATCCATGGCGCGCGGCGAGCCCGCATGCGTGAACGCGCGCTTTG-3') primers were used to generate the expression construct by PCR. The GC clamps (GGGGGG), BamHI site (GGATCC), and XhoI site (GGATCC) were introduced into the sense primer, while the antisense primer included XhoI site (CTCGAG) and FLAG epitope (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) sequence. The generated PCR product was digested with BamHI and XhoI, cloned into pCR II vector (Invitrogen), gel-purified, and then subcloned into BamHI- and XhoI-digested pcDNA3.1 and designated as pcDNA3.1/Rap1b. They were then transfected into MCs using LIPOFECTAmine™2000 reagent, and stable transfectants were selected by growing cells in 800 µg/ml G418. The surviving clone of cells were then propagated in the presence of relatively low concentration of G418 (100 µg/ml) and used for further studies. To assess the Rap1b expression in the cells, RT-PCR, immunoprecipitation, and Western blot analysis were performed (18, 22).

**PKC Inhibitor Studies**—The MCs cells were transfected with pcDNA3.1/Rap1b as described above. Both transfected and non-transfected MCs were maintained in a fresh serum-free Opti-MEM containing 5 or 30 mM d-glucose in the medium. Various PKC inhibitors, such as calphostin C and bisindolylmaleimide, were added to the culture medium at a concentration range of 10 nM, respectively. The PKC inhibitors were treated for 15 min before the addition of d-glucose. The viability of the PKC inhibitors was then assessed by WST-8 assay.

**Rap1b Mutagenesis Studies**—pcDNA3.1 vector lacking FLAG epitope was digested with XhoI and labeled with [35S]methionine. The labeled protein was loaded by SDS-PAGE autoradiogram. The Mutagenesis kit (Stratagene) following vendor instructions. In the first Rap1b mutagenesis, the following respective primers were used: 5'-GGAGGTGTTGGGAAGAATGCTCTGACTGTACA-3' and 5'-GGGAAGAATGCTCTGACTGTACCTTGATAGAAGACGCGCTTTG-3'. The generated PCR product was introduced into pcDNA3.1 vector lacking FLAG epitope and was generated as described above.

**Role of Rap1b in High Glucose-induced Fibronectin Synthesis**

**Glomerular Compartment**—Immunohistochemical studies revealed a mild Rap1b expression in the tubules of the kidneys of normoglycemic rats (arrowheads). The Rap1b expression also increased in kidneys of hyperglycemic rats, particularly in the outer medullary collecting tubules of the kidneys of normoglycemic rats (arrowheads). To confirm the Rap1b expression in glomerular compartment of the kidney, glomeruli from hyperglycemic rats with different blood glucose levels, ranging between 200 and 400 mg/dl, were isolated by the sieving method. Northern blot analysis revealed a faint ∼2.3-kb Rap1b mRNA transcript (Fig. 1D). In hyperglycemic rats, an increase in the Rap1b mRNA expression, proportional to the blood glucose levels (200–400 mg/dl), was observed (Fig. 1D). The β-actin expression was unaffected by the blood glucose levels (Fig. 1F). Similarly, immunoprecipitation studies revealed a dose-dependent increase in the intensity of the ∼21-kDa band, corresponding to the size of Rap1b protein, in SDS-PAGE autoradiograms (Fig. 1G), suggesting that both protein and mRNA expression increase in proportion to the rise in blood glucose levels in diabetic rats.

**Effect of HG on Rap1b and Fibronectin Expression in Mouse MCs**—Under basal conditions with 5 mM glucose concentration in the medium, a single faint ∼2.3-kb Rap1b mRNA transcript was detected by Northern blot analysis (Fig. 2A). Exposure of MCs to HG (10–30 mM) induced a dose-dependent increase in the expression of Rap1b, with a maximal response of ∼20-fold increase at 30 mM. The β-actin expression was unaffected (Fig. 2B). An increase in the protein expression of de novo synthesized Rap1b was also seen with 30 mM glucose in the culture medium by immunoprecipitation methods (Fig. 2D). To assess the specificity of HG response, MCs were exposed to 30 mM glucose. No increase in the expression was noted, and the
intensity of the ~21-kDa Rap1b band was similar to that of the

In a protocol similar to the assessment of Rap1b, the effect of HG on fibronectin mRNA was also determined. At 5 mM, a faint ~8-kb fibronectin mRNA transcript was seen (Fig. 2E). Exposure of MCs to HG for 24 h caused a significant dose-dependent increase in the fibronectin mRNA expression, which paralleled the increase in Rap1b mRNA expression observed under similar experimental conditions (Fig. 2, A and E). MCs exposed to HG also resulted in a significant increase in fibronectin protein expression as determined by Western blot analysis (Fig. 2F). Incubation of MCs with 30 mM L-glucose had no stimulating effect on fibronectin protein expression, and the intensity of the ~220-kDa fibronectin band was similar to that seen in the control cells treated with 5 mM D-glucose (Fig. 2H).

Effect of Calphostin C and Bisindolylmaleide on Glucose-induced Rap1b Expression in MCs—MCs were exposed to 5–30 mM D-glucose in the absence or presence of various concentrations of PKC inhibitors, calphostin C and bisindolylmaleide (Fig. 3). The treated cells were then processed for the isolation of total RNA and radiolabeled cellular proteins for the Northern blot analysis and immunoprecipitation procedures, respectively. An increase in the Rap1b mRNA and protein expression was observed in HG ambience (Fig. 3, A and D, lane 2 versus lane 1). Both the PKC inhibitors, calphostin C and bisindolylmaleide, caused a dose-dependent diminution in the Rap1b expression in cells treated with HG (Fig. 3, A and D, lanes 2–5). The Rap1b expression was almost undetectable at high concentrations of PKC inhibitors, i.e. 100 nM calphostin C and 20 μM bisindolylmaleide (Fig. 3, A and D, lane 5). The expression of β-actin remained unchanged in MCs treated either with calphostin C or bisindolylmaleide in HG ambience (Fig. 3, C and G, lanes 1–5).

Role of PKC in HG-induced Fibronectin Synthesis in MCs Over-expressing Rap1b—First a mammalian Rap1b cDNA expression construct was synthesized by cloning the wild type Rap1b cDNA into pcDNA 3.1 and designated as pcDNA3.1/Rap1b. The MCs were then transfected with pcDNA3.1/Rap1b, and stable trans-
Role of Rap1b in High Glucose-induced Fibronectin Synthesis

PKC in the induction of fibronectin expression.

Effect of Rap1b Mutation in HG-induced Fibronectin Synthesis—MCs were transfected either with pcDNA3.1 empty vector alone or pcDNA3.1/Rap1b (WT) or mutant (Rap1b/S17N, Ser → Asn) or (Rap1b/T61R, Thr → Arg) in the presence of low and high glucose in the culture medium. Then cellular expression of fibronectin and that secreted in the medium were determined by immunofluorescence and Western blotting procedures, respectively. Minimal cellular expression of fibronectin was ob-

FIG. 4. Characterization of Rap1b transfecant cell lines. A pcDNA 3.1/Rap1b recombinant plasmid was transfected into MCs, and a stable MC line was generated following G418 selection. The authenticity of stable transfectants expressing Rap1b was confirmed by RT-PCR, immunoprecipitation and Western blot methods. By RT-PCR, a 520-bp size product is observed in cells transfected with pcDNA3.1/Rap1b recombinant plasmid (A, Vector(VT−)), lane 3, and 285-bp product in non-transfected cells (A, Vector(VT+), lane 1). By immunoprecipitation and Western blot methods both the Rap1b recombinant and native Rap1b proteins were detected and expressed in Rap1b transfected cells (B and C, lanes 3, 4 compared with lanes 1, 2). The expression of Rap1b mRNA is significantly increased in the MCs exposed to HG ambience compared with cells (Rap1b(VT−)) is unaffected. B and F indicate the intactness of the RNA and their loading of equal amounts of total RNA in various lanes. D and H represent the expression of β-actin in the non-labeled and immunoprecipitated Rap1b protein in MCs in HG ambience and treated with PKC inhibitors. In control MCs, both the PKC expression, the protein expression increases in a dose-dependent manner in MCs exposed to HG ambience and decreases in a dose-dependent manner of PKC inhibitors (D and H).

FIG. 5. Role of PKC in high glucose-induced FN expression in non-transfected MCs and cells overexpressing Rap1b. The PKC inhibitors (calphostin C and bisindolylmaleide) in non-transfected MCs reduce the FN mRNA and protein expression in high glucose ambience in a dose-dependent manner (A, D, E, and H, lanes 6–8) compared with the control (A, D, E, and H, lanes 1 and 2). While in MCs overexpressing Rap1b, the PKC inhibitors did not significantly reduce the FN mRNA or protein expression (A, D, E, and H, lanes 6–8). The expression of β-actin is unaffected by high glucose treatment in both non-transfected or transfected MCs (C and G). B and F indicate the intactness of the RNAs and their loading of equal amounts of total RNA in various lanes.

Since activation of PKC by HG has been shown to mediate the stimulation of fibronectin synthesis both in vitro and in vivo models of diabetes (28–30), the role of PKC in HG-induced fibronectin expression in control MCs as well as MCs transfected with Rap1b was investigated. Both control MCs and Rap1b-transfected cells were exposed to 5–30 mM glucose for 24 h in the absence or presence of a PKC inhibitor calphostin C (10–100 nM) or bisindolylmaleide (5–20 μM), and fibronectin mRNA expression in the cells and protein expression in the culture medium were assessed by Northern and Western blot analyses, respectively. Similar to the previous observations (Fig. 2, E and H), HG induced a significant increase in both fibronectin mRNA and protein expression in control non-transfected MCs (Fig. 5, lane 2 versus lane 1). In control MCs, both the PKC inhibitors, calphostin C and bisindolylmaleide, inhibited the HG-induced stimulation of fibronectin mRNA and protein expression in a dose-dependent manner (Fig. 5, lanes 6–8). By contrast, both the PKC inhibitors had no significant effect in reducing HG-induced increase fibronectin mRNA and protein levels in cells overexpressing Rap1b (Fig. 5, lanes 3–5). The β-actin expression in MCs was unaffected by treatments with HG and PKC inhibitors (Fig. 5, C and G). These results suggest that PKC-dependent mechanism(s), potentially involving Rap1b, mediate HG-induced stimulation in fibronectin synthesis in cultured MCs, and also Rap1b may be downstream of
fibronectin protein expression in culture media of the MCs was seen with the transfection of wild type pcDNA3.1/Rap1b (WT) (Fig. 6G, lane 6 versus lane 2).

Effect of Rap1b Transfection on B-Raf and Raf-1 Expression—To delineate which one of the Raf pathways is involved in Rap1b-mediated responses, expression of B-Raf and Raf-1 in MCs exposed to HG was determined. The cells were transfected either with empty pcDNA3.1 vector (E-VE), or wild type pcDNA3.1/Rap1b (WT), or with Rap1b mutants Rap1b/S17N (S17N) and Rap1b/T61R (T61R) and exposed to HG, followed by the assessment of B-Raf and Raf-1 expression by Western blot analysis (Fig. 7). In B-Raf experiments, two bands of ~94 and ~68 kDa were observed in cells transfected with empty vector (E-VE) (Fig. 7A, lane 1). The ~68-kDa band was faint, but was detectable. This band disappeared in cells transfected with S17N or T61R Rap1b mutants, and the upper ~94 kDa band was unaffected (Fig. 7A, lanes 2 and 3). The transfecion of MCs with WT resulted in an increase in the intensity of both the bands (Fig. 7A, lane 4). However, there was a marked increase in the intensity of the ~68-kDa band, suggesting that the B-Raf pathway is affected by Rap1b-mediated responses in the presence of HG (Fig. 7A, lane 5 versus lane 2). In contrast to the results of B-Raf, the expression of Raf-1 was unaffected. In cells transfected with empty vector, a middle band was observed (Fig. 7B, lane 1). Transfection with the empty pcDNA3.1 vector resulted in a mild increase in the expression of Raf-1 compared with the MCs exposed to HG alone (Fig. 7B, lane 5 versus lane 2). Whereas, transfection with Rap1b/S17N or Rap1b/T61R mutants markedly diminished the FN expression (Fig. 7C, lanes D and G, S17N and T61R, lanes 3 and 4).

**Fig. 6.** Effect of Rap1b in mesangial cells. HG–induced cellular fibronectin was observed in cells transfected with empty vector (E-VE) (Fig. 6A) and Western blot analysis (Fig. 6B versus A) and Northern blot analysis (Fig. 6C versus A). Transfection with the empty pcDNA3.1 vector (E-VE) resulted in a marked increase in the cellular (E) as well as in the culture medium (G, E-VE, lane 5). While a marked increase in cellular fibronectin expression is seen with the transfection of pcDNA3.1/Rap1b (WT and G, WT, lane 6). Whereas, transfection with Rap1b/S17N or Rap1b/T61R mutants markedly diminishes the FN expression (G, E, S17N and T61R, lanes 3 and 4).

At high glucose (30 mM) concentration, its expression markedly increased, and it was seen mostly distributed in the cytoplasm (Fig. 6B, versus A). A remarkable reduction in the HG-induced cellular fibronectin was observed in cells transfected with mutant Rap1b/S17N or Rap1b/T61R (Fig. 6, C and D versus B). No distinguishable differences between the effects of these mutants on the reduction of fibronectin expression were observed. Transfection with pcDNA3.1/Rap1b (WT) resulted in a marked increase in expression of fibronectin compared with the MCs exposed to HG alone (Fig. 6, F versus B). However, transfection with pcDNA3.1 empty vector induced minimal increase in the fibronectin expression compared with the cells incubated with HG alone (Fig. 6, E versus B). Similar results were observed for the fibronectin secreted in the medium (Fig. 6G). An increased fibronectin expression was observed with the HG (Fig. 6G, lane 1 versus lane 2). A reduction in the expression was seen with transfection of the mutants (Fig. 6G, lanes 3 and 4 versus lane 2). A mild increase in the expression was observed with transfection of vector alone (E-VE) (Fig. 6G, lane 5), while a remarkable increase in the constitutive expression was observed with transfection of vector alone (E-VE) (Fig. 6G, lane 6 versus lane 2).
significant increase in the Rap1b activation after 30 min of exposure to 30 mM glucose. In contrast, the cells transfected with Rap1b mutants (S17N, T61R) had no significant effect on the transfection of MCs with WT Rap1b (Fig. 8A, lane 4 versus lane 1, arrows). The band intensity of ~68 kDa, however, is markedly increased (Fig. 8A, lane 3 versus lane 1, arrows). In contrast to the results of B-Raf, the expression of the Rap1~74-kDa band is not increased in cells transfected with the empty vector (E-VE), instead, a small decrease in its intensity can be seen in cells transfected with the empty vector (E-VE) (Fig. 8A, lane 4 versus lane 1, arrows). The intensity of the Raf-1 band is unaffected by transfection of the mutant S17N or T61R.

To further define the specificity of HG response to Rap1b, the activation of Rap1a, Rap2a, and Rap2b were assessed in MCs incubated with 5–30 mM glucose. No significant increase in the mRNA expression of Rap1a and Rap2b when compared with their expression in the MCs exposed to 5 mM glucose (Fig. 9, B and C). However, Rap1a, which exhibits ~80% homology to Rap1b, showed a significant increase in the mRNA levels in MCs treated with HG in a dose-dependent manner similar to Rap1b (Fig. 9A). Although there is a high degree of homology between the Rap1a and Rap1b, their transcript sizes substantially differ, i.e. ~2.3 and ~1.6 kb, respectively (34). The β-actin levels were unaffected by HG treatment (Fig. 9, D–F).

**DISCUSSION**

The clinical course of diabetic nephropathy is characterized by hyperfiltration, microalbuminuria progressing to overt proteinuria, and azotemia culminating into end stage renal disease. The pathologic changes that may correlate with the functional abnormalities include thickening of the glomerular basement membrane and increased deposition of mesangial matrix. During the late stages, changes in the mesangial matrix become markedly accentuated with the formation of intercapillary mesangial nodules in the renal glomerulus. These nodular lesions are believed to be the result of hyperplasia followed by hypertrophy of MCs accompanied with certain phenotypic alterations and accumulation of excessive ECM protein, and it belongs to a superfamily consisting many members that regulate cell proliferation, differentiation, intracellular vesicular trafficking, cytoskeletal rearrangement, cell cycle events, and glucose transport (35–37). In addition, they can modulate the expression of ECM proteins, e.g. oncogenic transformation of fibroblasts by v-Src and v-Ras is shown to be associated with down-regulation of fibronectin (38). Among the various family members, the amino acid sequence of Rap1b and Ras are homologous in their putative effector domain that includes a stretch of 32–40 amino acid residues (39). But Rap1b has been shown to antagonize the Ras functions, such as the Ras-induced transformation of NIH 3T3 cells (40) and activation of c-Raf-1 protein kinase-dependent MAP kinase cascade in Rat-1 cells (41). The findings that there is a concomitant increased expression of Rap1b and fibronectin under high glucose ambience (Fig. 2) would support the concept of antagonistic actions of Rap1b and Ras GTPases, since overexpression of the Ras results in down-regulation of fibronectin (see above). This effect of d-glucose seems to be specific, since the cells exposed to 1-glucose did not alter the expression of either of Rap1b or fibronectin (Fig. 2). The increased expression or activation of Rap1b can occur by a wide variety of stimuli, including various growth factors, phospholipase C, and second meso-
Fig. 8. Effect of HG on activation of Rap1b and Rap2b. A dose-dependent increase in Rap1b-GTP is seen in MCs exposed to 5–40 mM D-glucose with maximal stimulation at 30 mM D-glucose (A, panel a). The time course studies indicate the activation of Rap1b within 5 min of exposure to HG and increases over a period of 60 min (A, panel b). The activation of Rap1b was largely unaffected by anti-PDGF antibody (A, panel c). The Rap1a activation is much lower than that of Rap1b and at the highest concentration of 10 μg/ml in the presence of D-glucose (A, panel C). The Rap1b activation is much lower than that of Rap2b and at the highest concentration of 10 μg/ml in the presence of D-glucose (A, panel C). The Rap1a activation is much lower than that of Rap1b and at the highest concentration of 10 μg/ml in the presence of D-glucose (A, panel C). The Rap1b activation is much lower than that of Rap2b and at the highest concentration of 10 μg/ml in the presence of D-glucose (A, panel C).

**Withdrawn**
B-Raf have restricted distribution, it is likely that the latter two serve specialized functions in specific locations in the mammalian system (55, 56). Most of the extracellular signals, e.g. PDGF and EGF, which activate Rap1 also induce Raf-1 activation. The activation of the latter is related to the binding of Rap1 with the cysteine-rich and Ras binding domains of Raf-1, and such domain-specific interactions are believed to competitively inhibit Ras functions (57). On the other hand, Rap1 exhibits an additive effect on the Ki-Ras-stimulated B-Raf activity and downstream MAPK cascade (41), suggesting that these two small G proteins, i.e. Ras and Rap1, have differing actions with the involvement of different Raf isoforms. Interestingly, in other external stimuli, such as nerve growth factor, which affects both Rap1 and Raf-B activity, the effect is Ras-Raf-1-dependent, while the activation upon Rap1-B-Raf cascade induced ERKs may selectively involve Rap1-B-Raf pathway as exemplified by experiments with depolarization-mediated calcium influx in PC12 cells (60). Along these lines, it is clear that in the present studies with MCs the HG selectively induced the expression of B-Raf, while Raf-1 was unaffected (Fig. 7). In fact, a mild negative effect on the Raf-1 expression was observed upon transfection with wild type Rap1b. The notion that HG mediates B-Raf expression was strengthened by the mutational analysis where transfection with S17N and T61R Rap1 mutants resulted in the disappearance of the ~68-kDa band in Rap1b autoradiograms. The dominant negative effect of the S17N mutant on the B-Raf activation has also been reported in PC12 cells subjected EGF stimulation (50). The above discussion suggests that small G-proteins are activated in non-renal cells in response to a number of growth factors, thus the next critical question that needs to be addressed is whether glucose can directly or indirectly, e.g. via growth factors, induce the activation of Rap1b (Fig. 8).

The activation of small GTPases has been the subject of many recent reviews (36, 37, 51, 58, 61) and following discussion briefly summarizes certain features relevant to the activation of Rap1. A wide range of stimuli in different cell types has been reported to activate Rap1. The stimulus may be thrombin as in platelets, in lymphocyte by B-cell antigen receptor activation, and platelet-activating factor in neutrophils. In mesenchymal fibroblasts, Rap1 activation has been shown to occur by growth factors, such as EGF and PDGF. The Rap1 activation is usually rapid, and it is largely abolished by inhibitors of phospholipase C, while sparing the activation by cAMP. The second messenger generated by phospholipase C activation includes Ca^{2+} and DAG. Both exhibit a certain degree of cell specificity, i.e. Ca^{2+} is involved in thrombin-induced Rap1 ac-
activation in platelets, while DAG mediates B-cell antigen receptor-induced activation. Thus, these three distinct second messengers, Ca$$^{2+}$$, DAG, and cAMP, which activate Rap1, have been clearly identified, and they play a pivotal role in various signal transduction processes. Beside the stimuli described above, glucose could conceivably induce activation of Rap1b. Among the various second messengers, DAG may be responsible for its activation in MCs exposed to high glucose, since DAG is de novo synthesized in hyperglycemic state and, in part, also by the hydrolysis of phosphatidylcholine (62). DAG is known to activate PKC, which is followed by increased TGF-$$\beta$$, i.e. Ser$$^{37}$$ or Thr$$^{61}$$ are also critical for HG activation of Rap1b, since upexpression of dominant negative Rap1b/S17N and/ or T61R notably reduced the Rap1bGTP (Fig. 8A, panel d). Also, similar to the expression studies, the Rap1b activation was enhanced in cells overexpressing Rap1b. The HG-induced activation was GTPase-specific, since Rap2b was unaffected (Fig. 8B), suggesting that the latter may not involve the cAMP-dependent PKA pathway. This is also the case in studies with platelets where Rap2B, unlike Rap1a and Rap1b, was found not to be phosphorylated by PKA (74).

Analogous to the above findings, the expression studies revealed no change in Rap2a or Rab2b mRNA levels (Fig. 9) in MCs subjected to HG amebience. Interestingly, Rap1a expression exhibited a dose-dependent response to HG treatment, suggesting that the homologous Rap1a and Rap1b proteins respond to the HG milieu in a similar manner and add to the list of other Ras-related GTPases, e.g. Rad and Gem (75, 76), that are relevant to pathogenesis of diabetic complications. Moreover, Rad has also been shown to contribute to insulin resistance as well (77). Above all, these findings of this investigation support the notion that the GTP-binding proteins, like Rap1b, are relevant to pathogenesis and progression of diabetic nephropathy, characterized by excessive synthesis of ECM, proteinuria, and ultimately renal failure.

B. Activity of Rap1b/AKT and PKA in HG-induced up-regulation of ECM synthesis in MCs

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B. Activity of Rap1b/AKT and PKA in HG-induced up-regulation of ECM synthesis in MCs

Like Rap1b expression its activation was dose-dependent (Fig. 8A, panel a) and rapid, i.e. occurring within minutes (<5 min) of exposure to HG (Fig. 8A, panel b). Since Rap1 is localized in the perinuclear Golgi region, that is at a distance from the plasma membrane, and the de novo synthesis of cytokines, e.g. TGF-$$\beta$$, EGF or PDGF, would take some time to reach the perinuclear region, it is most likely that these cytokines may not be involved for Rap1b induction. Nevertheless, the role of PDGF in cellular proliferation linked to up-regulation of small GTPases has been elucidated in other studies, where an 2-fold increased expression of Rap1b was observed in response to the exposure of smooth muscle cells to PDGF (69, 70). With respect to PDGF, its up-regulation has been reported in glomeruli of diabetic rats as well as mesangial cells exposed to HG (71, 72). In human MCs, HG has been shown to induce an overexpression of TGF-$$\beta$$ through activation of PDGF loop, which in turn leads to cellular proliferation and mesangial ECM production (31, 73). Such effects were reversed in MCs treated with neutralizing anti-PDGF antibodies (31). However, in the present study the anti-PDGF antibody failed to reverse the HG-induced activation of Rap1b (Fig. 8A, panel c), suggesting that the PDGF is not involved in the Rap1b-B-Raf pathway that downstream modulates the ECM-fibronectin synthesis or expression. It is also interesting to note that like the results of Rap1b expression studies (Fig. 6) certain amino acid residues,
