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Suppression of Chemically Induced Apoptosis but Not Necrosis of Renal Proximal Tubular Epithelial (LLC-PK1) Cells by Focal Adhesion Kinase (FAK)

ROLE OF FAK IN MAINTAINING FOCAL ADHESION ORGANIZATION AFTER ACUTE RENAL CELL INJURY*

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Decreased phosphorylation of focal adhesion kinase (FAK) is associated with loss of focal adhesions and actin stress fibers and precedes the onset of apoptosis in renal epithelial cells caused by nephrotoxicants (Van de Water, B., Nagelkerke, J. F., and Stevens, J. L. (1999) *J. Biol. Chem.* 274, 13328–13337). The role of FAK in the control of apoptosis caused by nephrotoxicants was further investigated in LLC-PK1 cells that were stably transfected with either green fluorescent protein (GFP)-FAK or dominant negative acting deletion mutants of FAK, GFP-FAT, and GFP-FRNK. GFP-FAT and GFP-FRNK delayed the formation of focal adhesions and prevented the localization of endogenous (phosphorylated) FAK at these sites. GFP-FAT and GFP-FRNK overexpression potentiated the onset of apoptosis caused by the nephrotoxicant dichlorovinyl-cysteine. This was associated with an increased activation of caspase-3. GFP-FAT also potentiated apoptosis caused by doxorubicin but not cisplatin. The potentiation of apoptosis by GFP-FAT was related to an almost complete dephosphorylation of FAK; this did not occur in cells overexpressing only GFP. This dephosphorylation was associated with a pronounced loss of focal adhesion organization in GFP-FAT cells, in association with loss of tyrosine phosphorylation of paxillin. In conclusion, the data indicate an important role of cell-matrix signaling in the control of chemically induced apoptosis; loss of FAK activity caused by toxic chemicals results in perturbations of focal adhesion organization with a subsequent inactivation of associated (signaling) molecules and loss of survival signaling.

Apoptosis is a highly controlled type of cell death that may result from severe cellular injury such as due to ischemia/reperfusion injury, exposure to UV light, γ-irradiation, as well as treatment with toxic chemicals (1–3). The response of cells to such injury is crucial for the outcome of cell damage: cell survival or death. As a consequence of the cellular injury, various signaling pathways are (in)activated (4–6). Depending on the type of signaling cascade cell death is either stimulated or suppressed. Although some signal transduction cascades, *e.g.* growth factor signaling through PKB[1] and extracellular signal-regulated kinase, have been defined to play crucial roles in the regulation of the cellular response to damage (6, 7), the role of many other signaling pathways remains largely unknown. Thus, little is known about the role of disturbances of the signal transduction pathways mediated by interactions between cells and the extracellular matrix (ECM) in the cellular response to injury.

Cell adhesion to ECM through integrin receptors is critical for normal cell function (8, 9). Perturbation of proper cell-ECM interactions is observed in various acute and chronic pathological conditions including ischemia/reperfusion injury, cancer, as well as exposure to toxic chemicals (10–15). Loss of such interactions of normal cells results in the onset of apoptosis and is also referred to as anoikis (16, 17). Altered signaling as a result of loss of cell-ECM interactions is critical in this process, and focal adhesion kinase seems to play an important role (15, 18–21).

Engagement of integrins with the ECM results in activation of focal adhesion kinase (FAK), a 125-kDa non-receptor tyrosine kinase that is localized at focal adhesions, the closest contacts between cells and the ECM (22). FAK consists of an N-terminal band 4.1 JEF domain, a central kinase domain, and a C-terminal so-called focal adhesion targeting (FAT) domain (22, 23). Upon integrin binding to ECM, FAK is autophosphorylated on tyrosine residue 397, which is a docking site for the SH2 domain of Src family kinases as well as PI 3-kinase (22). As a consequence of Src binding other tyrosine residues of FAK, including Tyr^{576} and Tyr^{925}, are phosphorylated resulting in increased kinase activity of FAK and additional docking of other (cytoskeleton-associated) adapter and signaling molecules (22). This coordinated activation of FAK is critical in diverse cellular processes such focal adhesion formation/turnover, cell spreading and migration, cell proliferation, and control of apoptosis (15, 18–22, 24–27).

There is increasing evidence that FAK is involved in protection against apoptosis, in particular anoikis. For example, overexpression of constitutively active FAK prevents anoikis of epithelial cells (28). Furthermore, microinjection of peptides

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[1] The abbreviations used are: PKB, protein kinase B; AMC, 7-amino-4-methylcoumarin; FAK, focal adhesion kinase; FAT, focal adhesion targeting domain; FRNK, focal adhesion kinase-related non-kinase, LDH, lactate dehydrogenase; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; ECM, extracellular matrix; PTCs, proximal tubule epithelial cells; DPPD, diphenyl-phenylenediamine; DCVC, 1,2-dichlorovinyl-L-cysteine; PY, phosphotyrosine; PI, phosphatidylinositol; DEVD, Asp-Glu-Val-Asp.

that compete for FAK-integrin association, and also antibodies directed against FAK itself, can induce apoptosis in fibroblasts that are not yet fully spread (29). Moreover, treatment of tumor cells with FAK antisense oligonucleotides or overexpression of deletion mutants of FAK that only contain the FAT domain and compete for function of endogenous FAK leads to rounding up of cells followed by apoptosis (18, 30, 31). During apoptosis caused by a wide variety of stimuli FAK is cleaved in a caspase-dependent manner (15, 32–34). This FAK cleavage is seen in both adherent and non-adherent cell lines. FAK cleavage results in a C-terminal fragment that contains the FAT domain and lacks the central catalytic domain of FAK (34). This fragment resembles the protein focal adhesion kinase-related nonkinase (FRNK) that acts as a dominant negative of FAK and blocks FAK localization to focal adhesion as well as its phosphorylation (25, 34). Therefore, formation of this C-terminal fragment of FAK during apoptosis may block the anti-apoptotic function of endogenous FAK (34). Although the above data indicate an important role for FAK in anoikis, the role of FAK in chemically induced apoptosis is less well understood. Sonoda *et al.* (35) demonstrated that overexpression of FAK in HL60 cells inhibits apoptosis caused by hydrogen peroxide and etoposide; however, these cells were non-adherent and had very low expression levels of endogenous FAK. In addition, the same group demonstrated that hydrogen peroxide caused increased phosphorylation of endogenous FAK in T98G cells, which was related to protection against apoptosis (36, 37). However, these studies were also performed with suspended cells that lack normal focal adhesion complexes. Moreover, in these suspended T98G cells FAK phosphorylation levels are negligible, as expected (36). Thus it still is unknown whether endogenous FAK in adherent cells (the physiological situation) is critical for the control of chemically induced apoptosis and whether this is directly related to changes in the focal adhesion organization. Moreover, since many chemicals depending on the treatment conditions cause both necrosis and apoptosis, it remains unclear whether there is a difference in regulation of apoptosis and necrosis by FAK. These issues are part of the present investigations. For this purpose we used renal proximal tubular epithelial cells exposed to nephrotoxicants as a model.

Renal proximal tubule epithelial cells (PTCs) are an important target for a variety of nephrotoxic medicines, chemicals, and environmental pollutants as well as ischemia/reperfusion injury (38–40). Injury of PTCs is associated with detachment of viable PTCs from the extracellular matrix, both *in vitro* and *in vivo*, an effect that is related to redistribution of integrins, disturbances of focal adhesion organization, and F-actin organization (15, 41–47). To investigate the molecular mechanisms involved in these processes more precisely we have used primary cultures of rat PTC using the well characterized nephrotoxicant *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) (15, 43, 46–51). DCVC is metabolized by a cysteine conjugate β-lyase to a reactive acylating metabolite that covalently modifies cellular macromolecules (48, 52, 53); this bio-activation is required for the DCVC-induced cytotoxicity both *in vivo* and *in vitro*. DCVC induces both necrosis and apoptosis of PTC, depending on the treatment conditions (15, 49, 50, 54). It causes formation of reactive oxygen species that results in lipid peroxidation followed by necrosis (48, 54). When lipid peroxidation is blocked PTCs are protected from necrosis and only apoptosis is allowed (55). This apoptosis of PTC is preceded by dissolution of focal adhesion complexes in association with dephosphorylation of FAK (15, 54). These events are independent of caspase activation and, importantly, occur well before the onset of caspase-3 activity (15). Based on these findings and the literature data, we proposed that the loss of FAK activity after toxicant treatment is important for perturbations of focal adhesions organization as well as FAK-mediated cell survival signaling. To study this we created renal epithelial cell lines that stably overexpress dominant negative acting FAK deletion mutants, FAT and FRNK. Overexpression of these constructs did not block formation focal adhesion but interfered with endogenous FAK localization and phosphorylation at focal adhesions. The data indicate a role for endogenous FAK in the control of chemically induced apoptosis but not necrosis. This seems directly related to the role of FAK in focal adhesion organization and tyrosine phosphorylation of the FAK-associated adapter protein paxillin. These data show for the first time a role for FAK in the control of chemically induced apoptosis in adherent epithelial cells.

EXPERIMENTAL PROCEDURES

Materials and Plasmids

Dulbecco's modified Eagle's medium, fetal calf serum, G418, penicillin/streptomycin, and trypsin/EDTA were from Life Technologies, Inc. Bovine serum albumin fraction V, insulin, AMC, doxorubicin, and cisplatin were from Sigma. Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone was from Bachem (Bubendorf, Switzerland). Acyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was from Research Biochemicals Int. (Natick, MA). pEGFP expression plasmids (CLONTECH) encoding FAK, and the FAK deletion mutants FAT and FRNK coupled to green fluorescent protein (GFP) were kindly provided by Dr. Ilic (see Ref. 18 for detailed description of the constructs). Myc-FAK was kindly provided by Dr. Hanks. DCVC was synthesized as described (48).

Cell Culture and Preparation of Stable Transfectants

The porcine renal epithelial cell line LLC-PK1 was from American Tissue Culture Collection (ATCC) and used between passages 201 and 213. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin (complete medium) at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide. For preparation of stable cell lines, LLC-PK1 cells were transfected with 0.4 μg of DNA of pEGFP, pEGFP-FAK, pEGFP-FAT, or pEGFP-FRNK using LipofectAMINE Plus reagent according to the manufacturer's procedures (Life Technologies, Inc.). Stable transfectants were selected using 800 μg/ml G418. Individual clones were picked and maintained in complete medium containing 100 μg/ml G418. Clones were further analyzed for the expression of the various GFP chimeras using both flow cytometry, Western blotting, and immunofluorescence. For further experiments for each construct 2–4 individual stable cell lines were used. For some experiments pkGFP and pkGFP-FAT clones were transiently transfected with Myc-tagged FAK in a similar fashion as for the stable transfections.

Cell Treatment Conditions

For cytotoxicity experiments, cells were plated in 15-cm dishes ($2{\cdot}10^6$ cells) and grown to subconfluence in 3 days. Thereafter, cells were trypsinized and plated in collagen-coated 10- or 6-cm dishes at a density of $0.5–1.10^5$ cells/cm^2 in complete culture medium either with or without 10% (v/v) fetal bovine serum. In some experiments cells were plated in 24-well dishes containing coated glass coverslips (Corning Costar, Acton, MA). Cells were allowed to adhere and spread overnight. Cells were treated with DCVC in Hanks' balanced salt solution (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4{\cdot}7H_2O$, 0.4 mM $Na_2HPO_4{\cdot}2H_2O$, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 4 mM $NaHCO_3$, 25 mM HEPES, 5 mM D-glucose, pH 7.4) in the presence of the antioxidant diphenyl-phenylenediamine (DPPD; 10 μM), which prevents necrosis, for 6 h followed by recovery in complete medium containing 10 μM DPPD. In some experiments cells were treated with doxorubicin or cisplatin in complete culture medium without fetal bovine serum.

Determination of Cell Proliferation

For determination of cell proliferation cells were plated in 24-well dishes at a density of $3{\cdot}10^4$ cells/well, and complete culture medium was changed every day. At indicated times wells were rinsed twice with PBS, and after removal of PBS the culture plates were frozen at −20 °C. After thawing 200 μl of MilliQ water was added followed by incubation for 1 h at 37 °C. After freezing at −80 °C followed by thawing, to 50 μl of sample 50 μl of 20 μg/ml Hoechst 33258 in TNE (10 mM Tris, 2 M NaCl, 1 mM EDTA, pH 7.4) was added, and fluorescence was measured

using a plate reader (HTS 7000, PerkinElmer Life Sciences). Calf thymus DNA (Sigma) dissolved in TNE was used as a standard. Cell number was expressed as amount DNA/well.

Determination of Cell Death

Cell Cycle Analysis—Apoptosis was determined by cell cycle analysis as described (18). Briefly, floating and adherent cells that were trypsinized were pooled and subsequently fixed in 90% ethanol (−20 °C). After washing cells twice with PBS, 1 mM EDTA cells were resuspended in PBS/EDTA containing 7.5 μM propidium iodide and 10 μg/ml RNase A. After 30 min incubation at room temperature the cell cycle was analyzed by flow cytometry (FACS-Calibur, Becton Dickenson), and the percentage of cells present in sub-G_0/G_1 was calculated using the Cellquest software (Becton Dickenson).

Annexin V Staining—In some experiments apoptosis was determined by staining with fluorescent-labeled annexin V and propidium iodide. Briefly, floating as well as adherent cells that were trypsinized were pooled and centrifuged for 5 min at 500 × *g*. Pelleted cells were resuspended and allowed to recover for 30 min in complete culture medium. Thereafter, cells were centrifuged again for 5 min at 500 × *g* and resuspended in 100 μl of annexin-V binding buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2 \cdot 2H_2O$, pH 7.4) containing allophycocyanin-conjugated annexin-V followed by incubation on ice for 15 min. After addition of 100 μl of binding buffer containing 10 μM propidium iodide, cells were analyzed by flow cytometry. The percentage of cells that were either annexin-V-positive and propidium iodide-negative (AV+/PI−; *i.e.* apoptotic cells) or annexin-V-positive and propidium iodide-positive (AV+/PI+; *i.e.* necrotic cells) was calculated using the Cellquest software.

Lactate Dehydrogenase (LDH) Release—Necrotic cell death was measured by the release of LDH from cells in the culture medium as described (47). The percentage of cell death was calculated from the amount of LDH release caused by treatment with toxicants relative to the amount to that released by 0.1 w/v Triton X-100, *i.e.* 100% release.

Caspase activity was assayed as follows. Briefly, attached and detached cells were harvested and collected by centrifugation as above. The cell pellet was taken up in lysis buffer (10 mM HEPES, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, and 5 mM EGTA, pH 7.4) and subjected to three cycles of freezing and thawing. Equal amounts of cell proteins were used in a caspase assay using Ac-DEVD-AMC (25 μM; Research Biochemicals Int.) as a substrate. Fluorescence derived from release of the AMC moiety was followed using a fluorescence plate reader (HTS 7000; PerkinElmer Life Sciences). Caspase activity was calculated as pmol/mg cell protein·min using AMC as a standard.

Gel Electrophoresis and Immunoblotting

For Western blot analysis cells were scraped in ice-cold TSE (10 mM Tris, 250 mM sucrose, 1 mM EGTA, pH 7.4) plus inhibitors (50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) and pooled with the floating cells. Protein concentration was determined using the Bradford protein assay using IgG as a standard. Equal amounts of total cellular protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore). Blots were blocked with 5% (w/v) non-fat dry milk in TBS-T (0.5 M NaCl, 20 mM Tris-HCl, 0.05% v/v Tween 20, pH 7.4) and probed with the appropriate antibodies as follows: anti-PARP (monoclonal C2.10, 1:2,000; Enzyme System Products), anti-FAK (clone 77 directed against residues 354–533 of FAK, 1 μg/ml, Transduction Laboratories; or polyclonal antibody directed against the C-terminal residues 748–1053 of FAK, 1 μg/ml, Upstate Biotechnology, Inc.), anti-paxillin (clone 349, 0.5 μg/ml, Transduction Laboratories), phospho-state-specific anti-PY^{397}-FAK (0.25 μg/ml, BIOSOURCE Int.), and phospho-state-specific anti-PY^{118}-paxillin (0.25 μg/ml, BIOSOURCE Int.). For detection of FAK and paxillin, the appropriate horseradish peroxidase-coupled secondary antibodies (The Jackson Laboratories) and ECL reagent were used (Amersham Pharmacia Biotech). For phospho-state-specific antibody detection the Tropix kit was used (PerkinElmer Life Sciences).

Immunofluorescence and Imaging Techniques

For immunofluorescence studies cells were cultured on collagen-coated glass coverslips in 24-well dishes. Cells were fixed with 3.7% formaldehyde for 10 min followed by 3 washes with PBS. In some experiments cells were fixed in −20 °C methanol for 5 min followed by 3 washes with PBS. After cell permeabilization and blocking with PBS, 0.2% w/v Triton X-100, 0.5% (w/v) bovine serum albumin, pH 7.4 (PTB), cells were stained for FAK (mouse monoclonal clone 77, 1 μg/ml, Transduction Laboratories; or polyclonal antibody directed against the C terminus, 1 μg/ml, Upstate Biotechnology, Inc.) and PY^{397}-FAK (phospho-state-specific anti-PY^{397}-FAK, 1 μg/ml, BIOSOURCE Int.). Cells were also stained against paxillin (1 μg/ml, Transduction Laboratories) and PY^{118}-paxillin (phospho-state-specific PY^{118}-paxillin, 1 μg/ml, BIOSOURCE Int.) diluted in PTB. After transient transfection with myc-FAK cells were immunostained for Myc (mouse monoclonal clone 9E10, 1 μg/ml, Roche Molecular Biochemicals). For secondary staining Cy3-labeled goat anti-rabbit and Cy5-labeled goat anti-mouse antibodies were used (1 μg/ml, The Jackson Laboratories). Cells were mounted on glass slides using Aqua-Poly/Mount (Polysciences Inc., Warrington, PA). Cells were viewed using a modified Bio-Rad 600 MRC confocal laser scanning microscope, and GFP, Cy3, and Cy5 staining was analyzed using the 488, 543, and 633 nm lasers, respectively, in combination with the appropriate excitation/emission filter sets. For clarity negative images of the original collected images are shown.

Statistical Analysis

Student's *t* test was used to determine if there was a significant difference between two means ($p < 0.05$); statistical differences are indicated with an asterisk. When multiple means were compared, significance was determined by a one-way analysis of variance ($p < 0.05$) in conjunction with a Newman Keul's post hoc test. For analysis of variance, letter designations are used to indicate significant differences. Means with a common letter designation are not different; those with a different letter designation are significantly different from all other means with different letter designations.

RESULTS

Characterization of LLC-PK1 Cells Overexpressing GFP-FAK, GFP-FRNK, and GFP-FAT—To investigate the role of FAK in apoptosis of renal epithelial cells caused by nephrotoxic xenobiotics, we used the LLC-PK1 cell line, which is of renal proximal tubular cell origin. These cells are often used as a model to study mechanisms of renal cytotoxicity; the mechanism of toxicity of DCVC is similar in primary cultured renal proximal tubular cells and LLC-PK1 cells (49, 55). First we created stable cell lines of LLC-PK1 cells that overexpress deletion mutants of FAK, either FAT or FRNK (Fig. 1*A*). These mutants act as dominant negatives and compete for localization of FAK at focal adhesions (18). In addition, we created cell lines with stable overexpression of FAK. We used GFP expression constructs to facilitate clonal selection and characterization of the clonal derivatives. Furthermore, the use of GFP constructs allows us to follow focal adhesion organization and other biochemical events in living cells. GFP-FAT and GFP-FRNK chimeric proteins behave in a similar fashion as FAT and FRNK proteins (18). Four clonal derivatives of GFP, GFP-FAT, and GFP-FAK and two clonal derivatives of GFP-FRNK were obtained (Fig. 1). All clones expressed either GFP or GFP chimeras as determined by flow cytometric analysis, yet the mean GFP fluorescence intensity varied between the various constructs (Fig. 1*B*). Expression of GFP or GFP chimeras was in over 95% of the cells for all clones. The GFP-FAK, -FAT, and -FRNK chimeras had the expected molecular weight as detected by Western blotting using a polyclonal anti-FAK antibody directed against the C terminus of FAK (Fig. 1*C*) and an anti-GFP antibody (not shown). Importantly, the amount of transfected FAT and FRNK present in the cells was well above the levels of endogenous FAK. Overexpression of GFP-FAT or -FRNK did not affect the expression of endogenous FAK; somewhat increased FAK levels were found in pkGFP-FAK cells (Fig. 1*D*) possibly due to breakdown of GFP-FAK. None of the constructs affected expression of other focal adhesion-associated cytoskeletal proteins, including paxillin and $p130^{Cas}$. In addition, no difference in the expression of Pyk2 was observed between the different clones (Fig. 1*D*), although an increased expression in fibroblasts deficient in FAK has been reported (56).

GFP-FAT Delays Focal Adhesion Formation of LLC-PK1

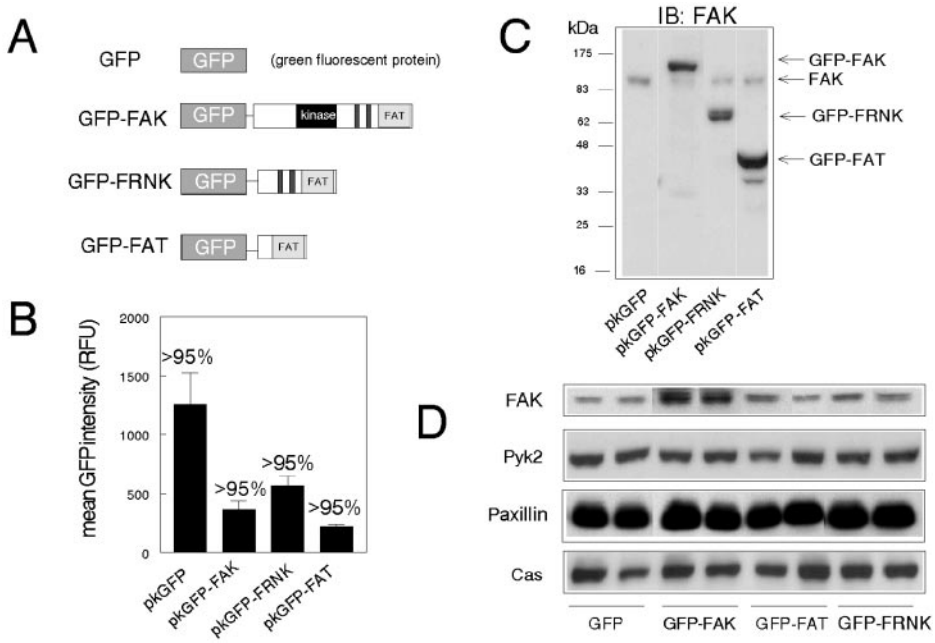

FIG. 1. **Characterization of LLC-PK1 cells stably expressing GFP, GFP-FAT, GFP-FRNK, and GFP-FAK.** *A,* LLC-PK1 cells were transfected with constructs containing GFP, GFP-FAT, GFP-FRNK, or GFP-FAK. *B,* stable clones were characterized by flow cytometric analysis for the average GFP fluorescence (*y* axes) and percentage of GFP-positive cells within the population (*numbers above bars*). Shown is mean ± S.D. of the relative mean GFP fluorescence intensity (FL1 detector) of four independent clones for each construct, except for GFP-FRNK where only two clones were obtained. *C,* size of the various constructs was checked by Western blotting (*IB*) using an anti-FAK antibody directed against the C terminus of FAK. Shown are representative clones for pkGFP (clone 7), pkGFP-FAK (clone 4), pkGFP-FRNK (clone 1), and pkGFP-FAT (clone 8). *D,* expression levels of FAK, paxillin, Pyk2, and $p130^{Cas}$ in pkGFP (clone 7 and 11), pkGFP-FAK (clone 4 and 5), pkGFP-FRNK (clone 1 and 2), and pkGFP-FAT (clone 6 and 8) were determined by Western blotting. For reasons of space only two clones for each construct are shown. Data shown are representative for the other clones.

Cells—The FAT domain of FAK is involved in the localization of FAK to focal adhesions through interactions with other focal adhesion-associated cytoskeletal proteins, including talin and paxillin (22). Previous studies in avian and mammalian fibroblasts and endothelial cells demonstrated that FRNK competes for the localization of FAK at focal adhesion and the organization of focal adhesion structures. Therefore, we tested whether GFP-FAT also inhibited cell spreading and focal adhesion organization. No statistical difference was observed between the percentage of spread cells for the GFP and GFP-FAT clones at 2, 4, or 24 h either in the presence or absence of serum (Table I). Next we determined whether GFP-FAT inhibited focal adhesion formation. At 4 h after plating GFP cells had formed focal adhesion structures as determined by accumulation of paxillin in structures that resemble focal adhesions (Fig. 2). Despite the fact that GFP-FAT was unable to block cell spreading, the early formation of focal adhesions was inhibited (Fig. 2). In pkGFP cells phosphorylated FAK (*i.e.* PY^{397}-FAK) was localized at focal adhesions. This was not observed in pkGFP-FAT cells. Several other focal adhesion-associated proteins, including paxillin, are located at focal adhesions and phosphorylated on tyrosine residues during cell spreading. Therefore, we also immuno-stained cells for phosphotyrosine (PY). In GFP cells PY staining was clearly visible at focal adhesions. PY staining was also observed in GFP-FAT cells, but it was not present in distinct sites resembling focal adhesions but rather as a broad band at the lamellipodia of spreading cells (Fig. 2).

GFP-FAT Competes for the Localization and Phosphorylation of FAK at Focal Adhesions—Previous studies of fibroblasts had indicated that FRNK only retards cell spreading and focal adhesion formation (25). Moreover, nullizygous FAK fibroblasts still form focal adhesions (56). We also determined whether FAT affected focal adhesion formation at later time points. In pkGFP cells 24 h after plating FAK and PY^{397}-FAK were clearly detectable at focal adhesions (Fig. 4*A*), which correlated with a phosphorylation of FAK as determined by Western blotting (Fig. 3). In pkGFP-FAT cells GFP-FAT was located at sites resembling focal adhesions. Despite the fact that there was still a considerable amount of PY^{397}-FAK present in pkGFP-FAT cells (Fig. 3), hardly any staining of FAK and PY^{397}-FAK was detected at focal adhesions as determined by immunofluorescent staining (Fig. 4*A*). Similar observations were made with either formaldehyde or methanol fixation or with different FAK antibodies (data not shown). Although the above indicate a displacement of FAK from focal adhesions by GFP-FAT, the possibility existed that GFP-FAT prevents immunofluorescent detection of FAK at these sites due to steric hindrance. To exclude this possibility we used two different approaches. First, cells were fixed with either formaldehyde or methanol followed by double staining for FAK with another focal adhesion-associated protein, either paxillin or vinculin. In pkGFP cells FAK and paxillin clearly co-localized at focal ad-

TABLE I
Effect of GFP-FAT on cell spreading

Cells were plated on collagen-coated coverslips and allowed to adhere in the presence or absence of 10% (v/v) fetal bovine serum. After 2, 4, or 24 h cells were fixed, and the percentage of spread cells was determined by phase contrast microscopy. Data shown are the mean ± S.D. of four independent clones ($n = 4$) and are representative for two different experiments. A–D indicate significant differences. Statistical differences were determined as described under "Experimental Procedures." ND, not determined.

	% cell spreading		
	2 h	4 h	24 h
GFP			
Plus serum	61 ± 4^{A}	80 ± 2^{B}	90 ± 1^{D}
Minus serum	ND	35 ± 7^{C}	73 ± 8^{D}
GFP-FAT			
Plus serum	49 ± 7^{A}	69 ± 3^{B}	87 ± 1^{D}
Minus serum	ND	28 ± 5^{C}	75 ± 7^{D}

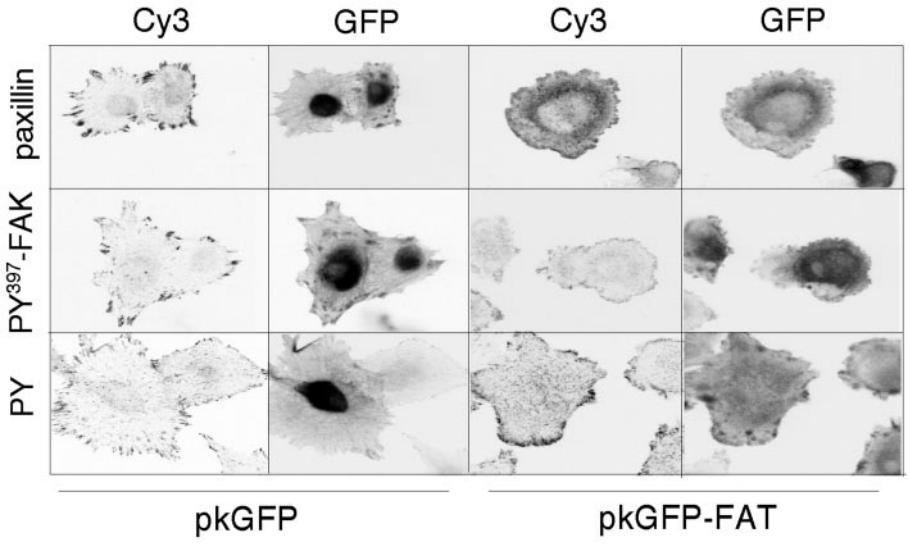

FIG. 2. **Effect of GFP-FAT on focal adhesion formation and FAK phosphorylation.** pkGFP and pkGFP-FAT cells were plated on collagen-coated coverslips in complete culture medium, and 4 h after plating cells were fixed and stained for either paxillin, PY^{397}-FAK, or phosphotyrosine (PY) followed by analysis by confocal laser scan microscopy. Shown are images from pkGFP (clone 7) and pkGFP-FAT (clone 8) that are representative for the other clones. Note the formation of focal adhesion (paxillin staining) in pkGFP cells but not in pkGFP-FAT cells. These focal adhesions contain PY397-FAK as well as phosphotyrosine. GFP-FAT cells contain concentrated regions of phosphotyrosine in lamellipodia but not in focal adhesion-like structures.

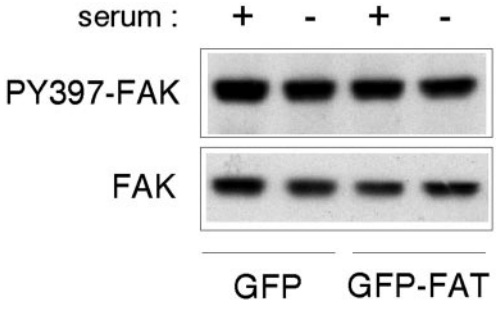

FIG. 3. **Effect of GFP-FAT on phosphorylation of FAK.** pkGFP and pkGFP-FAT cells were plated on collagen-coated 10-cm dishes followed by 24-h recovery in culture medium with or without 10% (v/v) fetal bovine serum. Thereafter, cells were scraped in TSE plus inhibitors, and equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis followed by transfer to polyvinylidene difluoride membranes and staining for FAK and PY^{397}-FAK. Shown are the data from pkGFP (clone 7) and pkGFP-FAT (clone 8), which are representative for other clonal derivatives.

hesions. However, despite the fact that in pkGFP-FAT cells paxillin was clearly present at focal adhesions, little FAK was observed at the same sites (Fig. 5*A*). Similar observations were made for vinculin staining (data not shown). In a second approach, both pkGFP and pkGFP-FAT cells were transiently transfected with Myc-tagged FAK, and after 24 h cells were fixed and stained for Myc and GFP. We anticipated that in pkGFP-FAT cells at least some of the expressed Myc-FAK protein would be localized at focal adhesions. If GFP-FAT would indeed cause steric locking thereby preventing immunodetection of antigens in the close proximity of GFP-FAT, then no Myc-FAK detection would be expected at the focal adhesions. However, in contrast, in pkGFP as well as pkGFP-FAT cells Myc staining was observed at sites resembling focal adhesions. Most important, in pkGFP-FAT cells Myc-FAK co-localized with GFP at focal adhesions (Fig. 5*B*). The FAT domain of FAK is required for the interactions with the adapter protein paxillin (22). FAK seems involved in the phosphorylation of paxillin on tyrosine residues 31 and 118 (57, 58). Although the above already indicated the correct localization of paxillin at focal adhesions in pGFP-FAT cells, the possibility existed of an altered phosphorylation of paxillin due to loss of FAK at focal adhesions in pkGFP-FAT cells. To this end we determined in pkGFP-FAT cells whether both paxillin and phosphorylated paxillin were still present at focal adhesions. Therefore, cells were co-stained for paxillin as well as PY^{118}-paxillin. Despite the fact that FAK was no longer present at focal adhesions in pkGFP-FAT cells, paxillin co-localized with GFP-FAT and was also still phosphorylated on tyrosine residue 118 (Fig. 4*B*).

In conclusion, the combined above observations indicate that overexpression of GFP-FAT in LLC-PK1 cells slows, but does not block, the formation of focal adhesions; it competes for the localization of FAK and, consequently, localization of PY^{397}-FAK on focal adhesions.

GFP-FAT Potentiates Apoptosis Caused by S-(1,2-Dichlorovinyl)-L-cysteine (DCVC)—Recently we showed that several nephrotoxicants, including DCVC, cause dephosphorylation of FAK, which precedes the onset of apoptosis, *e.g.* caspase activation and caspase-mediated cleavage of FAK (15). Since GFP-FAT competes for FAK localization and some of its function in LLC-PK1 cells (see above), we used these cells to investigate whether FAK is involved in controlling nephrotoxicant-induced apoptosis in renal epithelial cells. pkGFP and pkGFP-FAT cells were plated on collagen-coated dishes followed by overnight culturing to ensure that both cell lines had formed normal focal adhesion plaques (see above). Thereafter, cells were treated with DCVC and assessed for apoptosis. GFP-FAT overexpression clearly potentiated the DCVC-induced apoptosis of renal epithelial cells as determined by analyzing phosphatidylserine externalization using annexin V-allophycocyanin/propidium iodide staining (Fig. 6, *A–D*) or by cell cycle analysis (Fig. 6*E*). The onset of apoptosis in pkGFP-FAT cells was already observed after 8 h. After 24 h there was still a difference in the percentage of apoptotic cells between pkGFP and pkGFP-FAT cells (Fig. 6*E*).

Potentiation of DCVC-induced Apoptosis by FAT Involves Activation of Caspase-3—Caspase-3 is required for activation of endonucleases as well as the cleavage of various caspase substrates during apoptosis, including PARP. Therefore, we next investigated whether the potentiation of apoptosis as determined by flow cytometric analysis was associated with the more rapid onset of caspase-3 activity. Indeed, GFP-FAT overexpression accelerated the onset of caspase-3-like activity in a time- and concentration dependent manner (Fig. 7, *A* and *B*). This increased activity was directly related to a higher percentage of cells that was positive for active caspase-3 as determined by immunofluorescence staining (Fig. 7*C*; data not shown). The increased activity of caspase-3 in GFP-FAT cells was also associated with a more rapid onset of the formation of active caspase-3 and cleavage of the typical caspase-3 substrate PARP (Fig. 7*D*). In conclusion, the data indicate that GFP-FAT sen-

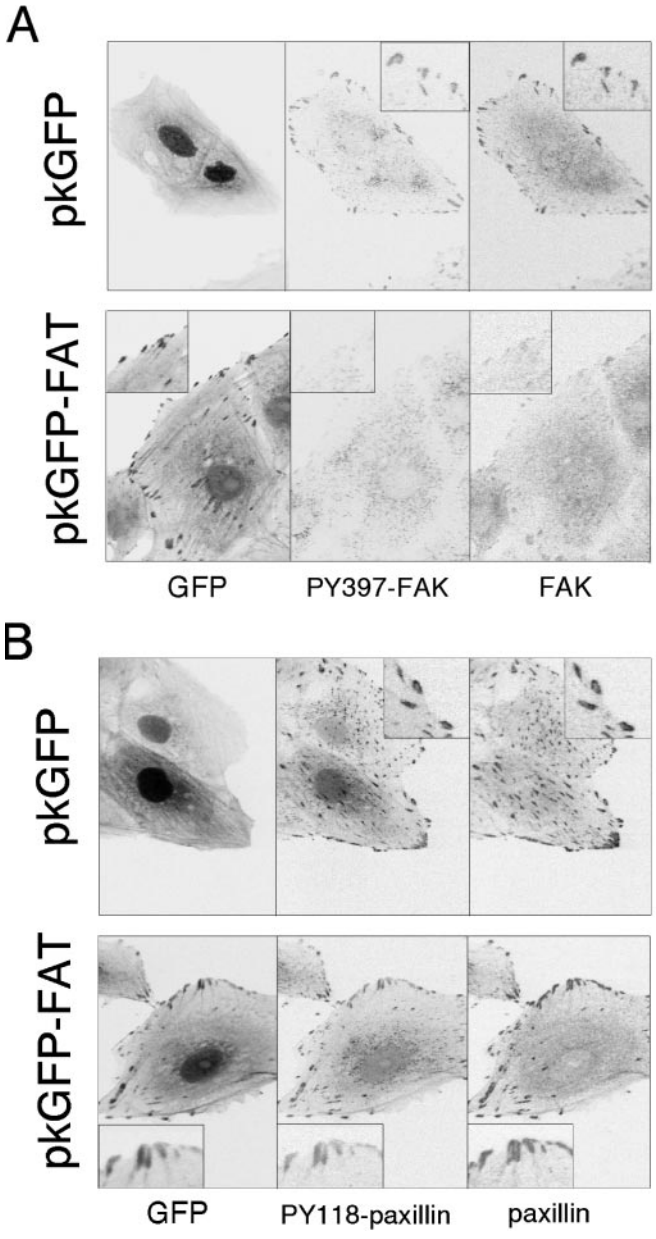

FIG. 4. **GFP-FAT prevents localization of FAK and PY^{397}-FAK at focal adhesions.** pkGFP and pkGFP-FAT cells were plated on collagen-coated coverslips and allowed to adhere and spread for 24 h. Thereafter, cells were fixed, followed by immunofluorescent staining for either FAK and PY^{397}-FAK (*A*) or paxillin and PY^{118}-paxillin (*B*). Localization of GFP or GFP-FAT (GFP; 488 nm laser), PY^{397}-FAK or PY^{118}-paxillin (Cy3; 543 nm laser), and FAK or paxillin (Cy5; 633 nm laser) were analyzed by confocal laser scan microscopy. Data shown are from pkGFP (clone 7) and pkGFP-FAT (clone 8) that are representative for the various pkGFP and pkGFP-FAT clones. Note that in GFP-FAT cells FAK and PY^{397}-FAK are no longer located at sites resembling focal adhesions (*A*). In addition, focal adhesions in pkGFP and pkGFP-FAT cells are normally formed, as judged by staining for paxillin and PY^{118}-paxillin (*B*).

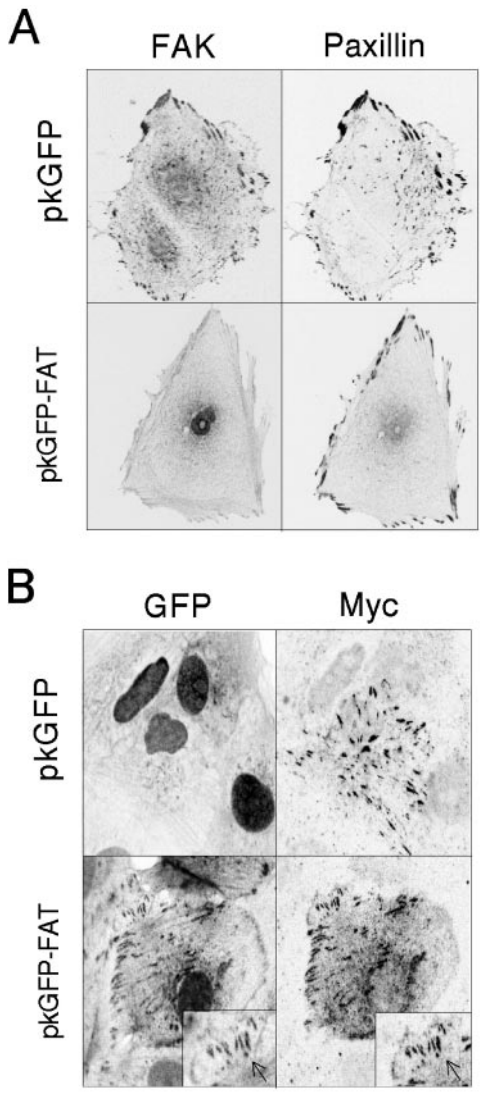

FIG. 5. **GFP-FAT does not disrupt paxillin and myc-FAK localization at focal adhesions.** *A,* pkGFP and pkGFP-FAT were plated on collagen-coated coverslips in complete medium and allowed to adhere for 24 h. Thereafter, cells were fixed and co-immuno-stained for FAK and paxillin followed by analysis by confocal laser scan microscopy. *B,* pkGFP and pkGFP-FAT cells were transfected with Myc-tagged FAK as described under "Experimental Procedures." 24 h after transfection cells were fixed and immunostained for Myc followed by confocal laser scan microscopy. Note the co-localization of Myc-FAK and GFP-FAT in pkGFP-FAT cells.

sitizes cells to undergo apoptosis by accelerating the activation of caspase-3.

FAK Signaling Is Not Involved in DCVC-induced Necrosis—DCVC-induced cytotoxicity is a continuum between necrosis and apoptosis (15, 55); both types of cell death share the same bio-activation pathway (15, 55). DCVC-induced necrosis involves the initiation of lipid peroxidation (48, 54). By inhibiting lipid peroxidation using a lipid radical scavengers, *i.e.* DPPD as in the above experiments, we follow apoptosis caused by DCVC more selectively (15, 49, 50, 55). Likewise, by omitting DPPD from the culture medium we can investigate the mechanisms of DCVC-induced necrosis. By using this switch we wanted to answer the question whether FAK signaling is involved in both DCVC-induced necrosis and apoptosis. Therefore, pkGFP and pkGFP-FAT cells were treated with DCVC in the presence or absence of DPPD. Necrosis was determined by measuring the release of LDH from the cells. DCVC clearly caused the release of LDH in pkGFP cells; as expected this was blocked by the antioxidant DPPD (Fig. 8). No significant difference was observed between the LDH release caused by DCVC in pkGFP and pkGFP-FAT cells (Fig. 8), indicating that FAK plays no role in controlling necrosis in chemically induced cytotoxicity.

GFP-FAK Overexpression Does Not Protect against DCVC-induced Apoptosis—The above investigations indicated that blocking FAK function by overexpressing GFP-FAT leads to increased sensitivity of LLC-PK1 cells toward DCVC-induced apoptosis. To investigate whether blocking FAK function by other means also results in an increase of DCVC-induced apo-

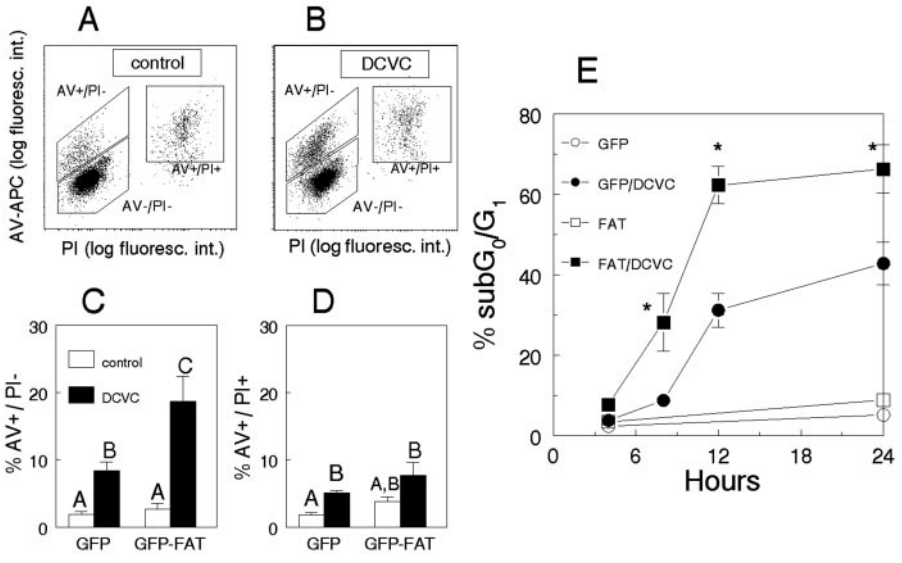

FIG. 6. **Effect of GFP-FAT on DCVC-induced apoptosis.** pkGFP and pkGFP-FAT cells were plated on collagen-coated 6-cm dishes in serum-free culture medium and after overnight attachment cells were treated with DCVC (0.25 mM) in Hanks'/HEPES buffer containing 10 μM DPPD for 6 h followed by recovery in complete medium containing 10 μM DPPD. After 8 h cell death was analyzed by annexin-V/propidium iodide (*AV*/*PI*) staining in untreated pkGFP-FAT (clone 8) cells (*A*) and cells treated with DCVC (*B*). The percentage of apoptotic cells (*AV*+/*PI*−) (*C*) and necrotic cells (*AV*+/*PI*+) (*D*) was calculated for the different GFP and GFP-FAT clones. For cell cycle analysis (*E*) samples were taken at 4, 8, 12, and 24 h after treatment with DCVC, and apoptosis was determined as described under "Experimental Procedures." Data shown (*C*–*E*) are mean ± S.D. of four independent clones for each construct ($n = 4$). The data are representative for two independent experiments.

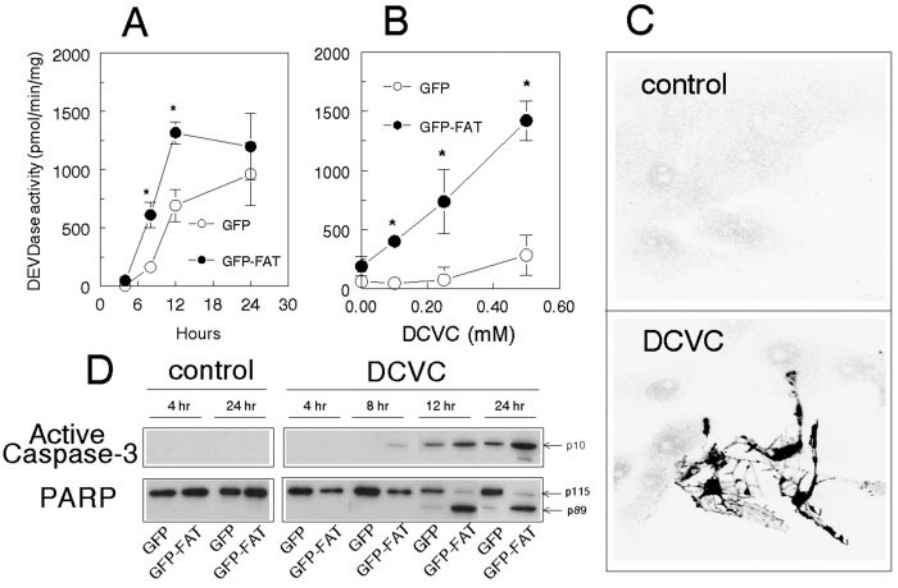

FIG. 7. **Effect of GFP-FAT on caspase-3 activation and PARP cleavage caused by DCVC.** pkGFP and pkGFP-FAT cells were treated with DCVC as described in Fig. 6. For DEVDase activity cells were treated with DCVC (0.25 mM), and samples were analyzed at the indicated time points (*A*). In another experiment cells were treated with different concentration of DCVC, and DEVDase activity was determined at 10 h (*B*). DEVDase activity shown is mean ± S.D. for four independent clones ($n = 4$). Data are representative for two different experiments. Caspase-3 activation was analyzed by immunofluorescence using an anti-active caspase-3 antibody (*C*). Cells that were plated on collagen-coated coverslips were either left untreated (*top panel*) or treated with DCVC (0.25 mM), 10 μM DPPD (*lower panel*) for 8 h. Thereafter, cells were fixed and processed for immunofluorescence followed by confocal laser scan microscopy. Shown is a representative field of pkGFP-FAT (clone 8). This clone is representative for other pkGFP-FAT clones. *D,* caspase-3 processing and PARP cleavage was determined by Western blotting. Cells were treated as in *A*. Polyvinylidene difluoride membranes were probed with either anti-active caspase-3 or anti-PARP. Data shown are for pkGFP (clone 7) and pkGFP-FAT (clone 8) and representative for other pkGFP and pkGFP-FAT clones, respectively. The data are representative for two different experiments.

ptosis and whether overexpression of GFP-FAK inhibits DCVC-induced apoptosis, cells expressing GFP-FRNK or GFP-FAK were treated with DCVC, and the effect on apoptosis was analyzed. Like GFP-FAT, overexpression of GFP-FRNK potentiated the induction of apoptosis caused by DCVC. Overexpression of GFP-FAK did not protect against apoptosis caused by DCVC (Table II).

Differential Regulation of Doxorubicin and Cisplatin-induced Apoptosis by FAK—To investigate whether modulation of apoptosis by FAK is a common mechanism for nephrotoxicant-induced apoptosis, pkGFP and pkGFP-FAT cells were treated with various concentrations of either doxorubicin or cisplatin. FAT overexpression significantly increased the sensitivity against doxorubicin-induced apoptosis of these cells (Fig. 9*A*). However, no significant difference was observed with regard to cisplatin-induced apoptosis between pkGFP and pkGFP-FAT cells (Fig. 9*B*).

GFP-FAT Potentiates Anoikis of LLC-PK1 Cells—FAK-mediated signals are central in the control of adhesion-mediated cell survival. Likewise, loss of cell adhesion results in loss of FAK signaling and induction of apoptosis, which is also termed anoikis. We hypothesized that perturbation of FAK function by overexpressing GFP-FAT should also enhance the sensitivity of cells toward anoikis. To test this hypothesis the various clones were trypsinized, and cells were plated on agarose-coated dishes to prevent cell adhesion; apoptosis was determined by cell cycle analysis. GFP-FAT expression significantly increased the induction of anoikis compared with GFP cells. Similar observations were made for GFP-FRNK cells (Table III). These data suggest that there is, at least in part, an overlap in the

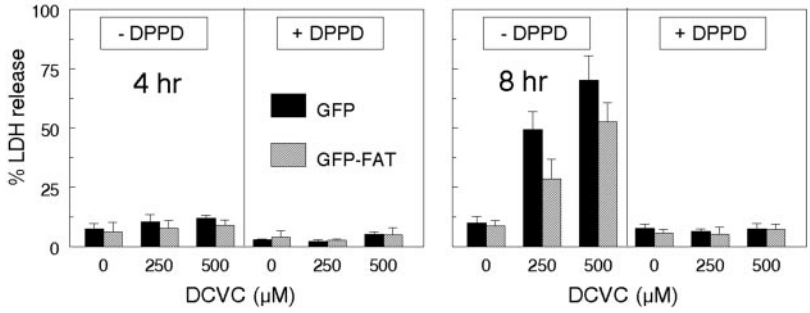

FIG. 8. **Effect of GFP-FAT on DCVC-induced necrosis.** pkGFP and pkGFP-FAT cells were treated with DCVC in Hanks'/HEPES buffer at the indicated concentration either with or without DPPD (10 μM). At 4 and 8 h the percentage of % LDH release was determined as described under "Experimental Procedures." Data shown are the means ± S.D. of four independent clone for each construct (n = 4). Data are representative for two independent experiments.

TABLE II

Effect of GFP-FAK and GFP-FRNK on DCVC-induced apoptosis

Cells were plated on collagen-coated dishes and allowed to adhere and spread overnight. Thereafter cells were treated with DCVC (0.25 mM) in Hanks'/HEPES buffer containing 10 μM DPPD for 8 h followed by 4 h of recovery in medium. Apoptosis was determined by cell cycle analysis using flow cytometry as described under "Experimental Procedures." Data shown are the mean ± S.E. of four different clones (n = 4) and are representative for two independent experiments. A–C indicate significant differences.

	% apoptosis	
	Control	DCVC
GFP	$2 \pm 1^{A\alpha}$	31 ± 4^{B}
GFP-FAK	3 ± 1^{A}	27 ± 4^{B}
GFP-FRNK	$4/6^{b}$	$46/50^{b}$
GFP-FAT	4 ± 1^{A}	62 ± 5^{C}

[a] Statistical differences were determined as described under "Experimental Procedures."

[b] Data shown are from two individual GFP-FRNK clones.

signals that control chemically induced apoptosis and anoikis of LLC-PK1 cells.

GFP-FAT Overexpression Potentiates Loss of FAK Phosphorylation and Focal Adhesion Organization—Focal adhesion organization is dependent on FAK phosphorylation. Although almost all of endogenous FAK was no longer present at focal adhesions there was still a substantial amount of FAK phosphorylation on Tyr^{397} (see Fig. 3). We reasoned that if GFP-FAT affects the function of endogenous FAK, then the potentiation of apoptosis caused by DCVC should be related to a more pronounced loss of FAK phosphorylation. Indeed, phosphorylation of endogenous FAK was almost completely lost in pkGFP-FAT cells after treatment with DCVC; this was not the case in pkGFP cells (Fig. 10).

In primary cultured renal proximal tubular cells DCVC-induced dephosphorylation of FAK is associated loss of focal adhesion organization (18). We hypothesized that if there is a direct relationship between loss of FAK phosphorylation and focal adhesion organization after cellular injury, then focal adhesion organization should be more affected by DCVC in pkGFP-FAT cells than in pkGFP cells. To investigate this pkGFP and pkGFP-FAT cells were double-stained for PY^{118}-paxillin and paxillin. PY^{118}-paxillin is primarily located at focal adhesion and, therefore, a good measure for focal adhesion organization. Treatment of pkGFP cells with DCVC for 8 h resulted in a minor loss of focal adhesion complexes as determined by PY^{118} paxillin/paxillin staining (Fig. 11). In contrast, in pkGFP-FAT cells treatment with DCVC resulted in an almost complete loss of focal adhesions (Fig. 11). These findings were confirmed by analyzing the levels of paxillin phosphorylation on Tyr^{31} and Tyr^{118} by Western blotting. Thus, tyrosine phosphorylation of paxillin was almost completely lost in pkGFP-FAT cells treated with DCVC, whereas most of it was still present in pkGFP cells after treatment with DCVC (Fig. 12).

DISCUSSION

The present investigations on the role of FAK in chemically induced perturbations of focal adhesion organization and onset of cell death (*i.e.* apoptosis and necrosis) allow us to draw several conclusions. First, the data indicate an important role for FAK in chemically induced apoptosis in adherent cells. Thus, overexpression of deletion mutants of FAK, namely FAT and FRNK, which both compete with endogenous FAK for localization to focal adhesions, accelerated the onset of apoptosis caused by DCVC. A similar finding was observed for doxorubicin that has a different mechanism of action than DCVC. In contrast, cisplatin-induced apoptosis of LLC-PK1 cells was not potentiated. Thus, FAK has a protective role against apoptosis caused by at least some, but not all, chemicals. To our knowledge this is the first report that indicates that FAK provides protection in chemically induced apoptosis in adherent cells. Second, FAK does not seem to play a role in the control of chemically induced necrosis. Although overexpression of FAT accelerated the onset and extent of DCVC-induced apoptosis, no effect was observed on the induction of necrosis. Both apoptosis and necrosis caused by DCVC require the same bioactivation by β-lyase (15, 48, 54, 55). The fact that the extent of necrosis is similar in pkGFP and pkGFP-FAT cells indicates that the degree of cell injury in both cell types is most likely the same, which would exclude the possibility that the increased apoptosis caused by DCVC in pkGFP-FAT is due to an increased bio-activation. Third, FAK seems important in the maintenance of focal adhesion organization after cellular injury. Treatment of LLC-PK1 cells resulted in loss of focal adhesion organization as assessed by staining of cells against paxillin and phospho-paxillin (Fig. 11). Although the organization of focal adhesion organization in untreated cells was not affected by GFP-FAT, treatment with DCVC caused a dramatic loss of focal adhesion in pkGFP-FAT cells but not in pkGFP cells. This effect strongly correlated with a rapid loss of the levels of FAK phosphorylation on tyrosine residue 397. Importantly, loss of focal adhesion organization occurred prior to activation of caspase-3. These observations are in accordance with our previous findings (15) in primary cultured rat renal proximal tubular epithelial cells where a complete loss of focal adhesion as well as FAK phosphorylation occurs prior to the onset of apoptosis. The combined data fit a model whereby toxicant-induced perturbation of FAK phosphorylation causes disturbances of focal adhesion organization and loss of phosphorylation of focal adhesion-associated adapter proteins, such as paxillin, most likely resulting in loss of signaling cascades that otherwise suppress apoptotic routes.

Our data indicate that GFP-FAT and GFP-FRNK delay the early but not the late formation of focal adhesions in LLC-PK1 cells. These data are consistent with those obtained by other investigations. Thus, transient overexpression of FRNK in avian fibroblast also delayed the formation of focal adhesions (25). In several breast tumor cell lines retrovirally mediated overexpression of a FAK C-terminal domain construct caused

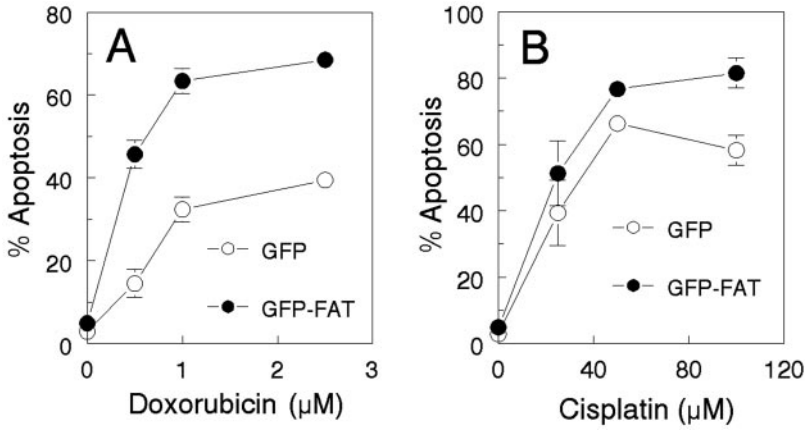

FIG. 9. **Effect of GFP-FAT on apoptosis of LLC-PK1 cells caused by either doxorubicin or cisplatin.** pkGFP and pkGFP-FAT cells were treated with the indicated concentrations of doxorubicin or cisplatin in complete culture medium. After 24 h apoptosis was analyzed by cell cycle analysis as described under "Experimental Procedures." Data shown are the means ± S.D. of four independent clone for each construct ($n = 4$). Data are representative for two independent experiments.

TABLE III
Effect of GFP, GFP-FAT, GFP-FRNK, and GFP-FAT on anoikis of LLC-PK1 cells

Cells were trypsinized and either plated on collagen- or agarose-coated 6-cm dishes. After 16 h cells were harvested, and apoptosis was determined by cell cycle analysis as described under "Experimental Procedures." Data shown are the mean ± S.E. of four different clones ($n = 4$). Data are representative for two independent experiments. A–C indicate significant differences.

	% apoptosis[a]	
	Collagen	Suspension
GFP	2 ± 1^{Aa}	19 ± 4^{B}
GFP-FRNK	3/4[b]	39/43[b]
GFP-FAT	3 ± 1^{A}	34 ± 4^{C}

[a] Statistical differences were determined as described under "Experimental Procedures."

[b] Data shown are from two individual GFP-FRNK clones.

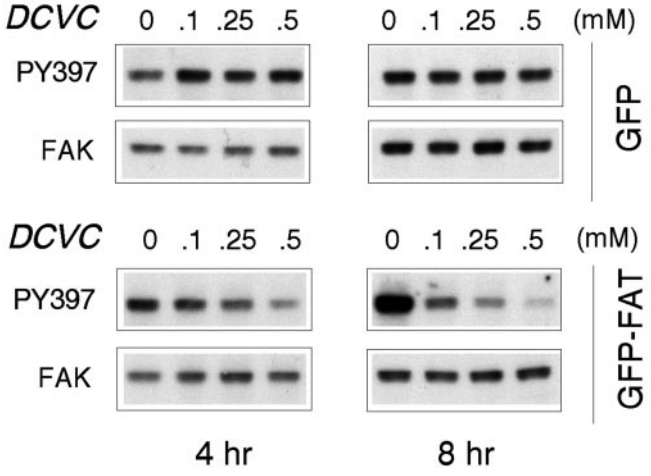

FIG. 10. **Effect of GFP-FAT on DCVC-mediated decreases of FAK phosphorylation.** pkGFP and pkGFP-FAT cells were treated with or without the indicated concentrations of DCVC in Hanks'/HEPES buffer containing DPPD (10 μM). At 4 and 8 h after treatment samples were taken and analyzed for FAK phosphorylation using Western blotting. Blots were stained for PY^{397}-FAK and after stripping stained for FAK. Shown are the data of pkGFP (clone 7) and pkGFP-FAT (clone 8) that are representative for other pkGFP and pkGFP-FAT clones. Data are representative for two independent experiments.

not only perturbations of focal adhesion organization but also led to rounding up of cells followed by apoptosis (31). This was associated with dephosphorylation of FAK (31). The fact that this was not observed in our system is most likely due to the fact that we have made stable cell lines that may have lower expression levels of dominant negative-acting FAK compared with transient retrovirally mediated transfection. Thus, most likely, only LLC-PK1 cells with relatively lower levels of GFP-FAT or GFP-FRNK that were still able to form focal adhesions at later stages and grow normally may have been selected. Despite the fact that FAK was no longer detectable at focal adhesion in pkGFP-FAT cells, the levels of PY^{397}-FAK as determined by Western blotting were comparable between pkGFP and pkGFP-FAT cells. This may explain the fact that no difference in the background apoptosis was observed between pkGFP and pkGFP-FAT cells.

We found that increased expression of GFP-FAK did not provide protection against chemically induced apoptosis. Similar findings were found in rabbit synovial fibroblast (18). In contrast, in HeLa cells overexpression of FAK protects almost completely against apoptosis caused by either hydrogen peroxide or etoposide (35). In Madin-Darby canine kidney cells overexpression of constitutively active FAK also protects against UV-induced apoptosis (21). These differences might be explained by the fact that HeLa cells themselves contain very low levels of endogenous FAK; moreover, HeLa cells are non-adherent cells. In contrast both the rabbit synovial fibroblast and our own LLC-PK1 cells are adherent cells that already possess endogenous FAK that is phosphorylated. Although in our hands pkGFP-FAK cells contain high levels of phosphorylated GFP-FAK, most of the phosphorylated GFP-FAK was not present in focal adhesion (data not shown). Moreover, importantly, our initial findings indicate that the levels of phosphorylation of PKB/Akt, which lies downstream from FAK and is involved in cell-ECM-mediated survival signaling, are similar in pkGFP and pkGFP-FAK cells.[2] This suggests that the levels of endogenous FAK are already sufficient to provide the maximal survival signals and that more FAK, *i.e.* GFP-FAK, does not necessary lead to increased survival. However, in pkGFP-FAT cells introduction of more FAK by transfection of wild type FAK might overrule the enhancement of the apoptosis caused by GFP-FAT. Unfortunately, such experiments seemed impossible since overexpression of Myc-tagged FAK by transient transfection in pkGFP-FAT cells did not alleviate localization of GFP-FAT from focal adhesions (Fig. 5*B*), thereby making the evaluation of an effect of FAK overexpression on GFP-FAT-mediated potentiation of apoptosis difficult.

Our previous studies (15) in primary cultured rat PTC indicated that toxicant-induced perturbations of the localization of FAK at focal adhesions coincided with an overall loss of FAK phosphorylation and focal adhesion disruption. A causal relationship between FAK dephosphorylation and focal adhesion organization could not be established (15). Our present data show that in pkGFP-FAT cells the DCVC-induced focal adhesion disruption occurs much faster than in pkGFP cells (Fig. 11). This is consistent with the fact that FAK levels at focal

[2] B. van de Water and I. Tijdens, manuscript in preparation.

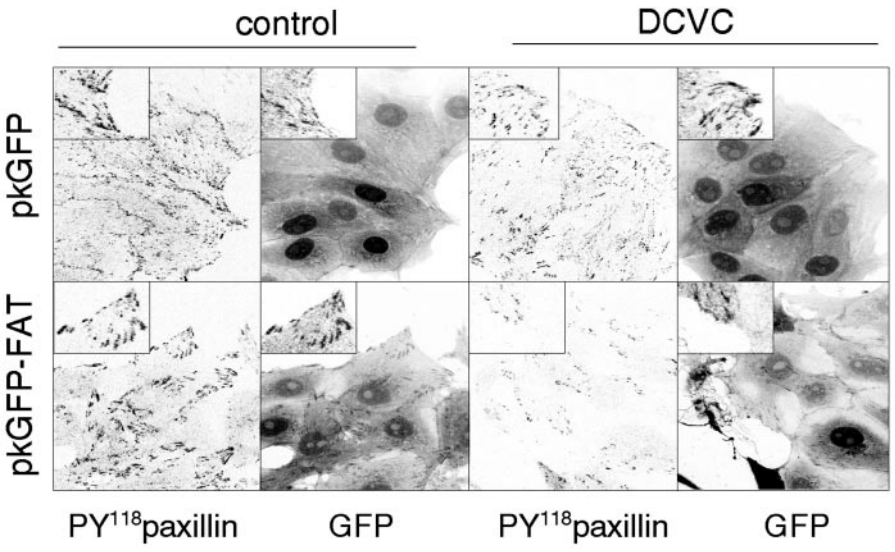

FIG. 11. **Effect of GFP-FAT on DCVC-induced focal adhesion re-organization.** pkGFP and pkGFP-FAT cells were plated on collagen-coated glass coverslips and after overnight attachment and spreading were treated with or without DCVC (0.25 mM) in Hanks'/HEPES containing DPPD (10 μM). After 8 h cells were fixed and immunostained for paxillin and PY^{118}-paxillin followed by confocal laser scan microscopy analysis of GFP, paxillin, and PY^{118}-paxillin. Data shown are from pkGFP (clone 7) and pkGFP-FAT (clone 8) and representative for other pkGFP and pkGFP-FAT clones, respectively. *Insets* in the panels of PY^{118}-paxillin pictures are enlargements of PY^{118}-paxillin staining, and the corresponding paxillin staining is shown as *insets* in the GFP pictures. Note that PY^{118}-paxillin staining between untreated pkGFP and pkGFP-FAT cells is comparable; in contrast, most of the PY^{118}-paxillin staining is lost after DCVC treatment in pkGFP-FAT cells but not in pkGFP cells.

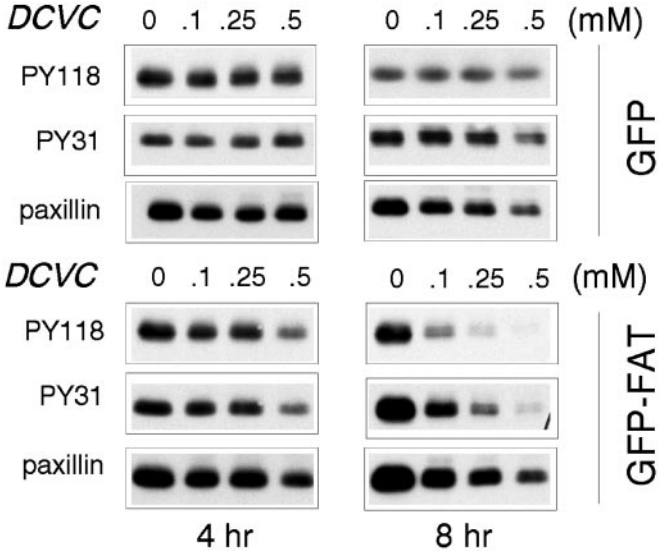

FIG. 12. **Effect of GFP-FAT on dephosphorylation of paxillin caused by DCVC.** pkGFP and pkGFP-FAT cells were treated with or without the indicated concentrations of DCVC in Hanks'/HEPES buffer containing DPPD (10 μM). At 4 and 8 h after treatment samples were taken and analyzed for paxillin phosphorylation using Western blotting. Blots were probed for PY^{31}-paxillin or PY^{118}-paxillin and thereafter stripped and stained and paxillin. Shown are the data of pkGFP (clone 7) and pkGFP-FAT (clone 8) that are representative for other pkGFP and pkGFP-FAT clones. Data are representative for two independent experiments.

adhesions in pkGFP-FAT cells are much lower than in pkGFP cells. A function of FAK in focal adhesion formation in LLC-PK1 cells is also supported by the fact that early focal adhesion organization is disrupted by GFP-FAT (Fig. 2). The increased disruption of focal adhesion in pkGFP-FAT treated with DCVC correlated with a drastic decrease of FAK phosphorylation on Tyr^{397}, suggesting a direct relationship between FAK phosphorylation status and focal adhesion organization.

Focal adhesion organization seems directly related to cell survival signaling. Thus, our previous (15) and present observations indicate that focal adhesion disruption occurs prior to the activation of caspase-3. We did not observe cells that were positive for active caspase-3 and still possessed normal focal adhesions. Rather, cells that were positive for active caspase-3 were fully fragmented (see Fig. 7). Also in other cell types apoptosis caused by transient overexpression of FAT or FRNK caused loss of focal adhesion independent of caspase activity and prior to the appearance of apoptotic cells (31). Given the importance of proper focal adhesion organization in the control of cell death, what then are the signaling cascades downstream from FAK that mediate protection against toxicant-induced apoptosis? FAK phosphorylation on Tyr^{397} results in a docking site for the SH2 domain of PI 3-kinase that seems important for ECM-mediated survival through PKB (59). Our preliminary data indicate that after DCVC treatment the phosphorylation of PKB is lower in pkGFP-FAT cells than in pkGFP cells, which seems directly related to the control of cell survival.[2] Alternatively, loss of FAK phosphorylation and consequently focal adhesion organization may lead to loss of paxillin phosphorylation on Tyr^{31} and Tyr^{118} (Fig. 11), which are important docking sites for other signaling molecules, including Crk, that plays an additional role in survival signaling (60).

Renal cell detachment plays an important role in the pathogenesis of acute renal failure caused by either ischemia/reperfusion injury or chemical exposure that is associated with apoptosis. Our present *in vitro* data support the notion that perturbations of cell-ECM signaling in renal epithelial cells lower the set point for onset of apoptosis. Preliminary data from our laboratory indicate that this may also be the case *in vivo*. Thus, DCVC-induced acute renal failure is more pronounced in heterozygous FAK mice than in wild type littermates.[3] This indicates that *in vivo* FAK-mediated signaling may be a serious option to modulate chemically induced renal injury; further studies are required to investigate whether this is a general phenomenon.

In conclusion, our study indicates a protective role for FAK in the control of chemically induced apoptosis of renal epithelial cells. Future studies should determine whether signal transduction cascades downstream from FAK promote cell survival

[3] B. van de Water and I. Tijdens, unpublished observations.

after renal cell injury and whether this is related to directly affecting components of the apoptotic machinery or indirectly through gene transcription regulation of pro- and/or anti-apoptosis molecules.

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