

# Dephosphorylation of Focal Adhesion Kinase (FAK) and Loss of Focal Contacts Precede Caspase-mediated Cleavage of FAK during Apoptosis in Renal Epithelial Cells\*

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**The relationship between focal adhesion protein (FAK) activity and loss of cell-matrix contact during apoptosis is not entirely clear nor has the role of FAK in chemically induced apoptosis been studied. We investigated the status of FAK phosphorylation and cleavage in renal epithelial cells during apoptosis caused by the nephrotoxicant dichlorovinylcysteine (DCVC). DCVC treatment caused a loss of cell-matrix contact which was preceded by a dissociation of FAK from the focal adhesions and tyrosine dephosphorylation of FAK. Paxillin was also dephosphorylated at tyrosine. DCVC treatment activated caspase-3 which was associated with cleavage of FAK. However, FAK cleavage occurred after cells had already lost focal adhesions indicating that cleavage of FAK by caspases is not responsible for loss of FAK from focal adhesions. Accordingly, although inhibition of caspase activity with zVAD-fmk blocked activation of caspase-3, FAK cleavage, and apoptosis, it neither affected dephosphorylation nor translocation of FAK or paxillin. However, zVAD-fmk completely blocked the cell detachment caused by DCVC treatment. Orthovanadate prevented DCVC-induced tyrosine dephosphorylation of both FAK and paxillin; however, it did not inhibit DCVC-induced apoptosis and actually potentiated focal adhesion disorganization and cell detachment. Thus, FAK dephosphorylation and loss of focal adhesions are not due to caspase activation; however, caspases are required for FAK proteolysis and cell detachment.**

Apoptosis or programmed cell death is critical for normal development and tissue homeostasis (1). However, uncontrolled apoptosis, as may occur after treatment with cytostatic chemicals, is a pathophysiological process and is associated with the occurrence of various human diseases (2, 3). Apoptosis is characterized by fragmentation of the nucleus, activation of nucleases, and importantly, activation of the caspase family of aspartate-directed proteases. The latter cleave a diverse set of cellular proteins, such as PARP, DNA-dependent protein kinase, protein kinase C- $\delta$ , gelsolin,  $\beta$ -catenin, GAS-2, fodrin, and

FAK<sup>1</sup> (4–15). Presumably, the change of function (gain or loss) caused by proteolysis of multiple target proteins contributes to apoptosis. Thus, the proteolysis of cytoskeleton-associated proteins is most likely involved in the morphological alterations observed during apoptosis *in vitro* and *in vivo*, i.e. membrane blebbing and “rounding up” of cells.

Maintenance of cell-matrix contact is an important cell survival factor (16–21) and loss of cell matrix and cell-cell contact, or rounding up, is a hallmark of apoptosis. Enforced loss of cell-matrix interactions of endothelial and epithelial cells, is sufficient to initiate a form of apoptosis that has been termed “anoikis” (18, 19). The fact that apoptotic cells detach from their substratum raises the possibility that the signaling events leading to anoikis are either the same as, or may overlap with signals that lead to other forms of apoptosis, for example, induced by chemicals or FasL/tumor necrosis factor.

Cell-matrix interactions occur at the closest contact between the cell and the substratum: the focal adhesions. This is a complex network of (cytoskeletal) proteins that links the filamentous actin (F-actin) cytoskeletal network through cell adhesion molecules, so called integrins, to the extracellular matrix (16, 17, 22, 23). Integrin engagement by the extracellular matrix results in activation of focal adhesion kinase (FAK). FAK is a 125-kDa protein tyrosine kinase that is critical in transmission of signals from the focal adhesion to the cytoplasm after cell attachment (24, 25). After activation, FAK interacts with a variety of focal adhesion (signaling) macromolecules, including the adaptor proteins paxillin and Grb-2, the cytoskeletal proteins tensin and talin, and signal transduction molecules such as Src, Fyn, and phosphoinositide 3-kinase (22, 23, 26–33). These interactions link integrin-mediated adhesion to downstream signaling cascades. For example, the SH2 domain of phosphoinositide 3-kinase binds FAK at the tyrosine autophosphorylation site (Tyr<sup>397</sup>) (34). Cell adhesion results in the activation of phosphoinositide 3-kinase and the downstream signaling molecule c-Akt/PKB (35, 36). Adhesion also leads to activation of the mitogen-activated protein kinase pathway, p70 ribosomal S6 kinase and protein kinase C (37–39). Activation of Akt/PKB, mitogen-activated protein kinase, or protein kinase C serve as anti-apoptotic stimuli (40–45). Although FAK is one of the few proteins that is phosphorylated on tyrosine residues upon cell adhesion, its role in the activation of all of the above signaling pathways is still under investigation. Nonetheless, it seems clear that FAK is important for focal adhesion signaling and cell attachment and migration.

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<sup>1</sup> The abbreviations used are: FAK, focal adhesion kinase; RPTE, renal proximal tubule epithelial; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; EBSS, Earl's balanced salt solution; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; AMC, 7-amino-4-methylcoumarine.

Although the downstream effector molecules responsible for FAK signaling are not completely known, there is increasing evidence that FAK is involved in anoikis and, perhaps, other forms of apoptosis (12–15, 46–50). For example, microinjection of peptides that compete for FAK-integrin association, and also antibodies directed against FAK itself can induce apoptosis in fibroblasts (48). Treatment of tumor cells with FAK antisense oligonucleotides also leads to apoptosis (47). Moreover, overexpression of constitutively active FAK prevents anoikis (46, 51). Furthermore, during apoptosis FAK is cleaved both in adherent and non-adherent cell lines (12–15), a process that depends on caspase activation since zVAD-fmk blocks FAK cleavage. Recombinant FAK is also cleaved by recombinant caspase-3 and -6 into fragments of approximately 44, 77, and 85 kDa corresponding to the fragments seen during FAK cleavage in apoptotic cells (13, 15). Despite the fact that FAK cleavage and/or inactivation provides an attractive explanation for the loss of cell-matrix interactions that occur during apoptosis, there is little or no evidence supporting this assumption, nor has the role of FAK signaling been addressed in chemically induced models of apoptotic cell death. Furthermore, the majority of studies have been performed in immortalized or transformed cells; little is known about the involvement of FAK signaling and cell detachment *in vivo* or in primary cultures of normal epithelial cells. Therefore, we have investigated the role of FAK in chemically induced apoptosis in primary cultures of renal proximal tubular epithelial cells.

The renal proximal tubule epithelial cells (RPTE) are an important target for a variety of nephrotoxic chemicals as well as ischemia/reperfusion injury (52, 53). Death of renal epithelial cells is associated with detachment of viable RPTE from the extracellular matrix, both *in vitro* and *in vivo*, an effect that is related to redistribution of integrins and loss of focal adhesion organization (54–59). Conditions that prevent attachment of the renal epithelial cell line Madin-Darby canine kidney also cause apoptosis, suggesting that there is a relationship between cell detachment and cell death (19, 46). Therefore, we investigated the role of FAK phosphorylation and its cleavage during loss of focal adhesion integrity and apoptosis of primary cultures of rat RPTE using the well characterized nephrotoxicant *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) (57, 58, 60–65). DCVC is metabolized by a  $\beta$ -lyase to a reactive acylating metabolite that covalently modifies cellular macromolecules (60–62); DCVC induces apoptosis of RPTE in culture (58, 65). Cell death of primary cultured RPTE caused by DCVC treatment is preceded by disorganization of the F-actin cytoskeletal network and dissolution of focal adhesions (57, 58). Thus, this compound is a useful agent to study the role of focal adhesion disturbance and cell detachment in apoptosis of primary cultures of kidney epithelial cells.

To study the relationship between focal adhesion assembly, FAK phosphorylation, and apoptosis and to investigate whether disturbances in FAK signaling may contribute to chemically induced apoptosis in RPTE, we determined FAK localization and phosphorylation. These effects were correlated with activation of caspases and the occurrence of apoptosis after DCVC treatment. Apoptosis of RPTE was preceded by an *early* tyrosine dephosphorylation of both FAK and the FAK-associated protein paxillin. Dephosphorylation was also associated with an *early* redistribution of FAK and paxillin from the focal adhesion. The cleavage of FAK was a *late* event and followed the activation of caspases. The general caspase inhibitor zVAD-fmk blocked the DCVC-induced increase in caspase activity, FAK cleavage, and apoptosis; however, FAK dephosphorylation and translocation still occurred. The data suggest that FAK dephosphorylation and dissolution of the focal adhe-

sions precede cleavage of FAK by caspases during chemically induced apoptosis of renal epithelial cells.

#### EXPERIMENTAL PROCEDURES

**Materials**—Fetal bovine serum was from Life Technologies (Grand Island, NY). Dulbecco's modified Eagle's medium/Ham's F-12, bovine serum albumin fraction V, cholera toxin, insulin, antibiotic were from Sigma. Epidermal growth factor was from Upstate Biotechnology Inc. (UBI, Lake Placid, NY). *N,N'*-Diphenyl-*p*-phenylenediamine (DPPD) was from Kodak (Rochester, NY). Benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was purchased from Bachem (Bubendorf, Switzerland). Rhodamine-phalloidin was from Molecular Probes (Eugene, CA). DCVC was synthesized as described (61).

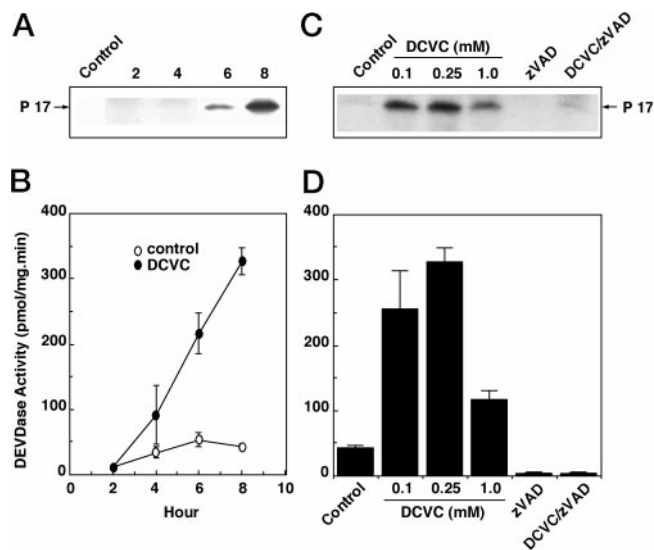
**Isolation, Culturing, and Treatment of Rat Renal Proximal Tubular Epithelial Cells (RPTE)**—RPTE were isolated by collagenase perfusion and separated by density centrifugation using Nycodenz as described (63, 66). Cells were cultured on rat tail collagen (Collaborative Research, Bedford, MA) coated dishes in Dulbecco's modified Eagle's medium/Ham's F-12 containing 1% (v/v) fetal bovine serum, 0.5 mg/ml bovine serum albumin, 10  $\mu$ g/ml insulin, 10 ng/ml epidermal growth factor, 10 ng/ml cholera toxin, and antibiotics as described (57, 67). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide and fed every other day. Cells were used after they had reached confluency 6 to 9 days after plating.

Confluent monolayers of RPTE in 24-well dishes containing coated glass coverslips, 6-well or 10-cm dishes, were washed with Earle's balanced salt solution (EBSS) twice. Thereafter cells were treated with DCVC in EBSS in a final volume of 1, 2, or 10 ml, respectively, for 4 h. To study events that are related to apoptosis, cells were treated with DCVC in the presence of DPPD (20  $\mu$ M; 10 mM stock in dimethyl sulfoxide) which blocks necrotic cell death but allows the onset of apoptosis (58, 65). The inhibitors zVAD-fmk (100  $\mu$ M; 100 mM stock in dimethyl sulfoxide) or Na<sub>3</sub>VO<sub>4</sub> (5, 10, 25, or 50  $\mu$ M; 100 mM stock in water) were added simultaneously with DCVC. Following treatment with DCVC for 4 h, cells were allowed to recover in complete medium containing DPPD (20  $\mu$ M) with or without the above inhibitors.

**Cell Death and Caspase Activation Assays**—Cell death was measured by the release of lactate dehydrogenase (LDH) in the culture medium as described (62). Percentage of cell death was calculated from the amount of LDH release caused by treatment with toxicants relative to the amount released by 0.1% (w/v) Triton X-100, *i.e.* 100% release. To determine the percentage of cell detachment, floating cells (supernatant) and adherent cells (obtained after trypsinization; see below) were collected separately. Cells were counted using a hemocytometer and the percentage of cell detachment was calculated as floating cells/total cells times 100%. DNA laddering was determined by agarose gel electrophoresis. Cells were harvested by scraping the adherent cells which were then combined with floating cells present in the culture medium. After centrifugation, the cell pellet was washed once with ice-cold PBS by centrifugation, lysed with lysis buffer (10 mM Tris, 1 mM EDTA, and 0.2% (w/v) Triton X-100, pH 7.4), and incubated on ice for 20 min. Cell debris was removed by centrifugation; the supernatant was treated with RNase (60  $\mu$ g/ml) for 1 h at 37 °C and then with proteinase K (120  $\mu$ g/ml) for 1 h at 50 °C. The DNA was precipitated with 0.5 M NaCl and an equal volume of isopropyl alcohol and separated by electrophoresis on 1% agarose gels. Nuclear fragmentation was determined in cells cultured on collagen-coated glass coverslips. After fixation with 3.7% (w/v) formaldehyde, cells were stained with 1  $\mu$ g/ml Hoechst 33258 in PBS. After washing with PBS, coverslips were mounted and viewed using a Nikon epifluorescence microscope. In some experiments nuclear staining was combined with immunofluorescence staining (see below).

Apoptosis was also determined by cell cycle analysis. Both floating and adherent cells that were trypsinized were pooled and fixed in 90% ethanol (–20 °C). After washing cells twice with PBS/EDTA (1 mM), cells were resuspended in PBS-EDTA containing 10  $\mu$ g/ml RNase A and 7.5  $\mu$ M propidium iodide. After 30 min incubation at room temperature the cell cycle was analyzed by flow cytometry (FACScan, Becton Dickinson) and the percentage of cells present in sub-G<sub>0</sub>/G<sub>1</sub> was calculated using the LYSIS software (Becton Dickinson).

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  $\beta$ -glycerophosphate, 50 mM NaCl, 2 mM MgCl<sub>2</sub>, and 5 mM EGTA) and subjected to three cycles of freezing and thawing. Equal amounts of cell proteins were used in a caspase assay using acetyl-DEVD-AMC (25  $\mu$ M; Research Biochemicals Int., Natick, MA) as a substrate. Fluorescence derived from release of the AMC moiety was followed using a fluores-

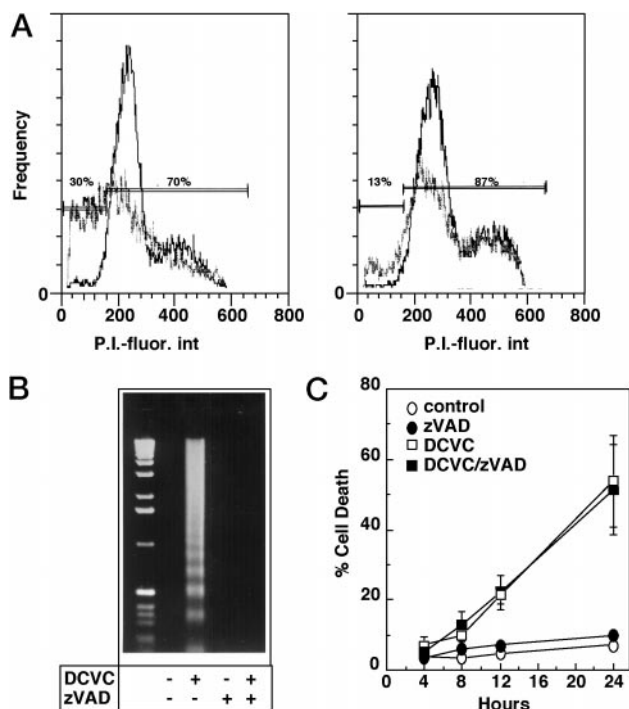


**FIG. 1. Effect of zVAD on caspase activation by DCVC.** RPTE were treated with DCVC in EBSS containing DPPD (20  $\mu$ M) and after 4 h cells were allowed to recover in complete medium containing 20  $\mu$ M DPPD for another 4 h ( $t = 8$  h). At 2, 4, 6, or 8 h after the addition of DCVC (0.25 mM) pro-caspase-3 cleavage into the 17 kDa caspase-3 fragment (A) and caspase activity (B) were determined. The concentration-dependent effect of DCVC on caspase cleavage (C) and caspase activity (D) as well as the effect of zVAD-fmk (100  $\mu$ M) on the caspase activation by DCVC (0.25 mM) was determined 8 h after the start of the incubation. Caspase-3 cleavage and overall caspase activity were determined as described under "Experimental Procedures." Caspase-3 cleavage shown is representative for four independent experiments. Caspase activity data shown are mean  $\pm$  S.E. of four independent experiments ( $n = 4$ ).

cence plate reader (HTS 7000 Bioassay reader; Perkin-Elmer, Norwalk, CT). Caspase activity was calculated as picomole/min/mg cell protein using AMC as a standard.

**Immunoprecipitation and Western Blotting**—For immunoprecipitation studies cells were plated in 10-cm Petri dishes coated with collagen. After treatment with toxicant cells were harvested in a Tris-sucrose-EGTA (TSE) buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM sucrose, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 1 mM  $\text{Na}_3\text{VO}_4$ , and 50 mM sodium fluoride. After sonication, 500  $\mu$ g of cell protein was incubated overnight with 1 volume of RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1.0% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100, and 0.1% (w/v) sodium dodecyl sulfate), with protease and phosphatase inhibitors as described for TSE buffer making a total volume of 1 ml. Thereafter samples were cleared by centrifugation and the supernatant was incubated with 4  $\mu$ g of FAK antibody (Transduction Laboratories, Lexington, KY) for 4 h followed by another 2-h incubation with protein G-coated Sepharose 4B Fast Flow beads (Sigma). Beads were pelleted by brief centrifugation and washed three times with low-salt buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, and 0.1% (w/v) Nonidet P-40 (pH 7.4), including protease and phosphatase inhibitors). Proteins were eluted from the beads by boiling samples for 5 min in 40  $\mu$ l of SDS-PAGE sample preparation buffer containing  $\beta$ -mercaptoethanol. Proteins in equal volumes of sample were separated by SDS-PAGE followed by Western blotting using standard techniques. In other experiments, cleared samples were used for the immunoprecipitation of tyrosine-phosphorylated proteins using agarose-conjugated anti-phosphotyrosine (4G10) (Upstate Biotechnology, Lake Placid, NY). Blots were probed with 1  $\mu$ g/ml mouse monoclonal anti-FAK antibody, 0.25  $\mu$ g/ml mouse monoclonal anti-paxillin (Transduction Laboratories), or 1  $\mu$ g/ml 4G10 anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology). For detection, horseradish peroxidase-conjugated anti-mouse secondary antibody and the ECL method were used (Amersham).

**Immunofluorescence and Imaging Techniques**—Immunofluorescence studies were carried out using cells cultured on collagen-coated glass coverslips in 24-well dishes. After treatment with toxicant cells were fixed with 3.7% formaldehyde for 10 min followed by 3 washes with PBS. After cell lysis and blocking with PBS, 0.2% (w/v) Triton X-100, 0.5% (w/v) BSA (pH 7.4) (PTB) cells were stained for FAK, PAX, or PY



**FIG. 2. Effect of zVAD on DCVC-induced cytotoxicity.** RPTE were treated with DCVC (0.25 mM) in EBSS containing 20  $\mu$ M DPPD in the presence or absence of zVAD-fmk (100  $\mu$ M) for 4 h followed by recovery. Cell cycle analysis (A, left panel: without zVAD-fmk; right panel, with zVAD-fmk) and DNA fragmentation (B) were determined 12 h after the start of the incubation as described under "Experimental Procedures." The data shown are representative for four independent experiments. Cell death (C) was determined by analyzing the relative release of LDH from cells in the culture medium compared with total LDH present and was expressed as % cell death. Data shown are mean  $\pm$  S.E. of four independent experiments ( $n = 4$ ).

using 10, 5, and 10  $\mu$ g/ml monoclonal antibody diluted in PTB as described previously. During the staining period of the cells with secondary fluorescein isothiocyanate-labeled anti-mouse antibody the F-actin cytoskeletal network was labeled with rhodamine-phalloidin (Molecular Probes, Eugene, OR; 0.3 unit/ml). After a final wash of the cells with PBS the DNA was stained with Hoechst 33258 as above. Cells were mounted on glass slides using Aqua-Poly/Mount (Polysciences Inc., Warrington, PA). Cells were viewed using a Bio-Rad 600 MRC confocal laser scanning microscope. Representative images for the different treatment conditions were printed using a Mitsubishi color video printer.

**Statistical Analysis**—Student's  $t$  test was used to determine if there was a significant difference between two means ( $p < 0.5$ ). When multiple means were compared, significance was determined by a one-way analysis of variance (ANOVA;  $p < 0.5$ ). For ANOVA analysis, 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. For example, a mean designated as A is significantly different from a mean designated B, but neither is different from a mean designated AB.

## RESULTS

**DCVC Causes Activation of Caspases and Cleavage of FAK in RPTE**—Apoptosis is associated with the activation of caspases. Treatment of RPTE with DCVC resulted in the cleavage of pro-caspase-3 (32 kDa) into the active caspase-3 fragment (17 kDa); this was associated with increased caspase-3 activity as determined using acetyl-DEVD-AMC as a substrate (Fig. 1). Activation of caspase-3 preceded DNA fragmentation and cell death as determined by formation of 180–200-base pair nucleosomal "ladders" and release of LDH into the medium (Fig. 2). A general caspase inhibitor, zVAD-fmk, completely blocked the DCVC-induced cleavage of caspase-3 and activation of DEV-

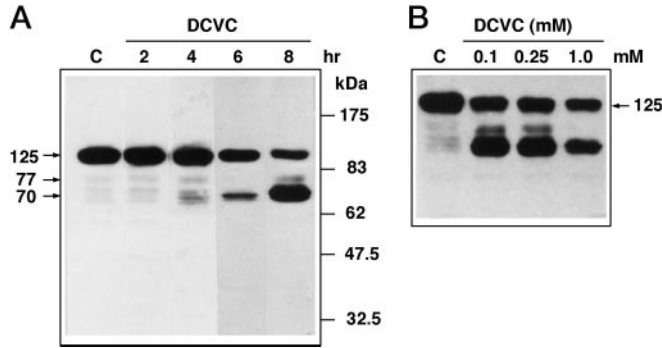
Dase activity (Fig. 1), DNA fragmentation, and the increase in apoptotic cells seen as a sub-G<sub>1</sub>/G<sub>0</sub> population by flow cytometry (Fig. 2). Although zVAD-fmk clearly blocked all the events that are characteristic for apoptosis, it did not block the overall cell killing as determined by the release of LDH from the cells (Fig. 2).

Since DCVC-induced apoptosis of RPTE is associated with impaired cell adhesion (57, 58, 65), and because FAK is cleaved

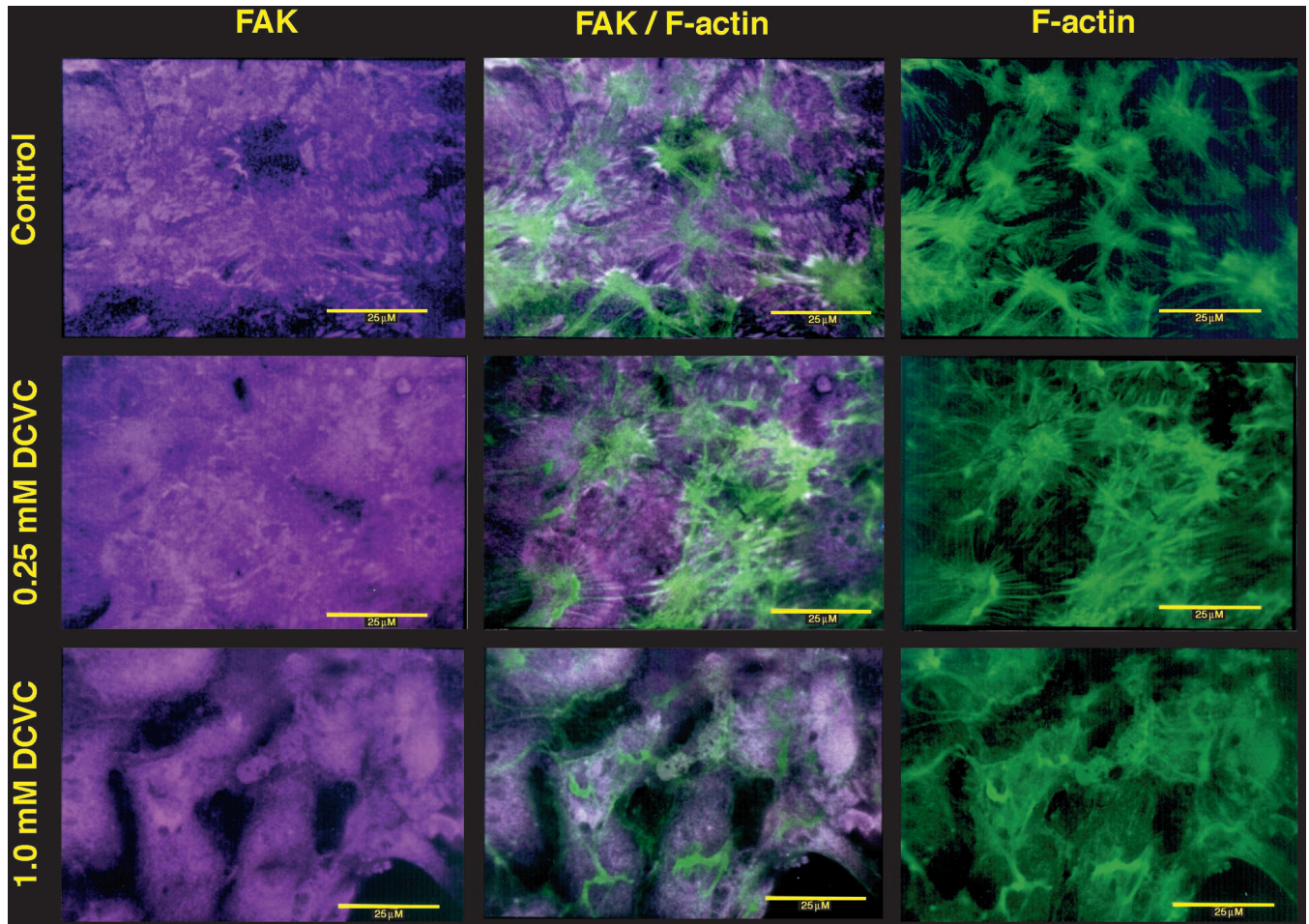
by caspases in cell-free systems as well as in intact cells *in vitro* (12–15), we determined whether DCVC-induced caspase activation is associated with FAK cleavage in RPTE. A major increase in the cleavage of FAK into two fragments with approximate sizes of 70 and 77 kDa was observed 6 h after DCVC treatment, but not before (Fig. 3). Cleavage of FAK correlated with activation of caspase-3 by DCVC (Fig. 1).

**DCVC-induced Translocation of FAK Precedes Caspase Activation**—We next investigated the fate of FAK during apoptosis at the cellular and biochemical level. First we determined whether cleavage of FAK was associated with altered localization of FAK by (immuno)staining of RPTE treated with DCVC for FAK and F-actin. Although FAK cleavage and caspase activation is first observed 6 h after DCVC treatment, FAK redistribution from the focal adhesions to the cytoplasm was observed after 2 h and was maximal 4 h after DCVC treatment (Fig. 4). Loss of FAK from focal adhesions was accompanied by an increased FAK staining in the cytosolic compartment and to some extent in the nucleus. Altered FAK localization was temporally associated with disruption and collapse of the F-actin cytoskeletal network, but FAK did not co-localize with F-actin (Fig. 4). Thus, altered FAK localization clearly preceded cleavage of FAK by caspases, suggesting that an altered focal adhesion ultrastructure is most likely not caused by caspase activation.

**DCVC-induced Translocation of FAK Is Associated with Loss of Tyrosine Phosphorylation**—The results indicated that DCVC-induced loss of FAK from focal adhesions was independ-

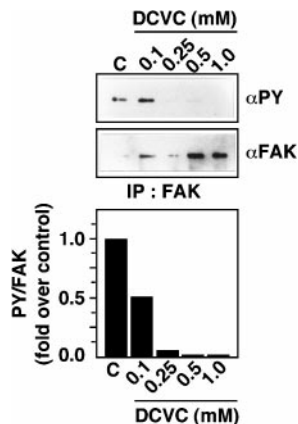


**FIG. 3. DCVC causes cleavage of FAK.** RPTE were treated with DCVC as described in the legend to Fig. 1 and FAK cleavage was determined by Western blotting as described under “Experimental Procedures.” *A*, to determine the time dependent effect of DCVC (0.25 mM) on FAK cleavage samples were taken at 2, 4, 6, and 8 h after start of the experiment. *B*, at 8 h after start of the experiment the concentration dependent effect of DCVC on FAK cleavage was determined. Data shown are representative for four independent experiments ( $n = 4$ ).



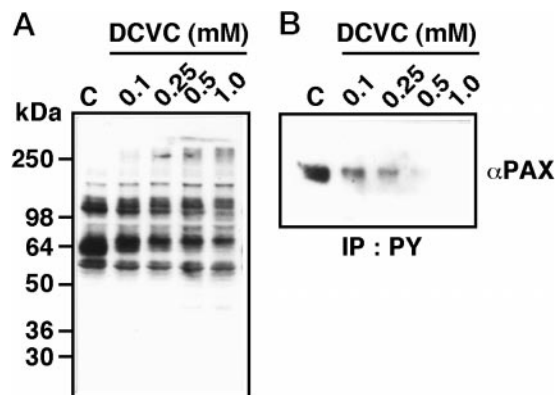
**FIG. 4. DCVC causes a redistribution of FAK.** RPTE were treated with DCVC (0.25 or 1 mM) as described in the legend to Fig. 1. After 4 h cells were fixated and double-stained for FAK and F-actin. Stained cells were viewed using a Bio-Rad confocal laser scanning microscope. Data are representative for three independent experiments ( $n = 3$ ). Note that FAK translocates to the cytosol and the nuclear compartment and does not co-localize with the collapsed F-actin network.

ent of caspase activation; therefore, other signals must be involved. Integrin engagement by extracellular matrix results in autophosphorylation of FAK and subsequent formation of focal adhesions (22–25). Since DCVC disrupts focal adhesions in

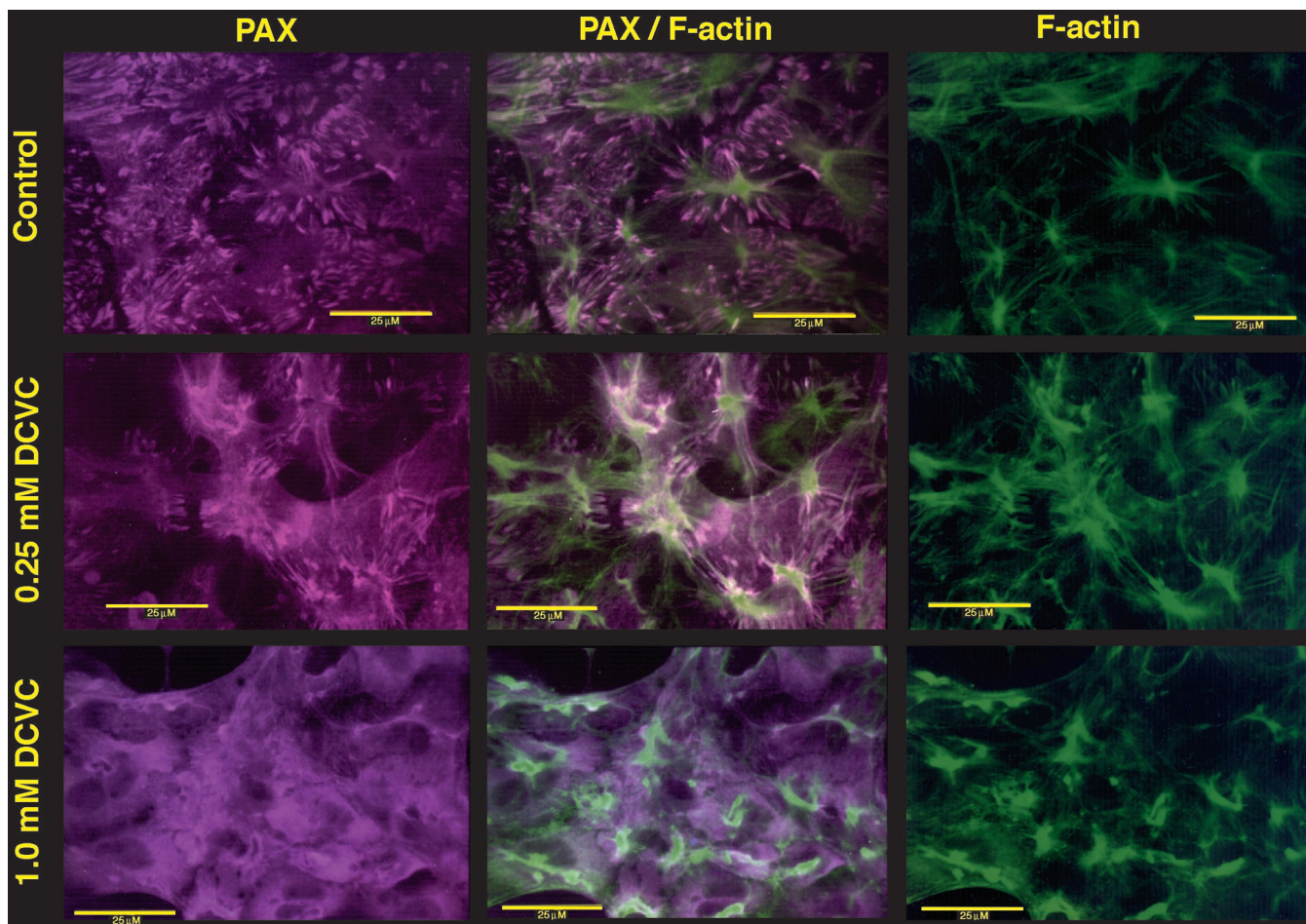


**FIG. 5. DCVC causes tyrosine dephosphorylation of FAK.** RPTE were treated with increasing concentration of DCVC (0.1, 0.25, 0.5, and 1.0 mM) in EBSS in the presence of DPPD (20  $\mu$ M). After 4 h cells were harvested and equal amounts of cell protein were immunoprecipitated with anti-FAK and immunoprecipitated proteins were separated by SDS-PAGE followed by Western blotting. Blots were probed for anti-phosphotyrosine (PY) and anti-FAK (A). Intensity of the bands were scanned by densitometry to determine the ratio of PY/pp125 FAK. The ratio was expressed as fold over control (B). Data are representative for three independent experiments ( $n = 3$ ).

RPTE (57, 58), we determined whether DCVC-induced loss of FAK from focal adhesions was associated with loss of tyrosine phosphorylation of FAK. Indeed, DCVC caused a dose-dependent dephosphorylation of FAK at tyrosine as demonstrated by immunoprecipitation of FAK followed by Western blotting with anti-phosphotyrosine and anti-FAK antibody. FAK was almost



**FIG. 6. DCVC causes a loss of paxillin phosphorylation.** RPTE were treated with increasing concentration of DCVC (0.1, 0.25, 0.5, and 1.0 mM) in EBSS in the presence of DPPD (20  $\mu$ M). After 4 h cells were harvested and total cell protein was separated by SDS-PAGE followed by Western blotting for anti-phosphotyrosine (A). Equal amounts of cell protein were immunoprecipitated with agarose-conjugated anti-PY followed by SDS-PAGE and Western blotting. Blots were probed with monoclonal anti-paxillin ( $\alpha$ PAX) (B). Results shown are representative of three independent experiments ( $n = 3$ ).



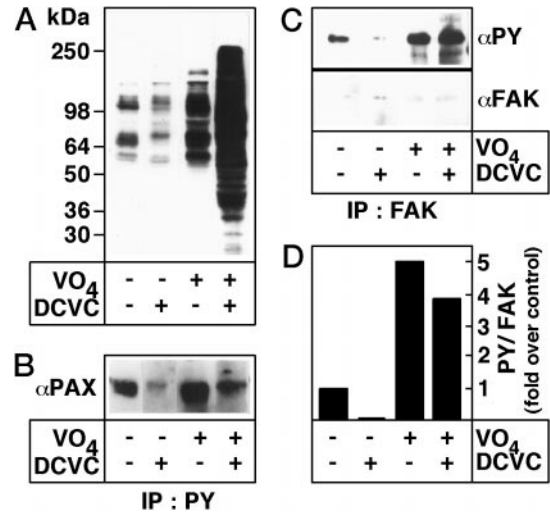
**FIG. 7. DCVC causes a translocation of paxillin from the focal adhesion to the F-actin stress fibers.** RPTE were treated with DCVC (0.25 or 1 mM) in EBSS in the presence of DPPD (20  $\mu$ M). After 4 h cells were fixed for 5 min with 3.7% formaldehyde. Cells were stained for paxillin and F-actin as described under "Experimental Procedures." Stained cells were viewed using a Bio-Rad confocal laser scanning microscope. Note the exact co-localization of paxillin with the collapsed F-actin network.

completely dephosphorylated after a 4-h treatment with 0.25 mM DCVC (Fig. 5), a time point at which cleavage of FAK had not yet occurred (Fig. 3). The increased intensity of FAK bands in the DCVC-treated cells (see Fig. 5) may be explained by a translocation of FAK from the cytoskeleton fraction to the cytosol, thus resulting in a higher recovery of FAK from these samples for immunoprecipitation.

**DCVC-induced Loss of FAK Phosphorylation Is Associated with Loss of Paxillin Phosphorylation**—The data suggested that focal adhesion disturbances and FAK dephosphorylation occur upstream of caspase activation. Many focal adhesion-associated proteins are also phosphorylated at tyrosine residues, and some are direct substrates for FAK. To investigate whether disturbances in tyrosine phosphorylation contributed to DCVC-induced impaired cell adhesion and cell death, we first evaluated the alterations in total cell protein-phosphotyrosine content. DCVC caused a dose dependent decrease in protein-phosphotyrosine from proteins of ~60–70 kDa and proteins with a molecular mass around 120–130 kDa (Fig. 6A). Paxillin is a 68-kDa cytoskeletal adaptor protein that co-localizes with focal adhesions in a variety of cell types and is heavily phosphorylated on tyrosine residues. Immunoprecipitation of phosphotyrosine-containing proteins with anti-phosphotyrosine antibody followed by electrophoresis and Western blotting for paxillin demonstrated that the broad protein band of ~60–70 kDa contained the cytoskeletal adaptor protein paxillin (Fig. 6B). Thus DCVC treatment causes a loss of protein phosphotyrosine from paxillin as well.

Next, we determined the effect of dephosphorylation on the cellular localization of paxillin. DCVC caused a rapid loss of paxillin from focal adhesions and an increase in paxillin staining in the cytosol (Fig. 7). Intense staining of paxillin was also observed in dots that co-localized with the collapsed F-actin cytoskeleton, suggesting that even though paxillin was lost from the focal adhesion, it remained associated with F-actin (Fig. 7). To further evaluate this translocation of paxillin to the F-actin network, cells were permeabilized just prior to fixation to determine cytoskeletal association. Within 2 h after DCVC treatment, co-localization of cytoskeleton-associated paxillin with the F-actin network was observed; after 4 h paxillin completely co-localized with large F-actin aggregates present in the cytosol (not shown). Paxillin did not co-localize with the cortical F-actin cytoskeletal network. A similar association with the F-actin network was observed with talin, another focal adhesion-associated cytoskeletal protein (data not shown). Although both paxillin and FAK co-localized in focal adhesions in control cells, FAK and paxillin no longer co-localized after DCVC treatment (compare Fig. 4 with Fig. 7).

**Inhibition of Protein Tyrosine Phosphatases Increases FAK and Paxillin Tyrosine Phosphorylation without Affecting Caspase Activation and Apoptosis**—The results indicated that nephrotoxicants cause dephosphorylation of both paxillin and FAK prior to loss of cell-matrix contact and cell death. To investigate the role of phosphatases in dephosphorylation of FAK and paxillin, cells were incubated with an inhibitor of protein tyrosine phosphatases, orthovanadate. As expected, treatment of RPTE with orthovanadate alone caused an overall increase of protein tyrosine phosphorylation. In addition, immunoprecipitation experiments indicated that both FAK and paxillin had approximately 4-fold more phosphotyrosine after orthovanadate compared with control cells (Fig. 8). Although DCVC alone caused a complete loss of phosphotyrosine on paxillin and FAK, FAK tyrosine phosphorylation after exposure to DCVC/orthovanadate remained approximately the same as orthovanadate-treated control cells. Thus, orthovanadate can block DCVC-induced dephosphorylation of both pax-



**FIG. 8. Orthovanadate prevents DCVC-induced tyrosine dephosphorylation of paxillin and FAK.** RPTE were treated with DCVC (0.25 mM) in EBSS containing 20  $\mu$ M DPPD in the presence or absence of  $\text{Na}_3\text{VO}_4$  (25  $\mu$ M). After 4 h cells were harvested. Total cell protein was separated by SDS-PAGE followed by Western blotting; blots were probed with anti-phosphotyrosine (PY). Note the increase of total PY-protein after treatment with  $\text{Na}_3\text{VO}_4$  (A). Equal amounts of protein were either immunoprecipitated with anti-PY or anti-pp125 FAK. Immunoprecipitated PY-proteins were separated by SDS-PAGE followed by Western blotting; blots were probed with anti-paxillin ( $\alpha$ PAX) (B). Immunoprecipitated pp125 FAK was also separated by SDS-PAGE followed by Western blotting; blots were probed with anti-PY ( $\alpha$ PY) and after stripping the blots were re-probed with anti-pp125 FAK ( $\alpha$ FAK) (C). Intensity of the pp125 FAK after staining for either anti-PY or anti-pp125 FAK, were scanned by densitometry, and the ratio PY/FAK was calculated and expressed as fold over control (D).

illin and FAK suggesting that an imbalance in protein tyrosine kinase and phosphatase activity is responsible.

To determine if preventing DCVC-induced dephosphorylation of paxillin and FAK was associated with loss of focal adhesion integrity or cell adhesion, we determined the effect of orthovanadate on DCVC-induced translocation of FAK and paxillin as well as cell attachment. Although orthovanadate itself had only a minor effect on FAK localization, it actually potentiated the DCVC-induced redistribution of FAK (Fig. 9). Similar results were obtained for the redistribution of paxillin (data not shown). Because DCVC-induced translocation of FAK is associated with impaired cell adhesion, we checked whether the potentiation of DCVC-induced FAK translocation by orthovanadate was associated with enhancement of RPTE detachment. Indeed, orthovanadate potentiated the cell detachment caused by DCVC (Table I). Thus, not only the phosphorylation itself but rather the cellular localization of FAK, in combination with tyrosine phosphorylation, seems important in the regulation of cell adhesion.

Although maintaining the phosphorylation of both FAK and paxillin with orthovanadate did not protect against cell detachment, the possibility existed that orthovanadate protected against cell death. To investigate this we determined the DCVC-induced caspase activation, a measure of the activation of the apoptotic machinery, as well as cell death. Orthovanadate did not inhibit the DCVC-induced activation of caspases (Fig. 10). Moreover, DCVC-induced release of LDH and formation of a population of cells with sub- $G_0/G_1$  DNA content, *i.e.* apoptotic cells, was unaffected (Fig. 10). Finally, although orthovanadate increased tyrosine phosphorylation of FAK (see above), this did not have an effect on the amount of FAK that was cleaved by caspases (Fig. 10).

**Caspases Mediate Cleavage of FAK and Cell Detachment Caused by DCVC**—Having characterized the temporal rela-

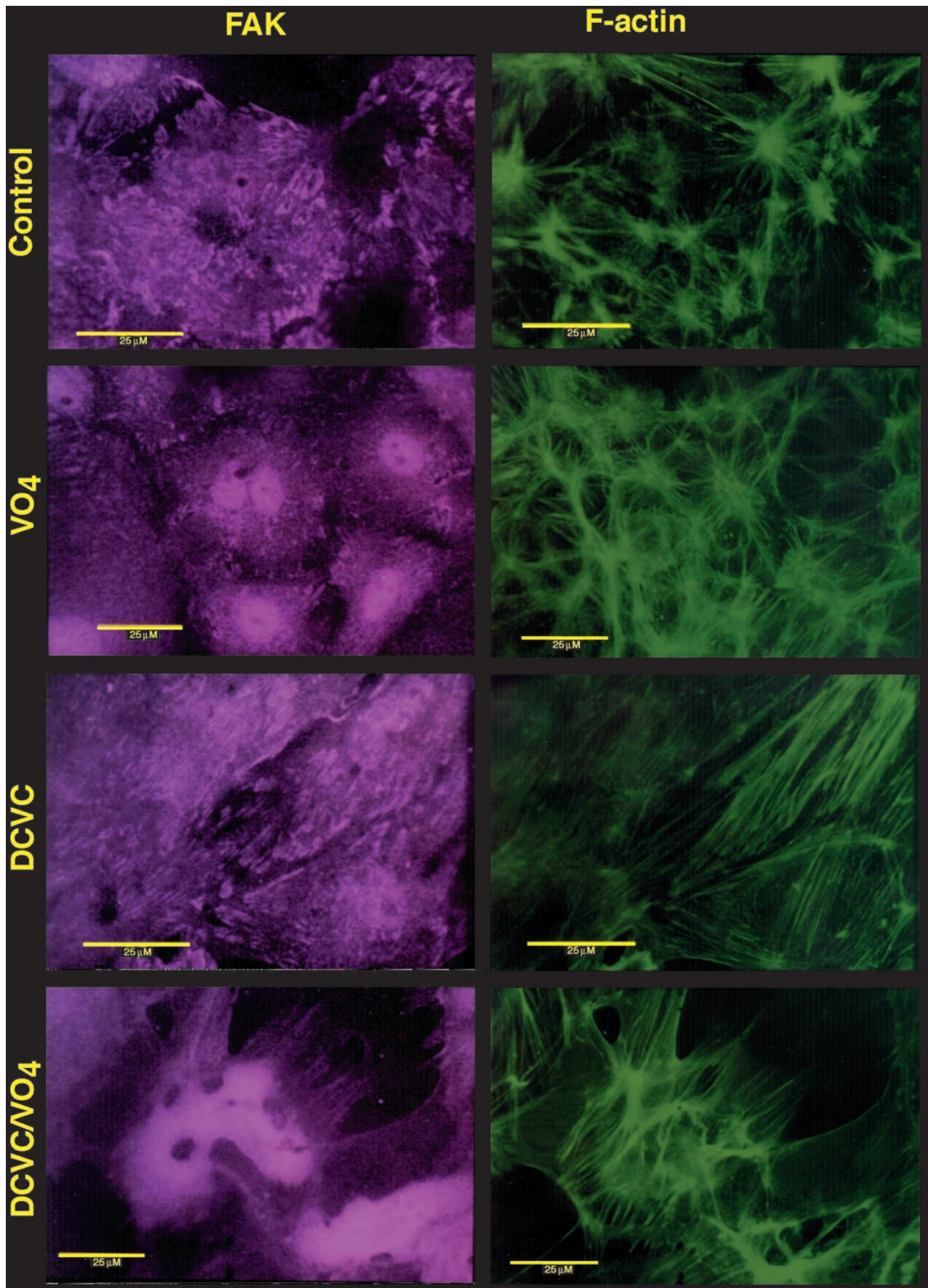


FIG. 9. Orthovanadate does not prevent translocation of FAK from the focal adhesions to the cytosol. RPTe treated with DCVC (0.25 mM) in EBSS containing 20 μM DPPD in the presence or absence of  $\text{Na}_3\text{VO}_4$  (25 μM). After 4 h cells were fixed and double-stained for pp125 FAK and F-actin. Stained cells were viewed using a Bio-Rad confocal laser scanning microscope. Data are representative for three independent experiments ( $n = 3$ ).

tionship between FAK and paxillin localization and dephosphorylation after DCVC treatment, we determined whether DCVC-induced activation of caspases is involved in the dephosphorylation of FAK and paxillin by determining the phosphotyrosine content of both proteins after DCVC treatment in the presence or absence of zVAD-fmk. As might be predicted from the disparity in the time dependence for FAK cleavage *versus* dephosphorylation, zVAD-fmk did not inhibit the DCVC-induced tyrosine dephosphorylation of cellular proteins (Fig. 11) including FAK and paxillin.

Next we determined whether the cleavage of FAK was dependent on caspase activity. Therefore cells were treated with DCVC in the presence of z-VAD-fmk. Inhibition of DCVC-induced caspase activation by z-VAD-fmk (see Fig. 1) was associated with a complete inhibition of FAK cleavage (Fig. 11). This indicates that the FAK cleavage is due to caspase activation.

A clear increase of floating RPTE was only observed after 8 h treatment with DCVC. This was also a time point when caspase activity and FAK cleavage was clearly increased. Therefore, the possibility existed that cell detachment was dependent on caspase activity. Inhibition of caspases with zVAD-fmk, which blocked the cleavage of FAK, did not have an effect on the DCVC-induced rounding up of cells. However, z-VAD-fmk clearly inhibited the cell detachment caused by DCVC (Fig. 11). To determine whether this was related to a protection against a disturbance of the focal adhesions, we analyzed the focal adhesion organization after DCVC treat-

ment in the presence of z-VAD-fmk. Z-VAD-fmk did not block any of the DCVC-induced alterations of the focal adhesions and F-actin network after 4 h treatment (not shown). Thus, caspase-mediated proteolysis of cellular (cytoskeletal) proteins, including FAK, does not mediate loss of the focal adhesion and F-actin network organization during apoptosis.

DISCUSSION

These studies on the temporal relationship between FAK dephosphorylation, translocation, and cleavage and the activation of the apoptotic machinery during apoptosis of primary cultured renal proximal tubular epithelial cells allow us to draw two important conclusions. First, FAK dephosphorylation caused by DCVC precedes activation of the apoptotic machinery, *i.e.* caspases, and is independent of caspase cleavage. Dephosphorylation also precedes the proteolysis of FAK. Although

TABLE I

Effect of orthovanadate on DCVC-induced cell detachment of RPTE

RPTE were treated with DCVC (0.25 mM) as described in the legend to Fig. 1 in the absence or presence of Na<sub>3</sub>VO<sub>4</sub> (25 μM). After 12 h the % detached cells was determined as described under "Experimental Procedures." Data shown are the mean ± S.E. of four independent experiments (n = 4).

Treatment	% Detached cells
Control	2.5 ± 0.9 <sup>A</sup>
DCVC	47.3 ± 1.6 <sup>B</sup>
Na <sub>3</sub> VO <sub>4</sub>	0.8 ± 0.4 <sup>A</sup>
DCVC/Na <sub>3</sub> VO <sub>4</sub>	62.1 ± 4.0 <sup>C</sup>

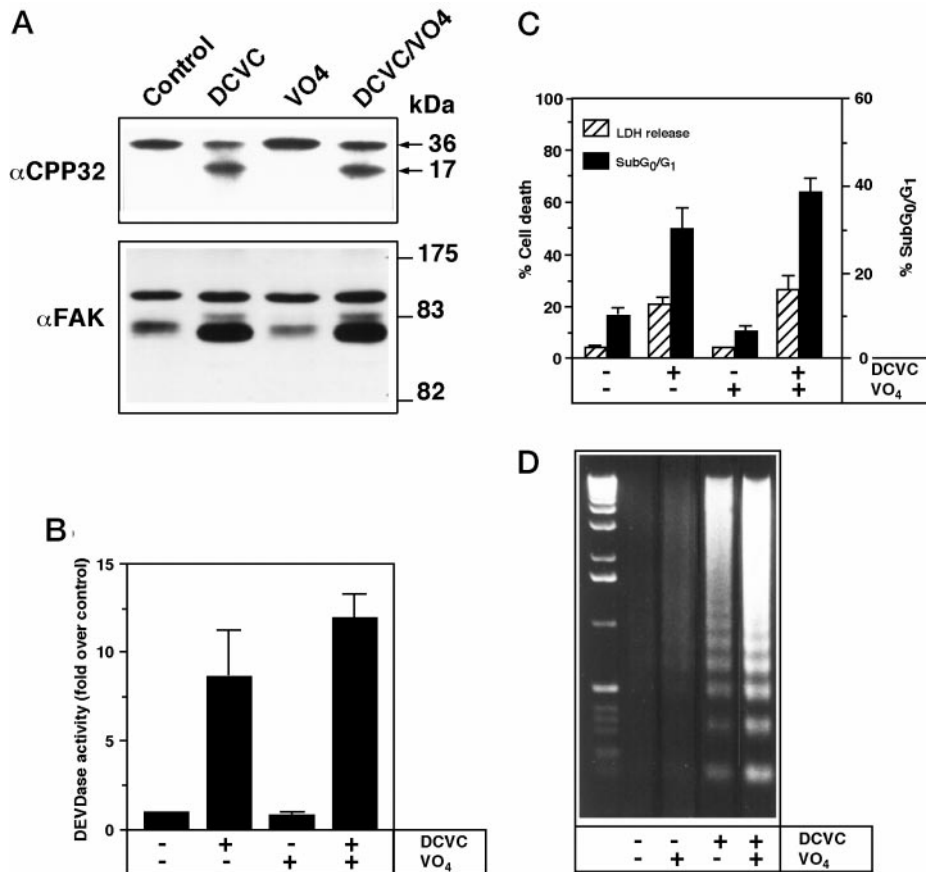


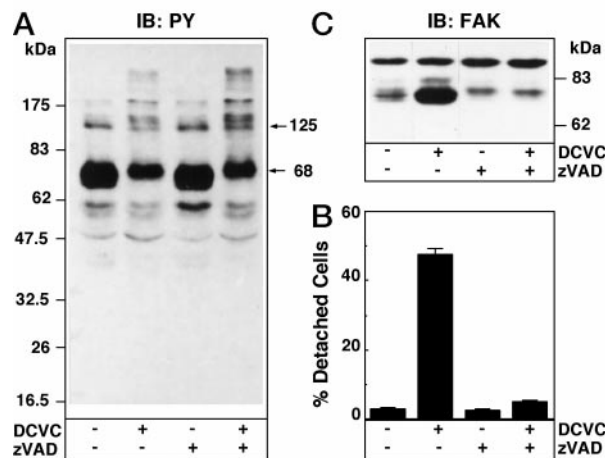
FIG. 10. Orthovanadate does not inhibit DCVC-induced cytotoxicity. RPTE were treated with DCVC (0.25 mM) as described in the legend to Fig. 1 in the absence or presence of Na<sub>3</sub>VO<sub>4</sub> (25 μM). After 8 h caspase-3 and FAK cleavage (A) and caspase activity (B) were determined as described in the legends to Figs. 1 and 3. After 12 h the % cell death as determined by the relative LDH release, and the % of cells present in the sub-G<sub>0</sub>/G<sub>1</sub> as measured by flow cytometry analysis, was determined (C) as described under "Experimental Procedures." The DCVC-induced DNA fragmentation was also analyzed after 12 h (D). Data shown are representative for four independent experiments (A and D) or mean ± S.E. (n = 4; C and D).



we have only described the effects of DCVC, similar observations were made with another nephrotoxicant, cis-platin, indicating that our observations are also important in other forms of nephrotoxicant induced cytotoxicity (59).<sup>2</sup> FAK cleavage has been reported before in other models of apoptosis (12–15), but the temporal relationship between FAK dephosphorylation, FAK translocation, and FAK cleavage has not been studied. Thus, to our knowledge, this is the first report that indicates that FAK dephosphorylation precedes activation of caspases and cleavage of FAK during apoptosis. Second, in this model, dephosphorylation of FAK is closely associated with loss of cell-matrix interactions, but even when FAK dephosphorylation is complete, cells remain attached to the substratum. Although caspase activity is not involved in the impairment of focal adhesion signaling, it appears that cleavage of FAK and other cytoskeletal proteins is required for irreversible detachment of cells from the substratum. Possibly, cleavage of FAK and other cytoskeletal proteins not only causes cell detachment but also prevents the reattachment and spreading of injured cells. This may have considerable consequences in renal injury, since blocking cell reattachment prevents the attendant tubular obstruction that occurs after renal injury (54, 55). Thus, our studies provide new insights into the role of focal adhesion signaling in renal injury.

Although disruption of focal adhesion signaling through FAK clearly precedes cell detachment after chemically induced apoptosis, the data also suggest that caspase activation is not responsible for the former. Rather, the data indicate that toxicant-induced dephosphorylation of focal adhesion proteins including FAK may contribute to loss of focal adhesions and cell-matrix interactions. Forced loss of cell-matrix interactions of RPTE, by keeping trypsinized RPTE in suspension, also results in caspase activation and apoptosis.<sup>3</sup> Therefore, the present observations fit a model in which loss of focal adhesion architecture and signaling is a trigger for chemically induced activation of caspases culminating in apoptosis; FAK may have a central role. Indeed, FAK and related family members play an important role in other models of apoptosis. Blocking the interaction of FAK with  $\beta$ -integrin during cell adhesion induces apoptosis (48). Expression of a constitutively active FAK, a CD2-FAK chimera that localizes to focal adhesions, but not chimeras containing FAK kinase-dead mutants, inhibit anoikis (46, 51). In contrast to the anti-apoptotic role of FAK, overexpression of proline-rich tyrosine kinase 2, also known as cellular adhesion kinase  $\beta$ , a FAK related non-receptor protein tyrosine kinase that also localizes at focal adhesion, induces apoptosis (50). Thus, different FAK family members may have opposing roles in regulating apoptosis. FAK activation seems a cellular survival factor and FAK dephosphorylation may lead to decreased activity of signaling cascades that otherwise would block the apoptotic machinery. For example, cell adhesion results in activation of Akt/PKB, which is involved in phosphorylation of the pro-apoptotic Bcl-2 family member Bad (35, 36, 40–42). This phosphorylation results in inactivation of the anti-apoptotic function of Bad and, as a consequence, protection against apoptosis (40–42). The relationship between chemically induced dephosphorylation and translocation of FAK and perturbation of downstream signal transduction cascades in the control of apoptosis warrants further investigations.

Cell detachment during apoptosis is likely to occur in two steps: loss of cell-to-cell contact and then loss of cell-substratum adhesion, *i.e.* the reverse of the steps in organizing an epithelial layer. Both steps seem important in controlling



**FIG. 11. zVAD-fmk blocks DCVC-induced FAK cleavage and cell detachment without affecting tyrosine dephosphorylation.** RPTE were treated with DCVC (0.25 mM) as described in the legend to Fig. 1 in the absence or presence of zVAD-fmk (100  $\mu$ M). After 4 h total cell lysates were separated by SDS-PAGE followed by Western blotting and blots were probed with anti-phosphotyrosine (PY) (A). FAK cleavage was determined after 8 h as described in the legend to Fig. 3 (B). Shown are representative immunoblots of four independent experiments. Cell detachment was quantified after 12 h as described under "Experimental Procedures" and expressed as mean of % detached cells  $\pm$  S.E. ( $n = 4$ ).

apoptosis (19, 46, 68). Our results indicate that FAK dephosphorylation and translocation as well as caspase-mediated FAK cleavage were associated with renal cell detachment during apoptosis. Yet, only caspase-mediated cleavage of FAK seems related to the final renal cell detachment: zVAD-fmk blocked cell detachment completely without affecting either the dephosphorylation and translocation of FAK or the disruption of focal adhesions. Thus, our data suggest that FAK dephosphorylation and loss of focal adhesions are closely associated with loss of cell-matrix contact, but not irreversible detachment which actually requires caspase activation since zVAD blocked detachment but not focal adhesion disruption. Thus, cells may still adhere to the substratum even though they have completely disrupted focal adhesions. This may be related to the fact that cell adhesion not only depends on cell-matrix but also on cell-cell interactions. Besides FAK, other cytoskeletal proteins that are involved in cell-cell interactions, *e.g.*  $\beta$ -catenin and fodrin (non-erythroid spectrin), have been identified as substrates for caspases (8, 11). Moreover, Gas2 and gelsolin, whose functions are directly related to the organization of the F-actin cytoskeletal network, are also caspase substrates (7, 9). Thus, the caspase-mediated cleavage of several cytoskeletal proteins, including FAK, may be required for the complete loss of cell-matrix as well as cell-cell interactions during apoptosis. Cleavage of these proteins may actually ensure that the cell does not reattach and proliferate at a distant site. Moreover, FAK cleavage will prevent (re)activation of anti-apoptotic signaling cascades, for example, through Akt/PKB, derived from focal adhesions thereby preventing survival of cells that are otherwise destined to die. Indeed, overexpression of the C-terminal fragment of FAK prevents autophosphorylation of endogenous FAK upon cell adhesion (15), and FAK cleavage is associated with loss of interaction between FAK and paxillin (14). Thus, these data indicate that FAK cleavage is linked to inhibition of focal adhesion-related signaling events as well as loss of protein-protein interactions.

The implications of cell reattachment in metastasis of tumor cells are well known. However, also during renal injury, it is clear that reattachment of dying or injured cells, through inte-

<sup>2</sup> B. Van de Water and J. L. Stevens, unpublished observations.

<sup>3</sup> B. Van de Water, unpublished observations.

grin-mediated signaling, has important pathophysiological implications since it contributes to the tubular obstruction that exacerbates renal injury (54, 55). In this scenario, pharmacological intervention of caspase activity could be used to block cell detachment after a renal insult and, thereby, alleviate tubular obstruction. It would be expected that such a treatment would reduce the severity of the renal injury. Thus, our studies provide new insights into possible modulation of acute renal injury.

Although it is clear that FAK dephosphorylation by itself is not sufficient to cause cell detachment, the exact role of caspase-mediated cleavage of FAK in this process needs further investigation. We studied the involvement of FAK in chemically induced apoptosis, however, also deregulation of other focal adhesion-associated signaling molecules that are implicated in focal adhesion regulation and/or control of cell death, e.g. c-Src, Fyn, and Crk, may be involved. Regardless of the role of other focal adhesion proteins, FAK cleavage during apoptosis will be important to prevent focal adhesion reassembly of cells that are otherwise supposed to die by apoptosis.

In summary, the present findings demonstrate that FAK dephosphorylation precedes toxicant-induced activation of caspases and subsequent cleavage of FAK. The fact that focal adhesion-mediated signal transduction is fundamental for cell survival of normal epithelial cells and that many focal adhesion-associated molecules have oncogenic potential, most likely by preventing anoikis (46), warrants future investigation on the role of other focal adhesion-associated signaling molecules in the control of (chemically induced) apoptosis.

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**Dephosphorylation of Focal Adhesion Kinase (FAK) and Loss of Focal Contacts Precede Caspase-mediated Cleavage of FAK during Apoptosis in Renal Epithelial Cells**

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