Role of Extracellular Matrix Renal Tubulo-interstitial Nephritis Antigen (TINag) in Cell Survival Utilizing Integrin αvβ3/Focal Adhesion Kinase (FAK)/Phosphatidylinositol 3-Kinase (PI3K)/Protein Kinase B-Serine/Threonine Kinase (AKT) Signaling Pathway*

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Tubulo-interstitial nephritis antigen (TINag) is an extracellular matrix protein expressed in tubular basement membranes. Combined mutations in TINag and nephrocystin-1 genes lead to nephronophthisis with reduced cell survival. Because certain extracellular matrix proteins are known to modulate cell survival, studies were initiated in Lewis rats lacking TINag to assess if they are more susceptible to cisplatin-induced apoptosis. In vivo and in vitro studies demonstrate an essential role of TINag in cellular survival to maintain proper tubular homeostasis utilizing integrin αvβ3 and downstream effectors.

This article has been withdrawn by the authors. The TINag immunoblot in Fig. 1D was inappropriately manipulated.

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2 The abbreviations used are: ECM, extracellular matrix; TINag, tubulo-interstitial nephritis antigen; CDDP, cisplatin; ILK, integrin linked kinase; S-Oligo, scrambled oligonucleotide; FAK, focal adhesion kinase; Wort, wortmannin; AKT, protein kinase B-serine/threonine kinase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; TBM, tubular basement membrane; RFU, relative fluorescence unit.
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ECM, particularly the basement membranes, have been long known to provide a scaffold for tissue organization by modulating cell/matrix signaling via integrin receptors, thus regulating the availability of growth factors and chemokines to maintain proper tissue homeostasis (10–13). Among the various ECM basement membrane proteins, the collagen, laminin, and proteoglycans are known to support cellular survival, their growth, differentiation, and proliferation during embryonic and adult life. The role of TINag in modulating cell/matrix interactions and cellular homeostasis remains to be explored.

TINag was initially identified as a nephritogenic antigen involved in tubulointerstitial nephritis mediated by the anti-TBM autoantibody present in sera of renal transplant recipients (14). In humans, it has been mapped to chromosome 6p11–6p12 (15). Mammalian TINag contains 11 exons and has a molecular mass of ~53 kDa. It has a signal peptide, von Willebrand factor domain, follistatin module, procathepsin and ATP/GTP binding domains, and six potential N-glycosylation sites (15). It does not have an RGD sequence, but it is believed to interact with laminin and type IV collagen (16). The TINag has been found to be genetically defective in pediatric patients with hereditary juvenile form of nephronophthisis (17–19), which is analogous to the genetically defective type IV collagen chains in the glomerular basement membranes of children with Alport syndrome (20), suggesting its role in renal development (21). In patients having mutations in another gene, nephrocystin-1 (Nphp-1), have also been found to have disrupted TBMs, a compromised cell survival, and progressively developed tubulointerstitial disease and chronic renal failure (19).

In view of the above interesting literature information, we proceeded to explore the role of TINag in cell survival and to delineate if this protein by itself plays any role in shielding the tubules from various forms of toxic injury, such as CDDP. A model of the Lewis rat kidney in which TINag seems to be undetectable in the tubular compartment, which perhaps is related to masking of its antigenic epitope or lack of protein expression, was used.

**EXPERIMENTAL PROCEDURES**

**Animal Model**—Six-week-old Wistar and Lewis (derived from parental Wistar stock) rats were purchased from Harlan Co. (Indianapolis, IN) and used for experiments with *cis*-diaminedichloroplatinum II (CDDP). Animals were housed in the animal facilities at 22 °C and 50% humidity with a 12-h light/dark cycle. The animals were given food and water *ad libitum*. After 2 days of acclimatization, the animals were divided into four groups (six in each group) as follows: Wistar control, Wistar-CDDP, Lewis control, and Lewis-CDDP. The CDDP groups received an intraperitoneal injection of cisplatin (5 mg/kg body weight; Sigma), dissolved in PBS. The control groups received PBS alone. The animals were sacrificed 72 h following cisplatin administration. Prior to sacrifice, blood samples were taken for determination of serum creatinine, using an assay kit (Exocell Inc., Philadelphia). Their kidneys were processed for various biochemical and morphological studies. All animal procedures used in this study were approved by the Animal Care and Use Committee of Northwestern University.

**Cell Culture**—HK-2 cells were obtained from ATCC (Manassas, VA) and grown in a keratinocyte serum-free medium (Invitrogen) in the presence of 5 ng/ml recombinant EGF and 0.05 mg/ml bovine pituitary extract. To assess the role of TINag and other ECM proteins, the HK-2 cells were seeded on sterile polystyrene Petri dishes coated either with filter-sterilized recombinant TINag (20 μg/ml) or type IV collagen (Sigma) or laminin or bovine serum albumin (BSA), as described previously (23). In addition, in some experiments wortmannin, a PI3K inhibitor, or CDDP was added to the culture media. For gene disruption the cells were transfected with various integrin receptor siRNAs. After 80% confluency, the cells were serum-starved for 12 h and processed for various studies.

**Morphology Studies**—Three-μm-thick sections were prepared from paraffin-embedded tissues and stained with hematoxylin and eosin. The degree of tubulo-interstitial injury was evaluated by examining 10 fields in randomly selected tissue samples. TINag expression in the Wistar and Lewis rat kidneys was also evaluated by immunohistochemistry. As described previously (23). Briefly, 4-μm-thick sections were deparaffinized as per the instructions of the vendor. The apoptotic bodies were identified as fluorescent nuclei with UV epifluorescence microscopy. For immunohistochemical analyses of p53 in kidney samples, avidin-biotin complex method was employed (23). The tissue sections were de-paraffinized, dehydrated with PBS, and treated with 0.3% H2O2; after that the tissue sections were subjected to an antigen retrieval procedure in citric acid buffer by following the vendor’s instructions (Prestige Medical, UK). After blocking with 5% normal goat serum in PBS, the sections were incubated with anti-p53 antibody conjugated with FITC (Sigma) or laminin or bovine serum albumin (BSA), as described previously (23).

Other morphological studies included the assessment of apoptosis in kidney tissues or HK-2 cells by using the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) method (24). De-paraffinized sections or frozen cryostat sections of the kidneys were digested with proteinase K (240 unit/ml, Promega, Madison, WI) for 30 min at 22 °C. After a rinse with PBS, the sections were incubated with TUNEL reagents (Roche Applied Science). The cells were processed for evaluation of apoptosis as follows. The HK-2 cells were fixed with 4% fresh paraformaldehyde for 20 min at 22 °C. They were permeabilized with 0.1% Triton X-100 at 4 °C for 1 min and then incubated with TUNEL reagents at 37 °C for 1 h, as per instructions of the vendor. The apoptotic bodies were identified as fluorescent nuclei with an UV microscope.
Nuclear morphology was also directly monitored by fluorescence microscopy following Hoechst 33342 (10 μg/ml; Invitrogen) staining, as described previously (24). Cellular apoptosis was characterized by visualizing nuclear condensation, fragmentation, and apoptotic bodies. Three fields, including 100 cells, were examined to semiquantitatively calculate the extent of apoptosis.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT) Assay—The cell viability was assessed with a colorimetric assay, where metabolically active cells are identified as having reduced dark purple formazan products derived from the yellow colored MTT by the action of mitochondrial succinate dehydrogenase. Following cisplatin treatment, 10 μl of MTT (5 mg/ml, Sigma) solution was added to 10⁻⁵ cells in 100 μl of media seeded in 96-well plates coated with various extracellular matrices (TINag, laminin, or collagen) or BSA. The cells were incubated for 4 h at 37°C in an atmosphere of 5% CO₂ and 95% humidity. The reaction was terminated by the addition of 100 μl of stop buffer (15% SDS in 40% dimethylformamide). After stabilization of the reaction for 2 h, the absorbance readings were recorded at a wavelength of 560 nm, which served as the background controls. The absorbance readings were also made at 630 nm, which served as the background controls. The relative viability of cells was calculated as A630−A630 test/A630 A630 control. The viability of control untreated cells was normalized as 100%.

Quantitation of Apoptosis by Annexin V FITC Staining and Flow Cytometry—Early stage apoptosis was examined by binding them with annexin V-FITC (Biomolecular Technologies, Inc., Mountain View, CA) and propidium iodide (Sigma) followed by FACS Canto II flow cytometer (BD Biosciences). For each sample, the cell suspension samples were analyzed by using a BD FACS Canto II flow cytometer (BD Biosciences). For each sample, 10,000 events were recorded.

Gene Disruption and Integrin Receptor Blockade Studies—Integrin α3 and αv containing a pool of three target-specific 20–25-nucleotide siRNA duplexes were purchased from Santa Cruz Biotechnology. In addition, scrambled sequence short RNA was purchased, which served as a control. Gene disruption of integrin α3 or αv was performed by transfecting in HK-2 cells with siRNA using Lipofectamine 2000 reagent (Invitrogen). The efficiency of gene knockdown was assessed by measuring the expression of integrin α3 and αv in the transfected cells. The effect of integrin α3 and αv gene disruption on cell viability, apoptosis, and expression of relevant apoptotic molecules in HK-2 cells maintained on TINag-coated culture dishes and treated with CDDP was monitored. Also, the effect of blockade of integrin αv receptor on CDDP-induced apoptosis in HK-2 cells was assessed following inclusion of anti-αvβ3 antibody (Abcam) into the culture medium.

Caspase-3/7 Activity Assays—Enzymatic activity of caspases was measured by using a pro-luminescent caspase-3/7 substrate, which contains a tetrapeptide (Asp-Glu-Val-Asp, DEVD) sequence (Promega, Madison, WI). Briefly, cellular proteins were extracted with 1% Triton X-100. The lysates containing 25 μg of protein were added to enzymatic reactions containing 50 μM DEVD substrate. After 1 h of incubation at 37°C, fluorescence at 360 nm (excitation)/530 nm (emission) was recorded, using a Synergy™ 2 multimode microplate reader (Biotek Instruments; Winooksi, VT). The fluorescence readings were normalized against 25 μg of protein/reaction and expressed as relative caspase activity.

TINag Protein Expression Studies—TINag protein expression in the kidney was evaluated as described previously (23). Briefly, kidney cortices were dissected and homogenized in an extraction buffer made up of 0.1 M Tris-HCl buffer, pH 7.5, containing 6 M guanidinium hydrochloride (Sigma) and protease inhibitors. The extraction buffer was used for 12 h at 4°C in an orbital shaker. The lysate was centrifuged at 15,000 × g for 20 min, and the supernatant was added with 8 volumes of absolute ethanol. The precipitate was centrifuged at 12,000 × g for 30 min. The precipitate was washed with 80% ethanol. Then, the precipitate was redissolved in 8 M urea with 1% Triton X-100, and the protein concentration was determined. Equal amounts of protein from various samples were loaded onto gels for SDS-PAGE analyses, transferred to nitrocellulose membranes. The blots were probed with rabbit anti-TINag antibodies, and then detected by chemiluminescence (ECL) system (Amersham Biosciences).

Extraction of Total Cellular, Mitochondrial, and Nuclear Proteins from Kidney Tissue and Cells for Expression Studies—For total cellular proteins, the kidney tissue or HK-2 cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Nonidet P-40) containing protease inhibitor mixture (Sigma) on ice for 30 min. The lysate was centrifuged at 12,000 × g at 4°C for 10 min, and the supernatant was collected for protein expression studies by Western blot analyses. For mitochondrial protein expression studies, first the mitochondria from HK-2 cells or kidney tissues were harvested by using a tissue/cellular mitochondrial isolation kit (Pierce) following the vendor’s instructions; the mitochondrial matrix proteins were then extracted for immunoblot analyses in an identical manner as described previously (24). For isolation of nuclei, the kidney tissues or cells were homogenized in sucrose buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM DTT, and 0.5 mM PMSF) in a Dounce glass homogenizer. The homogenate was spun down for 15 min at 800 × g at 4°C, and the pellet was collected and washed gently with the sucrose buffer. The pelleted nuclei were then resuspended in 50 μl of low salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 25% glycerol (v/v), 0.5 mM DTT, and 0.5 mM PMSF). To this, an equal volume of high salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 800 mM KCl, 0.2 mM EDTA, 25% glycerol (v/v), 1% Nonidet P-40, 0.5 mM DTT,
0.5 mM PMSF, and 4.0 μg/ml leupeptin, aprotinin, and pepstatin) was added very slowly while mixing with a wide bore pipette. The samples were then incubated for 45 min at 4 °C while rotating in an orbital shaker. The nuclear proteins were then recovered in the supernatants by centrifuging the extracts at 14,000 × g for 15 min.

Equal amounts of protein from various samples were subjected to SDS-PAGE and then transferred onto nitrocellulose membranes. Various blotts were individually probed with the following primary antibodies: polyclonal rabbit anti-Bcl-2, mouse monoclonal anti-Bax, mouse monoclonal anti-cytochrome c, rabbit polyclonal anti-FAK and -p-FAK, polyclonal rabbit anti-ILK and -p-ILK, polyclonal rabbit anti-AKT and -p-AKT, polyclonal rabbit anti-p53 and -p-p53, mouse monoclonal anti-β-actin, and rabbit polyclonal anti-TATA-binding protein antibodies. The blotts were then incubated with secondary antibodies, which included anti-rabbit or anti-mouse IgG antibody. The primary and secondary antibodies were purchased from Abcam, Santa Cruz Biotechnology, Sigma, and Cell Signaling. Finally, autoradiograms of the Western blotts were prepared using the ECL system.

Far Western Blotting Analyses—Far Western blotting was performed by modification of the methods described previously (25, 26). Equal amounts of recombinant TINag proteins (full-length and N- and C-terminal segments) were subjected to 10% SDS-PAGE and transferred onto the polyvinylidene fluoride (PVDF) membranes by electroblotting procedure. The PVDF membranes were probed with the primary antibody (0.1% Nonidet P-40, 10% glycerol, 0.1% Tween 20, 1 mM DTT, and 3% BSA) at 4 °C for 8 h. The kidney tissue protein extracts were then transferred to binding buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 1% Nonidet P-40, 10% glycerol, 0.1% Tween 20, 1 mM DTT, and 3% BSA) for 5 min. They were then transferred to a binding buffer containing 0.1% Nonidet P-40, 10% glycerol, 0.1% Tween 20, 1 mM DTT, and 3% BSA at 4 °C for 5 h. The kidney tissue protein extracts were then re-natured by incubating them in a Restore Western Blot Stripping buffer (Thermo Scientific, IL) for 15 min at 22 °C. The blotts were then re-probed with anti-TINag antibody as described above.

Solid Phase Binding Assays and Cell Morphological Features on ECM Substrata—To confirm the binding of TINag with αβ3 integrin, solid phase binding assays were performed as described previously (27). The binding affinity was compared with vitronectin, and the latter is known to bind αβ3 and mediate cell adhesion and migration via the PI3K/AKT pathway (28). Briefly, BSA, full-length TINag recombinant protein, and vitronectin (Millipore Corp., Billerica, MA) were reconstituted to a final concentration of 40 μg/ml in a 50 mM carbonate/bicarbonate buffer (1.59 g/liter Na2CO3 and 2.93 g/liter NaHCO3), pH 9.6. A 96-well MICROTEST assay plate (Falcon) was coated with 100 μl of various substrate proteins and allowed to adhere at 4 °C overnight. The wells were then briefly rinsed with TBST solution (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20). The protein-binding sites were blocked by adding 200 μl of blocking buffer (3% BSA in PBS) for 2 h at 22 °C. 100 μl of purified αvβ3 (Millipore) in a binding buffer (20 mM Tris, pH 7.4, 0.15 mol/liter NaCl, 1 mmol/liter CaCl2, 1 mmol/liter MgCl2) with varying concentrations of TINag were added and incubated at 14,000 × g for 12 h. This was followed by secondary antibody (anti-mouse IgG-horseradish peroxidase (HRP), 1:20,000 dilution) at room temperature (29). The HPR activity was detected by incubation of blotts or membranes with a mixture of 50 μl of 3,3',5,5'-tetramethylbenzidine for 30 min at 22 °C. The color development of the substrate was stopped by adding 50 μl of 1 mol/liter HCl. The absorbance in various wells of the MICROTEST plate was measured at 450 nm, and readings were plotted against various concentrations of integrin αβ3 used in the assay procedure.

To assess the morphology of cells on TINag and vitronectin substrata, the HK-2 cells were seeded on sterile polystyrene Petri dishes coated either with filter-sterilized recombinant TINag (20 μg/ml) or vitronectin (20 μg/ml), with BSA serving as a control, as described above. The cells were maintained for 24 h, followed by fixation with 4% paraformaldehyde. They were then stained with rhodamine phalloidin (1:50 dilution) for 20 min to visualize F-actin and counterstained with 300 nm 4′,6′-diamidino-2-phenylindole (DAPI) at 22 °C for 1 min to delineate nuclei. The cells were briefly washed with PBS, and after placing a drop of Antifade® solution (Invitrogen), they were coverslip-mounted and examined by a Zeiss microscope, equipped with UV epi-illumination.

Gene Expression Studies—For quantification of kidney injury molecule (Kim-1) expression, total RNA was extracted from the kidney tissue by using TRIzol reagent (Invitrogen), and contaminated DNA was removed with RNase-free DNase. Two μg of total RNA was then reverse-transcribed by using Superscript II reverse transcriptase (Invitrogen; 25 units/μl) and 24-mer oligo(dT) as the primer. The synthesized cDNA was analyzed in a sequence detection system (model 7000; Applied Biosystems, Foster City, CA) by using specific Kim-1 and 18 S RNA primers and absolute quantitative PCR SYBR Green mixtures (ABgene, Rochester, NY). The specific Kim-1 primers for real
Mitochondrial DNA (mtDNA) Studies

For long and short kDNA, the primers were as follows: 5′-CGC AGA GAA ACC CGA CTA AG (sense) and 5′-CAA AGC TCA GAG AGC CGT TA-3′ (antisense). The relative abundance of mRNAs was standardized with 18 S rRNA as the invariant control, and the expression was seen in the tubular basement membranes of cortical proximal and distal tubules (Fig. 1A). The expression was seen in the tubular basement membranes of cortical proximal and distal tubules (Fig. 1A, arrowheads), and it was absent in the medullary tubules as well as in the glomeruli (Fig. 1A, arrows). No expression was observed in the renal cortical or medullary tubules and glomeruli of Lewis rats (Fig. 1B, arrows and arrowheads). Northern blot analyses revealed TINag gene expression exclusively localized in the kidneys of Wistar rats although totally absent in the Lewis rats (Fig. 1C). Interestingly, genomic analyses of various TINag intron and exon regions by PCR amplification procedures revealed no significant differences in the nucleotide sequences between the Wistar versus Lewis rats (data not included). In line with the observations of mRNA expression, the Western blot analyses revealed the presence of TINag protein in kidneys of Wistar rats, although no protein product of relatively low or high molecular mass was visualized in Lewis rats (Fig. 1D). Suggesting that there are no alternatively spliced forms of TINag that may be present in the Lewis strain of rats.

Cisplatin-induced Kidney Injury in Wistar versus Lewis Rats—Three days following CDDP administration, the serum creatinine levels were increased in both the Wistar and Lewis strain of rats, as compared with the controls that received PBS only (Fig. 2A). However, the elevation of serum creatinine levels was significantly higher in Lewis versus Wistar rats, suggesting that the kidneys of Lewis rats are relatively more susceptible to injury induced by the administration of CDDP. The renal injury was also reflected in the mRNA expression of Kim-1, a marker of acute kidney injury. Significantly higher levels of Kim-1 were observed in the kidneys of Lewis rats compared with those of the parental Wistar strain following CDDP administration (Fig.

FIGURE 1. Status of TINag expression in kidneys of Wistar versus Lewis rats. By immunofluorescence microscopy, expression of TINag was exclusively confined to the basement membranes (TBMs) of cortical tubules in Wistar rat kidneys (A, arrowheads), and it was absent in glomeruli (A, arrows). Northern and Western blot analyses revealed TINag expression in kidneys of Wistar rats only (C and D). No expression was observed in the kidneys of Lewis rats (B–D).
By quantitative PCR analyses, the Kim-1 mRNA levels were detectable in control rats, but they were quite low. By light microscopy, no significant differences in the morphological features between these two strains were observed in control rats receiving PBS only (Fig. 2, D versus C). Cisplatin administration induced notable tubulointerstitial injury in both strains of rats in the form of necrosis of tubular cells with detachment from the underlying basal lamina and dilatation of the interstitial capillaries with influx of mononuclear cells (E and F, arrowheads). The studies were extended to see the magnitude of apoptosis between the two strains of rats. Under basal control conditions, i.e. animals receiving PBS, no discernible differences were observed in the extent of CDDP-induced renal injury in the medullary tubules or cortical glomeruli between Wistar and Lewis rats, although cellular changes were not readily discernible.

Magnitude of Cisplatin-induced Apoptosis in Kidneys of Wistar versus Lewis Rats—Relatively high susceptibility of the inner cortical region to CDDP-induced cellular injury was also reflected in apoptosis studies. Dual staining of TINag and TUNEL in kidney sections of Wistar rats receiving cisplatin showed apoptosis mainly confined to the inner cortex, whereas minimal apoptosis was observed in the medulla or outer cortex (Fig. 3, A–D, arrowheads). The studies were extended to see the magnitude of apoptosis between the two strains of rats. Under basal control conditions, i.e. animals receiving PBS, no discernible differences were observed in the extent of CDDP-induced renal injury in the medullary tubules or cortical glomeruli between Wistar and Lewis rats, although cellular changes were not readily discernible.

FIGURE 2. Cisplatin-induced kidney injury in Wistar versus Lewis rats. Serum creatinine levels increased in both Wistar and Lewis strain of rats 3 days following CDDP administration compared with controls (CON) (A). However, the elevation of serum creatinine levels was higher in the Lewis strain of rats. Similarly, the mRNA expression of Kim-1 was higher in the kidneys of Lewis rats that received CDDP (B). Light microscopy revealed no significant differences between the two strains in control rats receiving PBS only (C and D). Following CDDP administration, tubulointerstitial injury was observed in both strains of rats. However, high magnification photomicrographs revealed that the injury was more severe in Lewis strains of rats, and it was reflected by necrosis of tubular cells with detachment from the underlying tubular basal lamina and dilatation of the interstitial capillaries with emigration of mononuclear cells (E and F, arrowheads). (A, n = 6; *, p < 0.05, CDDP-treated Lewis versus Wistar rats.) (B, n = 6; *, p < 0.01, CDDP-treated Lewis versus Wistar strain.)
ible apoptosis, as ascertained by TUNEL method, was observed (Fig. 4, A and B), although many foci of apoptosis were seen following cisplatin treatment. At higher magnification, the foci of apoptosis were clearly visualized, and like the morphological changes (Fig. 2, E and F), the degree of apoptosis was relatively severe in Lewis rats compared with Wistar strain (Fig. 4, D versus C, arrows). Associated with apoptosis, cisplatin administration induced an increase in the expression of the pro-apoptotic protein Bax, and a decrease in anti-apoptotic protein Bcl-2, as assessed by Western blot analyses followed by densitometric analyses (Fig. 4, E–G), suggesting kidneys lacking TINag are more susceptible to cellular injury.

Status of Cisplatin-induced p53 Expression in Kidneys of Wistar versus Lewis Rats—The p53 protein was rapidly induced following DNA damage and was believed to be involved in the regulation of apoptosis and cell cycle arrest. No significant expression was observed in kidneys of Wistar or Lewis strains of rats by immunohistochemistry or Western blot analyses in control animals (Fig. 5, A, B, and E). Following cisplatin administration, an increased expression of p53 was observed in both Wistar and Lewis strains of rats. Nuclear localization of p53 could be readily seen in higher magnification photographs (Fig. 5, C and D, arrowheads). The region with maximal p53 expression appeared to be the inner renal cortex where notable apoptosis has been previously observed (Fig. 4, C and D). Interestingly, like apoptosis, the extent of p53 nuclear expression was relatively high in kidneys of Lewis rats receiving cisplatin (Fig. 5, D versus C). A high nuclear p53 expression in the Lewis strain of rats suggested that there is an increased phosphorylation of this protein leading to its translocation from the cytoplasm into the nucleus. This observation was reinforced by Western blot analyses, where bands with increased intensity were observed in blots probed with the phosphorylated form of anti-p53 antibody (Fig. 5E). The densitometric analyses confirmed the increase in the intensity of the bands (Fig. 5, F and G), thus authenticating a higher degree of nuclear damage in renal tubules of Lewis rats.

Relative Cell Survival and Apoptosis in HK-2 Cells Maintained on Various ECM Substrata following Cisplatin Treatment—To assess whether TINag plays a protective role in cisplatin-induced renal injury, in vitro studies using HK-2 cells were initiated. Viability of proximal tubular epithelial cells (HK-2 cells) maintained on various ECM substrata, including type IV collagen and laminin, was assessed and compared with cells on TINag, a tubular specific ECM protein. BSA served as a
control. No significant differences in the viability of HK-2 cells were observed under basal conditions where culture dishes were coated with various ECM proteins or BSA (Fig. 6A). Following addition of 20 μM CDDP in the culture media for 48 h, the cell viability in BSA-coated dishes was reduced to less than 50% (44.8 ± 5.1%). Compared with BSA, the viability was relatively high for cells seeded on TINag (70.6 ± 4.1%) or type IV collagen (75.8 ± 5.5%) substrata (Fig. 6A). The culture dishes coated with laminin alone exhibited a mild increase in the cellular viability (54.1 ± 3.8%). Interestingly, the cells grown on TINag + collagen IV or TINag + laminin had a marginal boost following CDDP treatment (F and G). TATA-binding protein (TBP) levels were unaffected. (n = 6; *, p < 0.01, protein expression of p53 and p-p53 in Wistar (WI) versus Lewis (LE) rats treated with CDDP.)
cells grown on TINag (16.5 ±percentage of cells exhibiting apoptosis was remarkably reduced in BSA-coated culture dishes for 48 h (Fig. 6, marked apoptosis in tubular epithelial cells maintained on (14.7/H11006 reduced (64.1/8.7%) dishes coated with BSA (Fig. 6, G)). A comparable reduction in the caspase3/7 activity in cells treated with CDDP observations made for the CDDP-induced activity of TINag and type IV collagen were further supported by the type IV collagen and TINag provide a similar degree of protec-
tive effect on mtDNA. The cells maintained on BSA-coated substrata had significantly less damage to the high molecular mass mtDNA damage, as reflected by an increase in the intensity of the bands (A, lanes 3 and 4, arrow). Laminin substratum yielded a marginal improvement (A, lane 5, arrow). Damage to high molecular mass mtDNA was also observed in both the strains of rats following cisplatin administration (B, lanes 5–8 versus 1–4, arrow). Interestingly, the mtDNA damage was relatively accentuated in Lewis strain compared with Wistar rats, as reflected by the attenuated density of the band (B, lanes 7 and 8 versus 5 and 6, arrow). The band intensity of low molecular mtDNA was unaffected by cisplatin administration (B, lanes 1–8, arrowhead).

FIGURE 7. Status of mtDNA in HK-2 cells maintained on various ECM substrata and in Wistar and Lewis rats following cisplatin treatment. The cells maintained on BSA-coated dishes exhibited remarkable reduction in the intensity of the band corresponding to high molecular mass mtDNA (~8 kb) following cisplatin treatment, suggesting significant damage to mtDNA (A, lane 2 versus 1, arrow). The low molecular mtDNA (~0.45 kb) was largely unaffected by the cisplatin treatment (A, lanes 1–5, arrowhead). Plating of cells on TINag or type IV collagen had significantly less damage to the high molecular mass mtDNA damage, as reflected by an increase in the intensity of the bands (A, lanes 3 and 4, arrow). Laminin substratum yielded a marginal improvement (A, lane 5, arrow). The low molecular mtDNA (~0.45 kb) was unaffected by the cisplatin treatment (Fig. 7A, lanes 1–5). Seeding of HK-2 cells on TINag or type IV collagen significantly reduced the high molecular mass mtDNA damage, as reflected by an increase in the intensity of the bands (A, lanes 3 and 4, arrow). Seeding of cells on TINag and type IV collagen had significantly less damage to the high molecular mass mtDNA damage, as reflected by an increase in the intensity of the bands (A, lane 2 versus 1, arrow). Seedling of cells exposed to cisplatin treatment, as reflected by the unaltered intensity of the bands (Fig. 7B, lanes 1–8, arrowhead).

Status of mtDNA in HK-2 Cells Maintained on Various ECM Substrata and in Wistar and Lewis Rats following Cisplatin Treatment—Because cells maintained on ECM substrata had reduced genomic DNA fragmentation, as reflected by attenuated apoptosis (see above), studies were extended to assess if TINag and other basement membrane proteins exert any protective effect on mtDNA. The cells maintained on BSA-coated dishes had remarkable reduction in the intensity of the band corresponding to high molecular mass mtDNA (~8 kb) following cisplatin treatment, suggesting significant damage to mtDNA (Fig. 7A, lane 2 versus 1, arrow). The low molecular

mitochondrial damage, as reflected by an increase in the intensity of the bands (Fig. 7A, lanes 1–5). Seeding of HK-2 cells on TINag or type IV collagen had significantly less damage to the high molecular mass mtDNA damage, as reflected by an increase in the intensity of the bands (Fig. 7A, lanes 3 and 4, arrow). Laminin substratum yielded a marginal improvement (Fig. 7A, lane 5, arrow). Damage to high molecular mass mtDNA was also observed in both the strains of rats following cisplatin administration (Fig. 7B, lanes 5–8 versus 1–4, arrow). Interestingly, the mtDNA damage was relatively accentuated in Lewis strain compared with Wistar rats, as reflected by the attenuated density of the band (Fig. 7B, lanes 7 and 8 versus 5 and 6, arrow). The low molecular mtDNA was unaffected by cisplatin administration (Fig. 7B, lanes 1–8, arrowhead).

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Status of mtDNA in HK-2 Cells Maintained on Various ECM Substrata and in Wistar and Lewis Rats following Cisplatin Treatment—Because cells maintained on ECM substrata had reduced genomic DNA fragmentation, as reflected by attenuated apoptosis (see above), studies were extended to assess if TINag and other basement membrane proteins exert any protective effect on mtDNA. The cells maintained on BSA-coated dishes had remarkable reduction in the intensity of the band corresponding to high molecular mass mtDNA (~8 kb) following cisplatin treatment, suggesting significant damage to mtDNA (Fig. 7A, lane 2 versus 1, arrow). The low molecular
length protein and its N- and C-terminal segments (figure not included).

Comparative Binding Affinity of TINag versus Vitronectin with Integrin αvβ3 and Assessment of Cytoskeletal Morphology of Cells Maintained on These Substrata—Solid phase binding assays were carried out to assess the comparative binding analyses between TINag and vitronectin, the latter exhibits a marked affinity for integrin αvβ3 receptor. BSA was used as a negative control. A remarkable binding of vitronectin with αvβ3 was observed, and it was saturable at a concentration of 2.5 μg/ml of the integrin receptor (Fig. 8C). A comparable binding of TINag with αvβ3 was observed, and it was also saturable at a similar concentration of integrin receptor, suggesting that TINag can serve as an authentic potential ECM ligand for αvβ3 (Fig. 8C). No significant binding of integrin αvβ3 with BSA was detected, indicating the specificity of ECM/integrin binding affinity, as observed in the solid phase assay experiments. Morphology studies revealed that cells maintained on BSA were viable; however, they had somewhat attenuated morphology, and their cytoskeletal elements were crowded in the cytoplasm (Fig. 8D). The cells maintained on vitronectin or TINag substrata had similar morphology. The cells were spread out and had flattened morphology, and their cytoskeletal elements, as stained with rhodamine phalloidin, were well organized and they could be distinctly visualized (E and F).

FIGURE 8. Authentication of TINag/integrin interactions and assessment of TINag and vitronectin binding with integrin αvβ3 and α3β1 receptors on HK-2 cells. Far Western analyses were performed to elucidate the interactions between the TINag proteins (full-length and N- and C-terminal segments) and their cytoskeletal elements (figure not included).

Comparative Binding Affinity of TINag versus Vitronectin with Integrin αvβ3 and Assessment of Cytoskeletal Morphology of Cells Maintained on These Substrata—Solid phase binding assays were carried out to assess the comparative binding analyses between TINag and vitronectin, the latter exhibits a marked affinity for integrin αvβ3 receptor. BSA was used as a negative control. A remarkable binding of vitronectin with αvβ3 was observed, and it was saturable at a concentration of 2.5 μg/ml of the integrin receptor (Fig. 8C). A comparable binding of TINag with αvβ3 was observed, and it was also saturable at a similar concentration of integrin receptor, suggesting that TINag can serve as an authentic potential ECM ligand for αvβ3 (Fig. 8C). No significant binding of integrin αvβ3 with BSA was detected, indicating the specificity of ECM/integrin binding affinity, as observed in the solid phase assay experiments. Morphology studies revealed that cells maintained on BSA were viable; however, they had somewhat attenuated morphology, and their cytoskeletal elements were crowded in the cytoplasm (Fig. 8D). The cells maintained on vitronectin or TINag substrata had similar morphology. The cells were spread out and had flattened morphology, and their cytoskeletal elements, as stained with rhodamine phalloidin, were well organized and they could be distinctly visualized (E and F).

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incorporation of TINag-integrin receptor antibody blocking experiments. Inclusion of transfection of α3. Interestingly, the change the extent of nuclear damage (Fig. 9C), although an increased nuclear condensation and fragmentation were observed with the transfection of αv siRNA (D). FACS analyses reinforced the above findings that nuclear damage (Fig. 9C, versus A). The α3-siRNA did not significantly enhance apoptosis. The apoptosis augmented by αv siRNA transfection notably augmented (35.3%) following CDDP treatment of HK-2 cells (H). The observed cellular effects seemed to be specifically related to the gene disruption of TINag-integrin/v as reflected by the siRNA experiments, although a minimal change was observed with scrambled siRNA group was designated as 100%. Finally, the status of translocation of apoptotic molecules, i.e. Bax and cytochrome c, was assessed following CDDP treatment and a decrease of cytochrome c from cytosolic (Cyto) to the mitochondrial (Mitochondrial (Mit)) compartment and vice versa, was mainly affected by αv siRNA transfection of CDDP-treated HK-2 cells (H). The observed changes in H are representative of three different experiments. (G, n = 5, *, p < 0.01, cellular viability following treatment with CDDP + siRNA-α3). Besides apoptosis, the MTT assays indicated that cell viability was also remarkably reduced with the gene silencing of α3 as reflected by the siRNA experiments (Fig. 9G). No significant change in the cell viability was observed with transfection of α3, rather it was marginally increased when compared with the scrambled siRNA-α3. The viability of cells in scrambled siRNA group was designated as 100%. Finally, the status of translocation of apoptotic molecules, i.e. Bax and cytochrome c, was assessed following CDDP treatment and transfection of αv siRNAs. There was an enhanced translocation of Bax from the cytosolic to the mitochondrial compartment and a decrease of cytochrome c in the cytosol following the transfection of αv siRNA in CDDP-treated HK-2 cells (Fig. 9H), thus suggesting that such a mitochondrial leakage led to the nuclear damage associated with CDDP-induced injury, which was augmented by down-regulating the TINag putative receptor, i.e. αvβ3. The above cellular effects were specifically related to the gene disruption of αvβ3 and not due to the ablation of α3β1 because expression of both the receptors was down-regulated following treatment with cisplatin (Fig. 9D). The observed changes in H are representative of three different experiments. (G, n = 5, *, p < 0.01, cellular viability following treatment with CDDP + siRNA-α3). Besides apoptosis, the MTT assays indicated that cell viability was also remarkably reduced with the gene silencing of α3 as reflected by the siRNA experiments (Fig. 9G). No significant change in the cell viability was observed with transfection of α3, rather it was marginally increased when compared with the scrambled siRNA-α3. The viability of cells in scrambled siRNA group was designated as 100%. Finally, the status of translocation of apoptotic molecules, i.e. Bax and cytochrome c, was assessed following CDDP treatment and transfection of αv siRNAs. There was an enhanced translocation of Bax from the cytosolic to the mitochondrial compartment and a decrease of cytochrome c in the cytosol following the transfection of αv siRNA in CDDP-treated HK-2 cells (Fig. 9H), thus suggesting that such a mitochondrial leakage led to the nuclear damage associated with CDDP-induced injury, which was augmented by down-regulating the TINag putative receptor, i.e. αvβ3. The above cellular effects were specifically related to the gene disruption of αvβ3 and not due to the ablation of α3β1 because expression of both the receptors was down-regulated following treatment with
FIGURE 10. TINag-integrin αv modulation of ILK, FAK, and p53 following Cisplatin Treatment of HK-2 Cells—The expression of both ILK and phosphorylated forms of ILK did not change significantly following siRNAs or CDDP treatments in cells maintained on TINag substratum while undergoing CDDP treatment and transfection of integrin αv-siRNA. Minimal expression of either p53 or p-p53 was observed in cells maintained on BSA- or TINag-coated dishes and treated with scrambled siRNA (Fig. 10, A, D, and E, lanes 1–3). A remarkable increase in the expression of both p53 and its phosphorylated form, p-p53, was observed upon treatment with CDDP (Fig. 10, A, D, and E, lane 5), a finding that is in line with the observations made in vivo in kidneys of Lewis rats (Fig. 5, D–G).

TINag Integrin αV Modulation of Signaling Events following Activation of FAK, i.e. AKT and Wortmannin-sensitive PI3K, Cell Viability, and Apoptosis in HK-2 Cells Undergoing CDDP Treatment—The cells maintained on TINag substratum had significantly increased expression of p-AKT (Fig. 11, A and B, lane 2 versus 1). The expression was remarkably reduced by the treatment of siRNA-αv or wortmannin (Fig. 11, A and B, lanes 3 and 4). Intriguingly, the CDDP treatment increased the expression of p-AKT; however, it was also reduced following the treatment with siRNA-αv or wortmannin (Fig. 11, A and B, lanes 7 and 8 versus 6). Along these lines, the CDDP-treated cells maintained on TINag substratum had reduced apoptosis and increased cell viability compared with those seeded on BSA-coated dishes (Fig. 11, C and D, lanes 5 versus 4). Both of these salutary effects of TINag were negated when cells were con-
comitantly treated with BSA (Fig. 11, A, n = 5; p < 0.01, expression of total FAK, p-FAK, and p-AKT in cells maintained on TINag substratum had increased expression of p-AKT (A and B, lane 2 versus 1). The expression was remarkably reduced by the treatment of siRNA-αv/β3 and wortmannin (A and B, lanes 3 and 4). Although CDDP-treated cells had increased the expression of p-AKT, it was also reduced by siRNA-αv/β3 or wortmannin treatment (A and B, lanes 7 and 8 versus 6). The CDDP-treated cells maintained on TINag substratum had also reduced apoptosis and increased cell viability compared to BSA control (CON) (C and D, lanes 5 versus 4). The expression of FAK and p-FAK were reduced in both strains of rats following CDDP treatment (A and D, n = 5; p < 0.01, expression of p-AKT in cells maintained on TINag versus BSA; and following treatment with Wortmannin, Wort.)

**FIGURE 11.** TINag integrin αv/β3 modulation of AKT and wortmannin-sensitive PI3K, cell viability, and apoptosis. THK-2 cells maintained on TINag substratum had increased expression of p-AKT (A and B, lane 2 versus 1). The expression was remarkably reduced by the treatment of siRNA-αv/β3 and wortmannin (A and B, lanes 3 and 4). Although CDDP-treated cells had increased the expression of p-AKT, it was also reduced by siRNA-αv/β3 or wortmannin treatment (A and B, lanes 7 and 8 versus 6). The CDDP-treated cells maintained on TINag substratum had also reduced apoptosis and increased cell viability compared to BSA control (CON) (C and D, lanes 5 versus 4). The expression of TINag were nullified when cells were concomitantly treated with PI3K inhibitor, wortmannin (C and D, lanes 6 versus 5). Although CDDP-treated cells had increased apoptosis and reduced cell viability (C and D, n = 5; p < 0.01, apoptosis and cell viability of cells maintained on TINag substratum versus seeded on BSA coated dishes or following treatment with Wortmannin, Wort.)

**FIGURE 12.** Status of FAK and AKT following cisplatin administration in Wistar versus Lewis rats. Western blotting followed by densitometric analyses revealed no significant differences in renal FAK or p-FAK in control (CON) Wistar versus Lewis rats (A–C). Their expression was reduced following CDDP treatment (A–C). However, the reduction in FAK and p-FAK expression was relatively more in kidneys of TINag-lacking Lewis rats treated with CDDP. No significant differences were observed in the expression of total AKT between control and CDDP-treated rats (A and D). Interestingly, p-AKT expression was decreased in Lewis rats compared with the Wistar strain in both CDDP-treated and control groups (A and E). Intriguingly, CDDP treatment led to a marginal increase in AKT and pAKT following CDDP treatment in Lewis strain of rats (A, D, and E), (n = 6; #, p < 0.01, expression of FAK, p-FAK, and p-AKT in Lewis versus Wistar strain of rats treated with CDDP.)

**DISCUSSION**

The role of ECM in the modulation of cellular behavior during embryonic development is known (30, 31). In general, the cell/matrix interactions modulated via integrin receptors are vital to the survival of the cells (10, 13). Each of the ECM proteins exhibits a certain degree of spatio-temporal specificity during embryonic and adult life, and thus their contribution...
toward the modulation of cell growth, differentiation, survival or apoptosis would be expected to be variable (11, 12). Because TINag is specifically expressed in the TBM, it is likely to influence the pathobiology of the tubules. In light of this, the contribution of TINag toward the tubular cell survival was investigated following an induction of acute injury in Lewis rats, a strain that conceivably lacks TINag protein, although the parental Wistar strain normally expresses this protein in the tubules.

The existence of TINag in the Lewis versus Wistar or other strain of rats is controversial (32–34). Utilizing autoantibodies, previous studies have indicated the absence of TINag staining in the kidneys of Lewis rats by immunohistochemistry, and this failure to detect it in the tubules was attributed to the masking of its antigenic epitopes by other basement membrane proteins (32–34). In our studies, we also failed to detect TINag with antibodies directed against the recombinant TINag (Fig. 1). To confirm its absence, Northern and Western blotting procedures were carried out, where renal cortices were extracted with strong chaotropic agents (14). Like the immunohistochemistry studies, both the biochemical procedures confirmed the absence of TINag in the tubules of Lewis rats although it was normally expressed in the parental Wistar strain (Fig. 1).

As indicated earlier, the TINag is a complex protein with several distinct domains having conceivable diverse potential functions (see above). However, not much is known about the clinical correlative studies that suggest that TINag could develop a juvenile form of nephronophthisis when mutation in TINag gene is associated with polycystic kidney disease in humans (18, 19). From these clinical studies, it is not yet delineated, and fortuitously the Lewis strain of rats provides an opportunity to elucidate its role in tubular homeostasis. Normally, Lewis rats do not exhibit any overt aberrations in tubular morphology or physiology. Therefore, we considered the clinical settings of nephronophthisis under conditions of dosage stress or under conditions of exposure, where the role of TINag was delineated. With the administration of CDDP, the serum creatinine and kidney Kim-1 mRNA, a marker of acute injury, increased in both the strain of rats, but they were significantly higher in Lewis rats (Fig. 2). Likewise, cellular damage and influx of inflammatory cells into the interstitial capillaries was more accentuated in Lewis rats, suggesting a relative susceptibility of this strain to CDDP-induced injury (Fig. 2). The damage to the tubular cells was accompanied by a higher degree of apoptosis. The apoptosis was mainly confined to the cortical tubules only where TINag is normally expressed (Fig. 3). Moreover, the extent of apoptosis and changes in the expression of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) molecules were much more accentuated in Lewis rats (Fig. 4), suggesting a potential role of TINag in cellular homeostasis. Conceivably, the apoptosis and Bax activation was in part mediated via p53-dependent pathway because its increased phosphorylation and nuclear localization was observed in kidneys of Lewis rats undergone CDDP stress (Fig. 5). Apoptosis in renal cells has been regarded as a critical event that leads to the kidney damage, and apparently it ensues following perturbation in the interactions between ECM proteins and cells lining the matrices (35, 36). The TINag plays a dynamic role in maintaining the morphology of proximal tubular epithelial cells by maintaining the cellular actin cytoskeleton organization via integrin receptors (23). In this regard, it is well known that the proper cell/matrix interactions between the glomerular podocytes and underlying basal lamina are crucial to the survival of these cells (37). The fact that all the parameters, reflective of renal injury, were relatively accentuated in TINag-deficient Lewis rats would suggest that TINag may provide certain degree of protection to shield the tubular cells from cisplatin-induced injury.

To elucidate the shielding effect of TINag relative to other ECM proteins in vitro experiments were carried out. Many of the ECM proteins besides providing support to the overlying cells also facilitate diverse cellular processes (31). Upon detachment of the cells from underlying ECM substrata, they undergo apoptosis or cell death, a process described as “anoikis” (38). Ancillary evidence for the role of ECM proteins in cellular survival has also been reported with kidney LLC-PK1 cells, a porcine tubular cell line, wherein detachment led to apoptosis of the cells associated with a significant decrease in type IV collagen, laminin and fibronectin expression (39). Under contexts cellular behavior of HK-2 cells on TINag substratum was investigated using a biomimetic surface. The cells maintained on culture dishes coated with bovine serum albumin (BSA) had significantly reduced cell survival and maintained cell morphology, whereas cell growth and proliferation were reflective of damage to the ECM proteins (16, 40). Besides the damage to nuclear protein/protein interactions may have an additive effect on the cellular homeostasis, as described in experiments with neuronal cells grown on biomimetic surfaces having motifs of different ECM proteins (16, 40). However, the cells maintained on combined substrata, TINag + type IV collagen or + laminin, did not exhibit any synergistic effect.

For cellular survival, the signals emanating from the ECM are transmitted via integrin receptors into the interior of cells to modulate various intracellular, mitochondrial, and nuclear events (10, 41–43). Far Western analyses were performed to establish protein/protein interactions between integrin receptors and various TINag recombinant proteins, which included the full-length and its N- and C-terminal fragments. The binding was mainly observed with αβ3, although the affinity for α3β1 was quite weak (Fig. 8). Interestingly, the binding was seen with full-length protein only, and it was comparable with that of vitronectin, a known ligand substrate for αv integrin. The binding was concentration-dependent and saturable for both the ligands (Fig. 8). The biological significance of such in vitro ligand/receptor interactions was apparent with the comparable improvement in the morphology of HK-2 cells seeded
Integrins are obligate heterodimers composed of two distinct glycoproteins, designated as α and β subunits. Various α and β integrin subunits can form more than 20 different heterodimers (10, 41–43). Their interactions with ECM proteins is divalent cation-dependent and peptide sequence-specific, and the substrate specificity is largely determined by the α-subunit, whereas the intracytoplasmic tail of β-subunit modulates various transductional events (42, 43). The specific combinations of α and β integrin subunits are also critical for the binding specificity of a given ECM ligand. For instance, type IIB procollagen N-propeptide can induce death of tumor cells via interactions with integrins αvβ3 and α3β1 (44), whereas collagen IV provides protection from cisplatin-induced apoptosis via integrin β1 signaling and the PI3K pathway (45). TINag protein does not have a typical RGDS domain, which is critical for binding with integrin; nevertheless, the ECM/integrin interactions do occur in the its absence (46). Because proximal tubules (HK-2 cells) express αvβ3 and α3β1, and the TINag includes the von Willebrand factor domain (15, 29), it is conceivable that the interactions can occur via this domain because it interacts with integrin αvβ3 (47). To elucidate that TINag yields its protective effect via these interactions in CDDP-induced injury, siRNA experiments were carried out. With the transfection of siRNA-αv or antibody blockade of αvβ3-increased survival and DNA fragmentation, translocation of Bax and a reduced cell survival were observed (Fig. 8). These studies are supportive of the observations made with HK-2 cells, which indicate that the ligand-dependent nature of the underlying substratum is required to establish the peritubular epithelia of angiogenic blood vessels (48).

One of the major downstream molecules that is recruited following ECM/integrin interactions is FAK (Fig. 8). FAK is known to be involved in many downstream cellular events essential for the survival of cancer cells. Decreased phosphorylation of FAK has been known to be related to the loss of cellular focal adhesions and stress fibers preceding the onset of caspase-mediated apoptosis by nephrotoxicant, which apparently disrupts cell/matrix interactions (49). Moreover, nephrotoxicant-induced cell injury is further potentiated with the transfection of LLC-PK1 cells with a dominant negative deletion mutant of FAK (50). In support of the role of FAK in cell survival are our observations where the expression and phosphorylation of FAK in the HK-2 cells treated with cisplatin was also found to be modulated by TINag/αvβ3 interactions (Fig. 10). Seeding of cells on TINag substratum mostly increased the phosphorylated form of FAK without influencing the total FAK expression. The exposure of HK-2 cells to cisplatin decreased the phosphorylation of FAK, which was accentuated with αv-siRNA treatment, suggesting that the disruption of TINag/αvβ3 interactions also adversely affect the FAK phosphorylation, and thereby the cell survival.

Another captivating aspect of pathobiology of FAK relates to the contention that nuclear accumulation of FAK promotes cell proliferation and survival by p53 ubiquitination (51–53). This notion is supported by the studies demonstrating interactions of the N-terminal domain of FAK with the transactivation domain of p53 (54). These studies demonstrated that FAK and p53 interactions suppress p53-mediated apoptosis and inhibit transcriptional activity of p53. Moreover, proper cell/matrix interactions in anchorage-dependent cells are needed to suppress p53-mediated apoptosis by modulating survival signals transduced by FAK (55). Thus, perturbation in cell/matrix or TINag/αvβ3 interactions by siRNA or deficiency of substratum TINag would attenuate the FAK expression and concomitant increased expression or phosphorylation of p53, as observed in cell culture experiments (Fig. 10) and in kidneys of CDDP-treated Lewis rats (Fig. 5). Because activated p-FAK is known to bind directly with p53, it would suggest that this may be one of the pathways whereby TINag/αvβ3 interactions could modulate apoptosis and cellular survival following a tubular cell injury (supplemental Fig. 2, pathway I).

One may also consider other pathways that may be involved following the activation of FAK because it can mediate signaling of many downstream cellular events that modulate cell migration, cell cycle progression, and cell survival (56). For instance, the activation of c-Src known to mediate the regulation of another wortmannin-sensitive kinase known PI3K and thereby the AKT pathway. The PI3K/AKT pathway is responsible for the cellular events to guide directly or indirectly whether or not a given cell will undergo apoptosis (Fig. 11). Various experimental studies became supportive of the notion that decreased expression and increased cellular viability on ECM-free substrata were annulled by the inhibition of AKT using wortmannin (Fig. 11). The wortmannin treatment led to a decreased expression of p-AKT, suggesting that ECM/integrin interactions modulate events involved in the PI3K/AKT pathway. The AKT is a downstream effector of PI3K that is known to regulate diverse cellular pathobiological processes, by targeting ECM substrates (58). The fact that the CDDP-treated or -untreated cells maintained on the TINag substratum had increased expression of p-AKT, which was significantly reduced with the siRNA-αv transfection or inclusion of wortmannin in the culture medium, would suggest the involvement of the FAK/PI3K/AKT pathway in the signaling events initiated upon TINag/αvβ3 interactions (Fig. 11). It is worth mentioning here that the AKT-mediated events may be differentially modulated by various ligands or cytokines, and they may also be cell-specific because survival of certain cells like osteoclasts is not dependent upon AKT activation (59, 60). Nevertheless, our in vitro findings corroborated with in vivo studies where Lewis rats lacking the TINag ECM ligand showed a remarkable reduction in the expression of both p-FAK and p-AKT, following CDDP administration (Fig. 12).

In summary, it appears that FAK expression and its phosphorylation followed by activation of the PI3K/AKT pathway are central to the generation of cellular survival signals in two different interrelated pathways under the homeostatic state of proper cell/matrix interactions. The fact that a relatively high attenuation in the expression or phosphorylation of FAK and AKT and increased p53 expression and cellular apoptosis was observed in CDDP-treated Lewis rats that lack ECM-TINag would support the above alluded intricate cellular signaling biologic precepts (supplemental Fig. 2, pathway II).
