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Cadmium Affects Focal Adhesion Kinase (FAK) in Mesangial Cells: Involvement of CaMK-II and the Actin Cytoskeleton

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ABSTRACT

The toxic metal ion cadmium (Cd^{2+}) induces pleiotropic effects on cell death and survival, in part through effects on cell signaling mechanisms and cytoskeletal dynamics. Linking these phenomena appears to be calmodulin-dependent activation of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II). Here we show that interference with the dynamics of the filamentous actin cytoskeleton, either by stabilization or destabilization, results in disruption of focal adhesions at the ends of organized actin structures, and in particular the loss of vinculin and focal adhesion kinase (FAK) from the contacts is a result. Low-level exposure of renal mesangial cells to $CdCl_2$ disrupts the actin cytoskeleton and recapitulates the effects of manipulation of cytoskeletal dynamics with biological agents. Specifically, Cd^{2+} treatment causes loss of vinculin and FAK from focal contacts, concomitant with cytoskeletal disruption, and preservation of cytoskeletal integrity with either a calmodulin antagonist or a CaMK-II inhibitor abrogates these effects of Cd^{2+}. Notably, inhibition of CaMK-II decreases the migration of FAK-phosphoTyr925 to a membrane-associated compartment where it is otherwise sequestered from focal adhesions in a Cd^{2+}-dependent manner. These results add further insight into the mechanism of the CaMK-II-dependent effects of Cd^{2+} on cellular function. J. Cell. Biochem. 114: 1832–1842, 2013.

KEY WORDS: FOCAL ADHESION KINASE; MESANGIAL CELL CYTOSKELETON; CAMK-II; CADMIUM TOXICITY; VINCULIN

Cadmium (Cd) is an important occupational and environmental toxic metal [Järup and Åkesson, 2009] that causes apoptosis [Liu and Templeton, 2007], oxidative stress [Thévenod, 2009], and cytoskeletal disruption [Wang and Templeton, 1996; Wang et al., 1996]. For the general population, environmental exposure to Cd through food or drinking water is an important health issue due to its long biological half-life and accumulation in various target organs such as the kidney [Järup and Åkesson, 2009; Nordberg et al., 2012]. Though the proximal tubules of the kidney are major sites of toxicity [Prozialeck and Edwards, 2012], the glomerulus has also been shown to be affected [Roels et al., 1993; Järup et al., 1995; L'Azou et al., 2007].

The mesangial cell of the glomerulus is a smooth-muscle-like pericyte that plays a central role in maintaining the structural integrity of the glomerulus and can alter the glomerular filtration rate by modifying luminal surface area. It is particularly susceptible to the effects of Cd^{2+} due to lack of an underlying glomerular basement membrane, resulting in exposure to toxic substances in the plasma [L'Azou et al., 2007]. Unlike other divalent metal cations, Cd^{2+} selectively disrupts the filamentous (F-)actin cytoskeleton in rat mesangial cells without a subsequent increase in globular (G-)actin monomers [Wang and Templeton, 1996], which is partly due to translocation of gelsolin to the cytoskeleton [Apostolova et al., 2006] and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) activation and actin binding [Liu and Templeton, 2013].

CaMK-II is a Ca^{2+}-dependent serine/threonine kinase that becomes activated upon binding to Ca^{2+}/calmodulin and undergoes autophosphorylation followed by autonomous downstream kinase activity. The specific isoform found in mesangial cells is CaMK-IIδ [Xiao et al., 2005]. In mouse mesangial cells, Cd^{2+} activates CaMK-II leading to increased phosphorylation and

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contributing to Cd^{2+}-induced apoptosis [Liu and Templeton, 2007]. Recently, we showed that in rat mesangial cells CaMK-II is activated and CaMK-IIδ associates with actin filaments upon Cd^{2+} treatment; association with F-actin is abrogated by inhibition of CaMK-II [Liu and Templeton, 2013].

Focal adhesions anchor the cytoskeleton to the extracellular matrix and play a critical role in regulating cell proliferation, apoptosis, and migration. Focal adhesions are composed of several proteins including paxillin, vinculin, α-actinin, and focal adhesion kinase (FAK). Both the structural integrity of the actin scaffold and the intracellular membrane environment determine the assembly and disassembly of focal adhesions [Wehrle-Haller, 2012]. FAK is the main non-receptor protein tyrosine kinase involved in focal adhesion formation. Upon integrin clustering, FAK becomes autophosphorylated at Tyr397, resulting in recruitment of Src kinases that phosphorylate other tyrosine residues leading to subsequent activation of several downstream signaling cascades [Katz et al., 2003].

Focal adhesions have previously been shown to be disrupted by toxic elements [Chatzizacharias et al., 2008] such as arsenic [Yancy et al., 2005] and lead [Giuliani et al., 2005], which alter the structural integrity of the actin cytoskeleton. We recently showed that Cd^{2+} may potentially disrupt focal adhesions in rat mesangial cells with loss of vinculin from focal contacts, and that inhibition of CaMK-II prevents this effect [Templeton and Liu, 2013]. In the present study, we sought to determine the mechanism of focal adhesion disruption by Cd^{2+}. We report that Cd^{2+} causes a loss of both vinculin and FAK from focal contacts and that this process is partly due to changes in the actin cytoskeletal dynamics. Additionally, we have found that Cd^{2+} increases the phosphorylation of FAK-Tyr925 in a time-dependent manner, but does not increase phosphorylation at the autophosphorylation site, Tyr397. Finally, we show that FAK-phosphoTyr925 is increased in a membrane fraction by treatment of cells with Cd^{2+}, dependent upon activation of CaMK-II. Thus, this study shows that Cd^{2+}-mediated focal adhesion disruption is due at least in part to a CaMK-II-dependent effect on FAK localization and actin cytoskeletal integrity.

MATERIALS AND METHODS

MATERIALS

Fetal bovine serum (FBS) and RPMI-1640 culture medium were purchased from Wisent Biocenter (Quebec, Canada). Mouse monoclonal anti-vinculin (#V9139) and anti-β-actin (#A1978) antibodies were purchased from Sigma–Aldrich (St. Louis, MO). Rabbit polyclonal anti-FAK antibody (#06-543) was purchased from EMD Millipore (Billerica, MA). Rabbit polyclonal anti-FAK-phosphoTyr397 antibody (#ab4803) was purchased from Abcam (Cambridge, UK). Rabbit polyclonal anti-FAK-phosphoTyr925 (#3284S) and HRP-conjugated secondary antibodies were from Cell Signaling Technology (Beverly, CA). Mouse monoclonal anti-transferrin receptor (TfR; #136800), anti-secondary FITC-conjugated antibodies (#A11001, #A11034), and rhodamine-phalloidin were purchased from Invitrogen (Danvers, MA). KN93 was from Calbiochem (Billerica, MA). Trifluoroperazine (TFP) was from Sigma. Cytochalasin D and jasplakinolide were purchased from Molecular Probes (Burlington, ON).

TISSUE CULTURE

Rat mesangial cells were cultured as previously described [Wang et al., 1996]. Cells were grown in 10% FBS in RPMI-1640 medium in a humidified atmosphere of 5% CO_2 at 37°C and used between passages 7 and 15. Cells were passaged 1:4 and grown overnight before starvation in 0.2% FBS for 48 h to render them quiescent. During $CdCl_2$ treatment, cells were washed once and incubated with serum-free (SF) medium and $CdCl_2$ with parallel SF controls included. For inhibitor studies, cells were pre-treated with 10 μM KN93 or 5 μM TFP in SF medium for 1 h followed by co-treatment with $CdCl_2$ for 6 h. For some studies, cells were treated with cytochalasin D (200 or 500 nM) or with 50 nM jasplakinolide in SF medium for 1–6 h.

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Cells were plated on 12 mm cover-slips, grown overnight, and starved for 48 h with 0.2% FBS in RPMI-1640. After treatment with $CdCl_2$, cells were washed with chilled PBS, fixed with 4% paraformaldehyde, and permeabilized with 100 mM PIPES, pH 6.9, containing 0.5% Triton X-100 lysis buffer, 1 mM EGTA, and 4% polyethyleneglycol 8000. Permeabilized cells were blocked in 5% BSA/PBS and incubated overnight with primary antibody (1:30 or 1:50) at 4°C. Cells were washed three times with 0.2% BSA/PBS followed by incubation with rhodamine-phalloidin (1:100) and FITC-conjugated secondary antibody (1:30) for 1 h at room temperature. Coverslips were mounted on glass slides with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlington, ON) to stain the nuclei. Images were taken using a Nikon fluorescent microscope.

CELL FRACTIONATION

Total cell lysate. Cells were washed twice with chilled PBS and lysed by one freeze–thaw cycle in 50 mM HEPES, pH 7.4, with 0.5% Nonidet P-40, containing protease and phosphatase inhibitors (1 mM Na_3VO_4, 25 mM NaF, 1 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). Lysates were then sonicated twice for 5 s and centrifuged (15,000*g*, 15 min). The supernatant was collected as total cell lysate.

Cytosol-cytoskeletal fractionation. Cells were washed twice with chilled PBS and lysed with 10 mM Tris–HCl, pH 7.4, with 2 mM $MgCl_2$, 138 mM KCl, and 0.2% Triton X-100, containing protease and phosphatase inhibitors. The lysate was centrifuged (10,000*g*, 15 min) and the supernatant was designated the cytosolic fraction. The detergent-insoluble pellet was washed once with chilled PBS and resuspended in 5 mM Tris–HCl, pH 8.0, with 0.2 mM $CaCl_2$, and 200 μM ATP, sonicated three times for 5 s and centrifuged (10,000*g*, 5 min). The supernatant was designated the cytoskeletal fraction.

Cytosolic and membrane fractionation. Differential detergent fractionation was used to isolate subcellular fractions according to Biederbick et al. [2006]. Briefly, cells were washed twice with chilled PBS and pelleted (400*g*, 5 min). The cell pellets were resuspended in 0.007% Digitonin in 5 mM Tris–HCl, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, and protease

inhibitors. The suspension was agitated for 8 min on ice and centrifuged (1,800*g*, 8 min), and the supernatant was further clarified (15,000*g*, 20 min) and designated the cytosolic fraction. The pellet was washed twice with chilled PBS and resuspended in 20 mM Tris–HCl, pH 7.4, with 2 mM $MgCl_2$, 138 mM KCl, and 0.5% Triton X-100, containing protease and phosphatase inhibitors, and incubated on ice for 30 min. The suspension was centrifuged (8,000*g*, 10 min) and the supernatant collected as the membrane fraction. The cytosolic fraction was shown to be free of the mitochondrial marker apoptosis inducing factor (AIF), and the membrane protein TfR, while these two proteins were localized in the membrane fraction.

WESTERN BLOTTING

Equal amounts of protein were mixed with 5× SDS loading buffer, heated for 5 min at 95°C and resolved on 8% SDS–PAGE gels. Proteins were transferred to nitrocellulose membranes and blocked with 5% BSA for 1 h, prior to incubation with primary antibody overnight at 4°C. Antibody dilutions were as follows: anti-vinculin (1:10,000); anti-FAK (1:2,000); anti-FAK-phosphoTyr397 (1:1,000); anti-FAK-phosphoTyr925 (1:2,000); anti-TfR (1:10,000); anti-AIF (1:1,000). Anti-β-actin antibody (1:10,000) was used to visualize β-actin as a loading control. Membranes were washed with TBS-T and incubated with HRP-conjugated secondary antibody (1:10,000) for 1 h. Quantification of bands was performed using Image J 1.42q (NIH, USA).

STATISTICAL ANALYSIS

Multiple measurements are reported as mean ± SD with hypothesis testing for significance reported as a *P*-value for measurements derived from one-way ANOVA, followed by Tukey's post hoc test when multiple treatment comparisons are considered. Calculations were performed with InStat Software (GraphPad, San Diego, CA).

RESULTS

CADMIUM DISRUPTS ACTIN–VINCULIN CONTACTS

Vinculin is an integral component in the assembly of the focal adhesion complex, serving to anchor F-actin to the contacts, and it might be expected that Cd-induced disruption of the actin filaments would destabilize the complex. In preliminary studies [Templeton and Liu, 2013] we showed that Cd treatment leads to a loss of localization of vinculin at contact surfaces. This is demonstrated by dual staining of vinculin and F-actin (Fig. 1). At 10 μM $CdCl_2$, actin filaments are less prominent and vinculin localization to focal adhesions is diminished. With 40 μM Cd, focal adhesions as revealed by vinculin staining are all but absent.

To demonstrate further the role of F-actin in maintaining localized vinculin contacts, cytochalasin D was used to disrupt the actin polymer. After 1 h in 200 nM cytochalasin D, localization of vinculin to the contacts was disrupted, while in 500 nM cytochalasin D actin filaments were also significantly disorganized (Fig. 2A–I). Interestingly, the F-actin stabilizing agent, jasplakinolide, also resulted in a progressive loss of vinculin localization at focal adhesions, suggesting F-actin polymerization–depolymerization dynamics are more important than filament integrity in sustaining vinculin localization (Fig. 2N,O).

CaMK-II IS INVOLVED IN Cd-DEPENDENT DISRUPTION OF FOCAL ADHESIONS

Because we showed previously that inhibition of CaMK-II prevented cytoskeletal disruption and protected against Cd-induced apoptosis [Templeton and Liu, 2010], translocation of CaMK-IIδ to the cytoskeleton [Liu and Templeton, 2013], and vinculin delocalization [Templeton and Liu, 2013], we investigated more fully the role of CaMK-II in focal contact disruption. A 6 h exposure to 10 μM $CdCl_2$ was chosen as the basal condition, as it showed extensive loss of vinculin from focal adhesions (Fig. 3A,B). The calmodulin antagonist TFP was largely protective of vinculin localization to focal adhesions (Fig. 3C), as was the CaMK-II inhibitor KN93 (Fig. 3D). Even at a much higher concentration of $CdCl_2$ (40 μM) that caused extensive disruption of actin filaments and vinculin localization (Fig. 3E), KN93 preserved both F-actin integrity and localization of vinculin to the ends of the filaments (Fig. 3F).

FOCAL ADHESION KINASE (FAK) LOCALIZATION IS AFFECTED BY Cd

FAK is a key signaling molecule involved in the recruitment, assembly, and consequent signaling of the focal adhesion complex [Wehrle-Haller, 2012]. Total FAK localization to the anchored ends of actin filaments mirrors that of vinculin (Fig. 4A), and is likewise disrupted by 6 h $CdCl_2$ treatment at 10 μM (Fig. 4B) and 40 μM (not shown) where KN93 is protective at both concentrations (Fig. 4C,D). Again, comparable to their effects on vinculin (Fig. 2), cytochalasin D (500 nM) and jasplakinolide (50 nM) both result in loss of total FAK staining from focal contacts (Fig. 4E,F).

TRANSLOCATION OF FAK TO THE CYTOSKELETAL FRACTION IS STIMULATED BY Cd

To determine more quantitatively whether Cd favors degradation or redistribution of vinculin and FAK, we performed Western blotting of cytosolic and cytoskeletal fractions of Cd-treated cells. There were no discernible changes in vinculin in either compartment after treatment with up to 40 μM $CdCl_2$ (Fig. 5A), suggesting that disruption of the focal contacts does not result in degradation or significant recompartmentalization of vinculin. However, with increasing Cd concentration up to 40 μM, there is a dose-dependent increase in total FAK in the cytoskeletal compartment (Fig. 5B), indicating enhanced association of FAK with actin filaments despite its loss from the termini of the filaments. This translocation of total FAK to the cytoskeletal fraction was abrogated by KN93 (Fig. 5C).

CADMIUM STIMULATES SITE-SPECIFIC PHOSPHORYLATION OF FAK

Because inhibition of CaMK-II maintained FAK in the focal adhesion and suppressed its Cd-dependent translocation to the cytoskeletal fraction, we examined the possible role of Cd-dependent phosphorylation of FAK in this phenomenon. Activation of FAK results in its autophosphorylation at Tyr397, whereas subsequent recruitment of Src to the focal adhesion complex achieves additional phosphorylation on Tyr925 [Westhoff et al., 2004; Wehrle-Haller, 2012]. Cd (10 μM) does not stimulate significant phosphorylation at Tyr397,

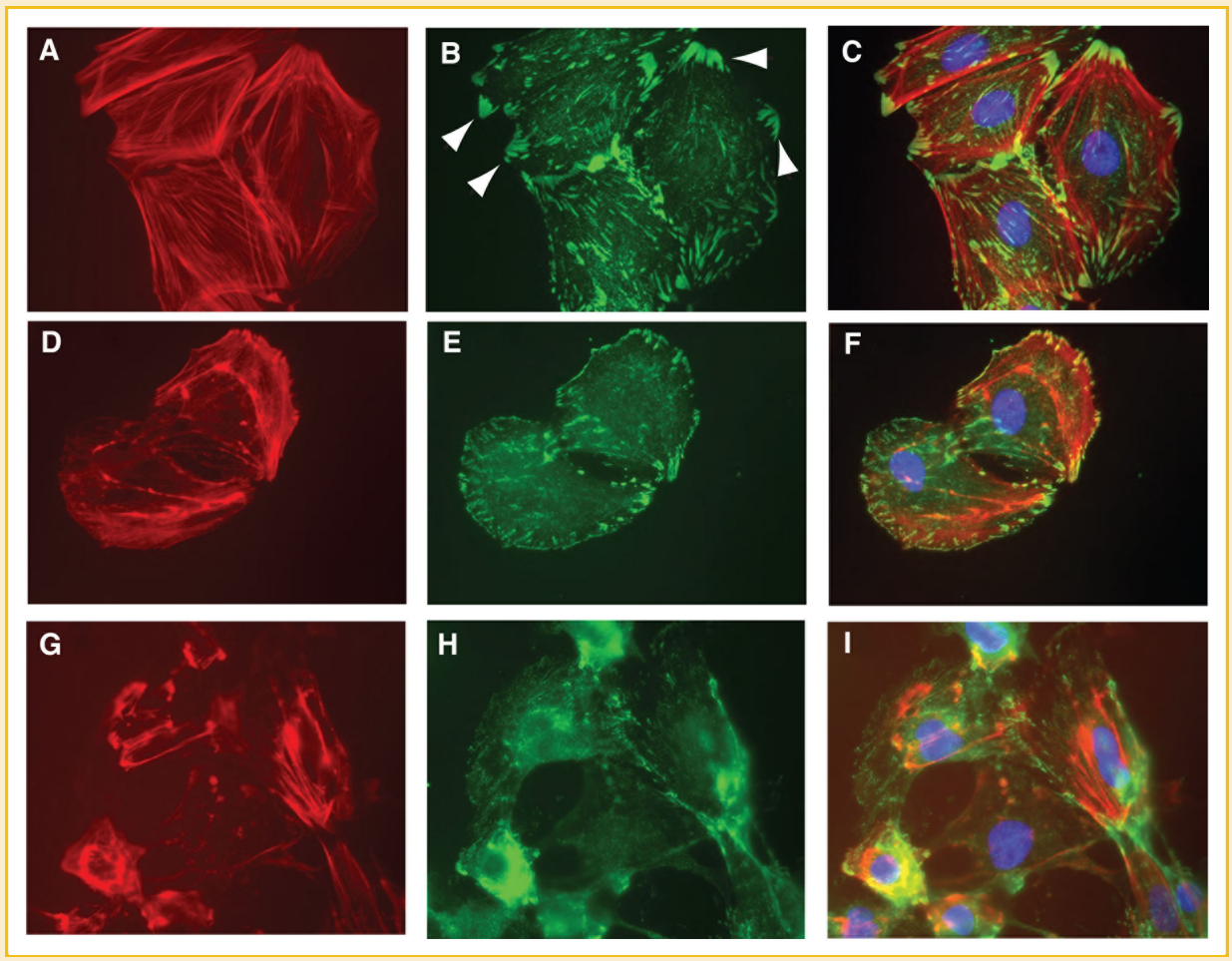

Fig. 1. Cadmium-dependent loss of vinculin from focal contacts. Subconfluent cultures of rat mesangial cells were incubated in serum-free medium (top row, panels A–C) or treated with $CdCl_2$ at either 10 μM (middle row, panels D–F) or 40 μM (bottom row, panels G–I) for 6 h. Cells were stained with rhodamine-conjugated phalloidin (red) to visualize F-actin (panels A, D, G) or FITC-conjugated anti-vinculin antibody (green, panels B, E, H). Localization of vinculin to focal contacts in the untreated cells is indicated by the arrowheads (panel B). Red and green channel overlays (panels C, F, I) show localization of the vinculin-rich contacts at the ends of F-actin filaments (panel C), lost upon Cd treatment (panels D, I). Nuclei are stained blue with DAPI in the overlays. All micrographs are 400× magnification.

but does induce a rapid (by 30 s) and sustained (up to at least 6 h) phosphorylation of Tyr925 (Fig. 6). KN93 is without effect on phosphorylation at either site (Fig. 6), indicating that the effect of CaMK-II on preservation of the FAK content of the focal adhesion is not due to a direct effect on FAK as a substrate for a CaMK-II-dependent kinase cascade. However, KN93 does protect against loss of phosphorylated Tyr925 from focal adhesions (Fig. 7), indicating further that CaMK-II, while influencing cytoskeletal assembly, does not act on focal adhesions through FAK phosphorylation. Neither cytochalasin D nor jasplakinolide affects cellular levels of total FAK or FAK-phosphoTyr925 as demonstrated by Western blotting although, consistent with Figure 2, both cause loss of FAK-phosphoTyr925 from focal adhesions as seen by immunostaining (data not shown). However, Cd^{2+} significantly increases the association of FAK-phosphoTyr925 with a cell membrane fraction, with no change in total FAK (Fig. 8A). KN93 attenuates the Cd-dependent increase in membrane-associated FAK-phosphoTyr925 (Fig. 8B).

DISCUSSION

Cd has pleiotropic effects on cells, including cytoskeletal disruption, mutagenesis, and both inhibition and induction of apoptosis [Jin et al., 2003; Gunawardana et al., 2006; Xiao et al., 2009; Liu and Templeton, 2010]. Previously, we observed that Cd causes loss of localization of vinculin to focal contacts, an effect that was dependent on CaMK-II [Templeton and Liu, 2013]. In this study, we further investigated the mechanism of this effect. We present evidence that Cd disrupts focal contacts by disrupting the localization of the structural protein vinculin and the tyrosine kinase FAK (Figs. 1 and 4). This phenomenon appears to be dependent on actin dynamics, because disrupting F-actin using cytochalasin D and stabilizing it with jasplakinolide both resulted in disruption of focal contacts (Figs. 2 and 4). This is in agreement with previous data showing a change in focal contacts in the presence of cytochalasin B and mycalolide B, both agents that disrupt the F-actin cytoskeleton [Bongiorno-Borbone et al., 2002]. Since Cd

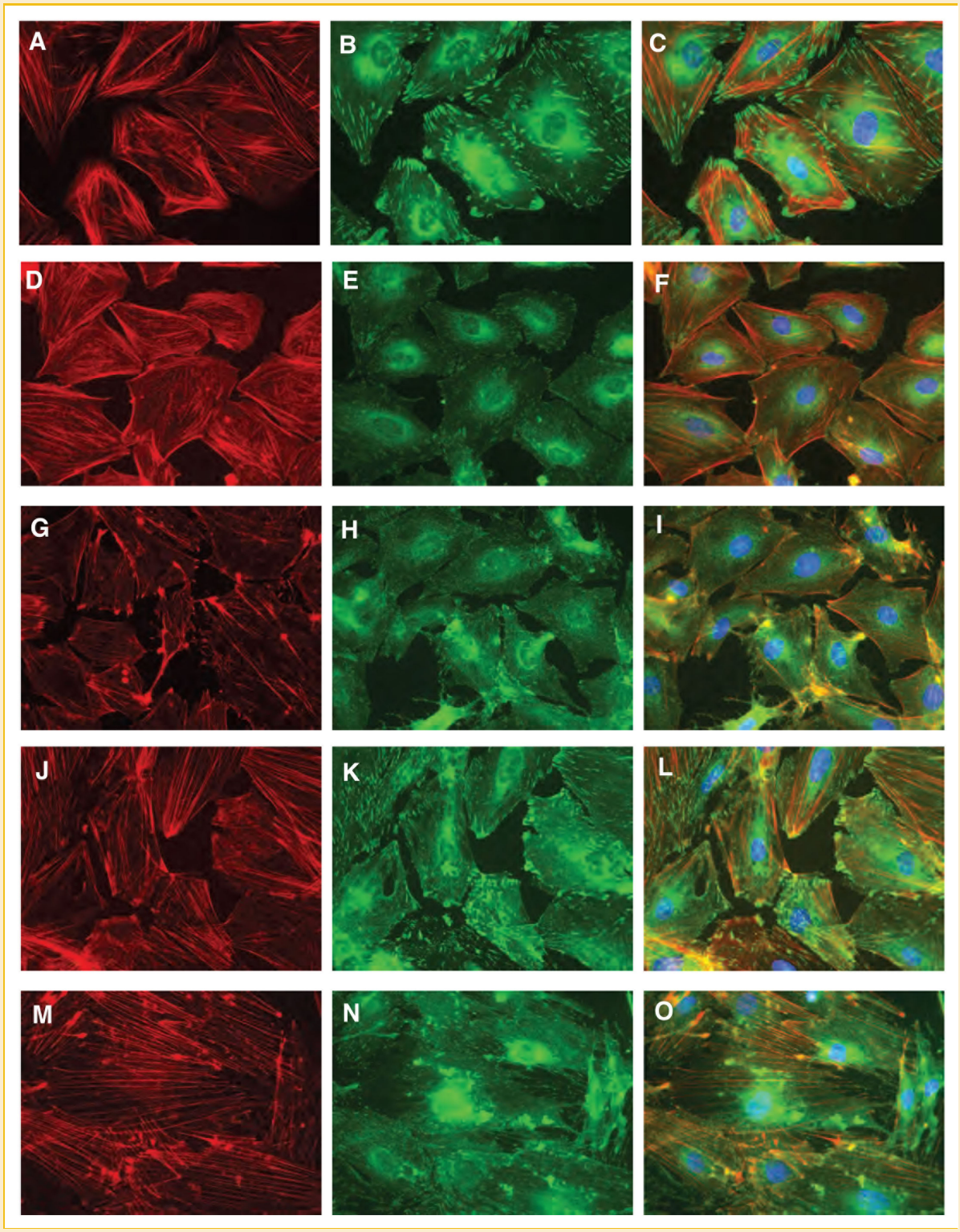

Fig. 2. Effect of cytoskeletal manipulations on vinculin localization to contacts. Cells were stained with rhodamine-phalloidin (staining F-actin red, left-hand column) and FITC-anti-vinculin antibody (green, middle column), as in Figure 1. Red-green overlays with blue nuclear DAPI staining are shown in the right-hand column. Untreated cells in serum-free medium (first row, panels A–C) show vinculin-enriched focal adhesions as in Figure 1B. Cells were then treated with the F-actin-disrupting agent cytochalasin D for 1 h at 200 nM (panels D–F) or 500 nM (panels G–I). Alternatively, the F-actin stabilizer jasplakinolide was used at 50 nM for 1 h (panels J–L) or 6 h (panels M–O). All micrographs are 200× magnification.

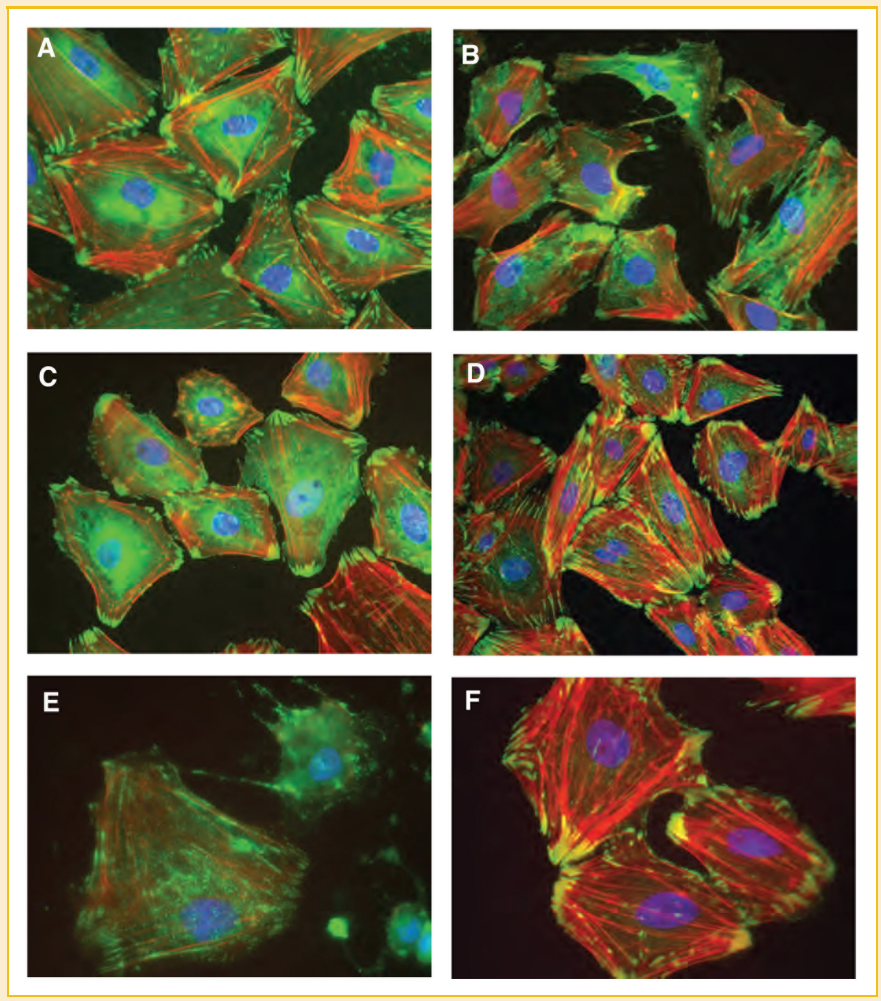

Fig. 3. Effect of calmodulin and CaMK-II antagonism on Cd-dependent focal contact disruption. Cells were stained with rhodamine-phalloidin (red), FITC-anti-vinculin antibody (green) and DAPI (blue) as in Figs. 1 and 2, and only the three-color overlays are shown. Panel A: control cells in serum-free medium. Panel B: Cells treated with 10 μM $CdCl_2$ for 6 h. Panel C: Cells treated with Cd as in panel B but with the calmodulin antagonist TFP (5 μM). Panel D: Cells treated with Cd as in panel B but with the CaMK-II inhibitor KN93 (10 μM). Panel E: Cells treated with 40 μM $CdCl_2$ for 6 h. Panel F: Cells treated with 40 μM $CdCl_2$ and 10 μM KN93. Panels A–D, ×200. Panels E, F, ×400.

also disrupts normal actin dynamics leading to a more G-actin-rich, depolymerized state [Wang and Templeton, 1996] this may be a potential mechanism for Cd-mediated disruption of focal adhesions.

Calcium/calmodulin dependent protein kinase-II has been implicated in mediating several toxic effects of Cd, including apoptosis [Liu and Templeton, 2007; Liu and Templeton, 2008] and cytoskeletal disruption in mesangial cells [Templeton and Liu, 2013]. Cd activates CaMK-II and increases the association of both CaMK-II and cleaved gelsolin with actin, thus promoting cytoskeletal disruption [Liu and Templeton, 2013]. CaMK-II is autophosphorylated upon binding of Ca^{2+}/calmodulin, generating a Ca^{2+}/calmodulin-independent species [Hudmon and Schulman, 2002; Chen et al., 2011]. Antagonism of calmodulin with TFP, or inhibition of CaMK-II with KN93, maintained cytoskeletal integrity and focal contacts upon Cd treatment (Figs. 2 and 4). Chen et al. [2011] have shown that CaMK-II inhibition prevented the decrease in cell spreading associated with Cd treatment in neuronal cells, an observation also in agreement with Szabo et al. [2007] who showed an increase in cell spreading and focal contacts in fibroblasts upon CaMK-II inhibition. As CaMK-II has been implicated in mediating cytoskeletal disruption in mesangial cells, inhibition by KN93 could abrogate this effect by returning actin dynamics to normal levels.

FAK has also been shown to regulate focal contacts and the actin cytoskeleton, as FAK-null cells have larger focal contacts that alter actin polymerization through a Rho kinase-dependent pathway [Westhoff et al., 2004; Fabry et al., 2011]. To study the mechanism of the effects of Cd and CaMK-II on focal adhesion integrity, we investigated the changes of vinculin and FAK levels and localization in mesangial cells. Previous work by Siu et al. [2009] showed a decrease in total FAK levels with Cd treatment associated with a disruption of the rat blood–testis barrier. However, in whole cell lysates, we found that both vinculin and FAK levels were unchanged with Cd treatment (data not shown). This discrepancy may be due to differences in tissue type and model systems between the studies. We

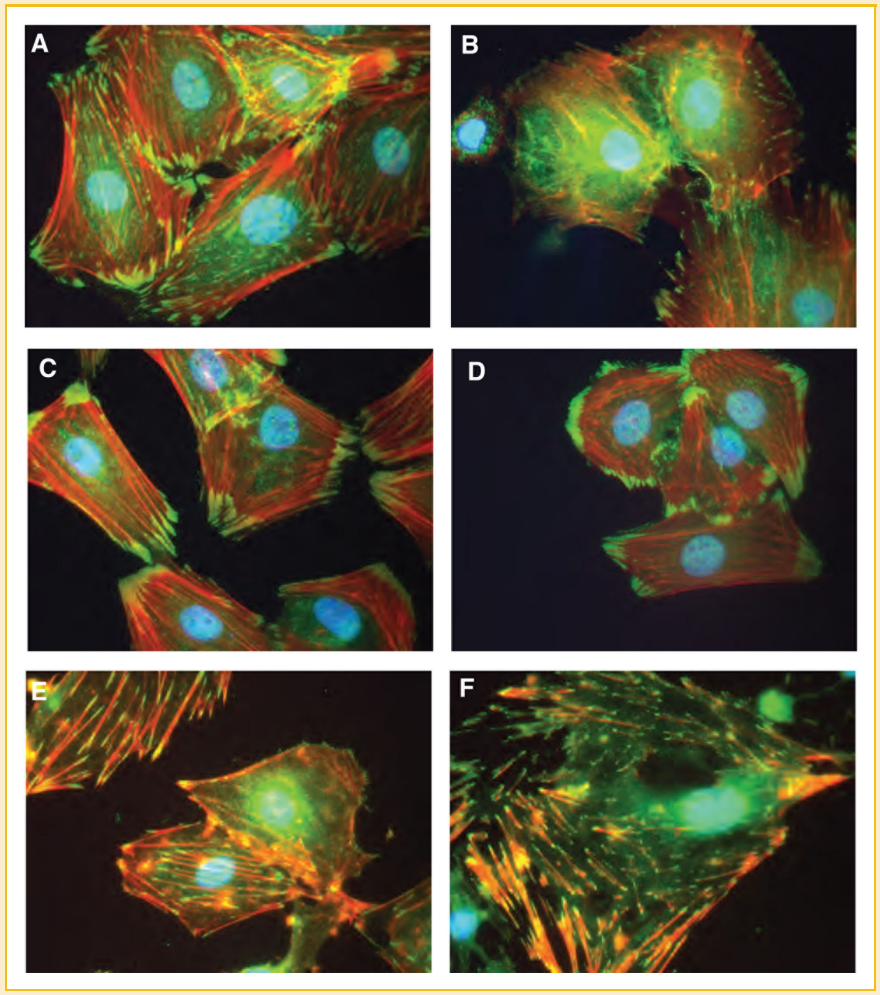

Fig. 4. Localization of focal adhesion kinase (FAK). Cells were stained with rhodamine-phalloidin (red), FITC-anti-FAK antibody (green), and DAPI (blue) and the three-color overlays are shown. Panel A: control cells in serum-free medium, with FAK showing a similar localization to vinculin in contacts at the termini of actin filaments, as in Figure 1. Panel B: After treatment with 10 μM $CdCl_2$ (6 h), less FAK is localized in contacts. Panel C: In the presence of 10 μM $CdCl_2$ and 10 μM KN93 localization of FAK to the contacts is preserved. Panel D: As in panel C but with 40 μM $CdCl_2$. Protection by KN93 persists even at 40 μM $CdCl_2$. However, as with vinculin, both cytochalasin D (500 nM, panel E) and jasplakinolide (50 nM, panel F) cause loss of FAK from the contacts. All micrographs are ×400 magnification.

then sought to determine how subcellular localization of vinculin and FAK are affected by Cd. Vinculin levels remained unchanged in the cytoskeletal fraction with Cd treatment. In contrast, Cd stimulates FAK translocation to the cytoskeleton in a dose-dependent manner, an effect that is dependent on CaMK-II (Fig. 5). This result apparently contradicts the current paradigm of focal adhesion formation, as previous studies have shown that focal contact formation results in FAK-actin association [Cezar-de-Mello et al., 2006]. However, Ohmori et al. [2000] have shown that in activated platelets, FAK is associated with the cytoskeleton upon activation in an aggregation-dependent manner, resulting in FAK cleavage and loss of kinase activity. Given that FAK cleavage was not detected in the present study (data not shown), this is unlikely to be an effect of Cd-mediated cytoskeletal translocation. Whether the cytoskeletal translocation of FAK stimulated by Cd in mesangial cells has a functional downstream effect or is an inconsequential effect associated with Cd-mediated cell death remains to be investigated.

Cd also increases the phosphorylation of several other kinases in mesangial cells, including p38, Erk1/2, Src, and CaMK-II [Liu and Templeton, 2008; Xiao et al., 2009]. FAK becomes autophosphorylated at Tyr397, resulting in recruitment of Src which subsequently phosphorylates other tyrosine residues on FAK [Westhoff et al., 2004; Wehrle-Haller, 2012]. Under basal conditions, FAK was constitutively autophosphorylated at Tyr397, and this remained unchanged with Cd treatment (Fig. 6). This result is in agreement with other studies that have shown constitutive phosphorylation of Tyr397 in several cell types [Katz et al., 2003; Hamadi et al., 2005]. In contrast, Cd causes a rapid increase in phosphorylation of Tyr925 beginning at 30 s and sustained over time, an effect that is independent of CaMK-II (Fig. 6). This is believed to occur by inhibition of phosphatases through oxidative

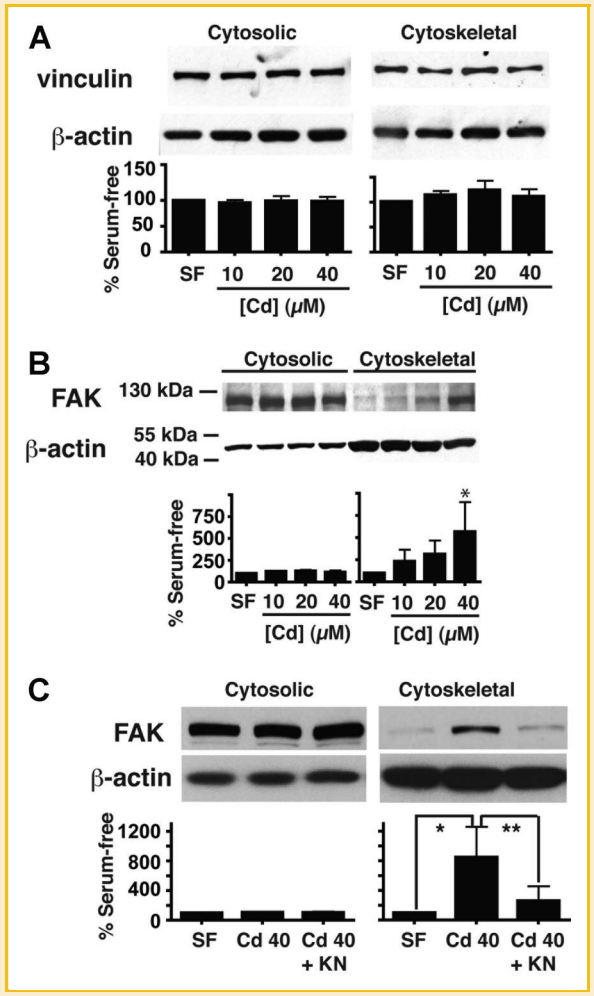

Fig. 5. Cytoskeletal localization of vinculin and FAK upon Cd treatment. The photographs show Western blots of vinculin (panel A) and FAK (panel B, C) of extracts from cytosolic (left side) and cytoskeletal (right side) fractions, as indicated. Immunostains of β-actin are included as protein loading controls. The histograms under each set of blots are the mean ± SD of densitometric scans of either the vinculin or FAK signals from several independent experiments, representative blots being shown. Signals are expressed relative to the fractions from serum-free (SF) control cells taken as 100%. Panel A: Cells were either held in serum free conditions or treated for 6 h with 10, 20, or 40 μM $CdCl_2$. Values are from n = 3 independent experiments and there are no significant differences. Panel B: Treatments were as in panel A and Western blotting of FAK is performed. The signals from n = 4 independent experiments are quantitated and * indicates a significant increase above SF control ($P < 0.05$). Panel C: Cells were treated with 40 μM $CdCl_2$ without (Cd 40) or with (Cd 40 + KN) KN93. Values from n = 5 independent experiments show an increase in cytoskeletal localization of FAK with Cd treatment ($^{*}P < 0.01$) that is abrogated by KN93 ($^{**}P < 0.05$).

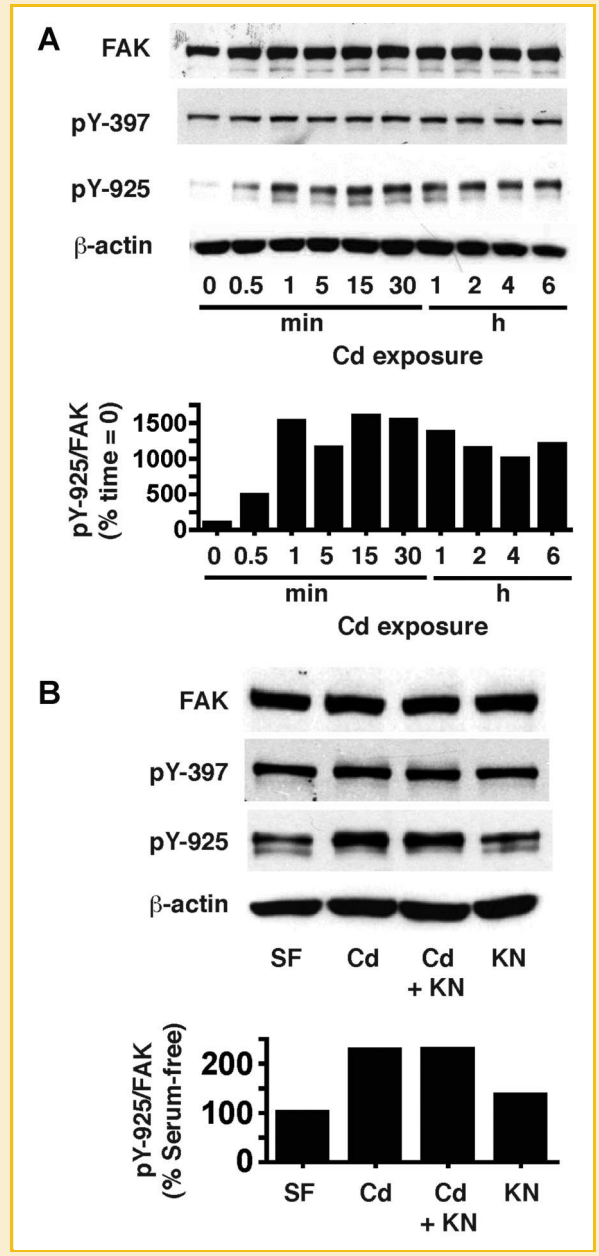

Fig. 6. Cadmium-dependent Tyr phosphorylation of FAK. Western blots are shown with antibodies to total FAK and phospho-specific antibodies to FAK phosphorylated at Tyr397 (pY-397) and Tyr925 (pY-925), with anti-β-actin as a protein loading control. Panel A: Signal as a function time of treatment with 10 μM $CdCl_2$. The blots are representative of two experiments and the histogram shows the results of densitometry of the ratio pY-925 FAK blot to total FAK, normalized to time 0 as 100%. Panel B: Lanes from the left show serum-free control cells not treated with Cd (SF), cells treated for 6 h with 10 μM $CdCl_2$ (Cd), and cells treated with 10 μM $CdCl_2$ in the presence of 10 μM KN93 (Cd + KN). The right-most lane shows cells treated with KN93 alone, to rule out possible increases in phosphorylation brought about by the inhibitor alone. The histogram shows the ratio of pY-925 FAK to total FAK as in Panel A, normalized to the SF control taken as 100%.

stress [Chen et al., 2008], although the rapid rise in phosphorylation likely involves activation of another signaling cascade as Cd would seem to be insufficient to induce oxidative stress so rapidly. Interestingly, Tyr925 is usually unphosphorylated in vivo, as mutating Tyr925 had no effect on the overall tyrosine phosphorylation status of FAK [Katz et al., 2003]. Additionally, phosphorylation of FAK at Tyr925 leads to increased focal adhesion turnover

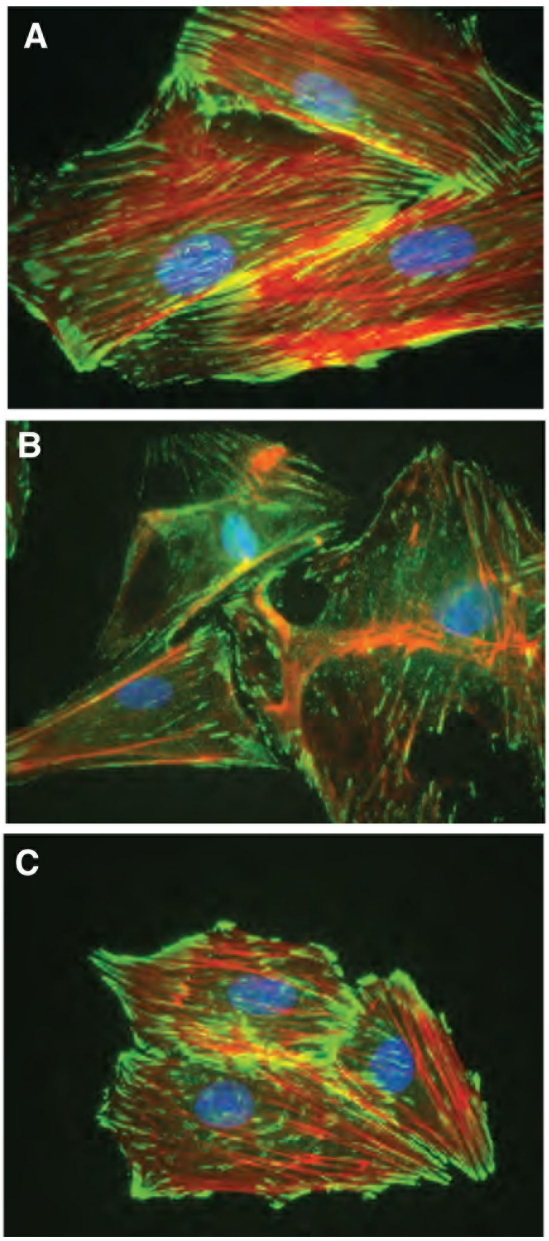

Fig. 7. Effect of Cd-treatment on localization of FAK-phosphoTyr925. Overlay immunofluorescent staining rhodamine phalloidin (red), FITC-anti-FAK-phosphoTyr925 (green), and DAPI (blue) are shown (×400 magnification). Panel A: Control cells in serum-free medium. Panel B: Cells treated for 6 h with 10 μM $CdCl_2$, showing significant loss of F-actin and less prominent localization of FAK-phosphoTyr925 to focal contacts. Panel C: Cells treated for 6 h with 10 μM $CdCl_2$ in the presence of 10 μM KN93, showing preservation of F-actin and FAK-phosphoTyr925 in focal contacts.

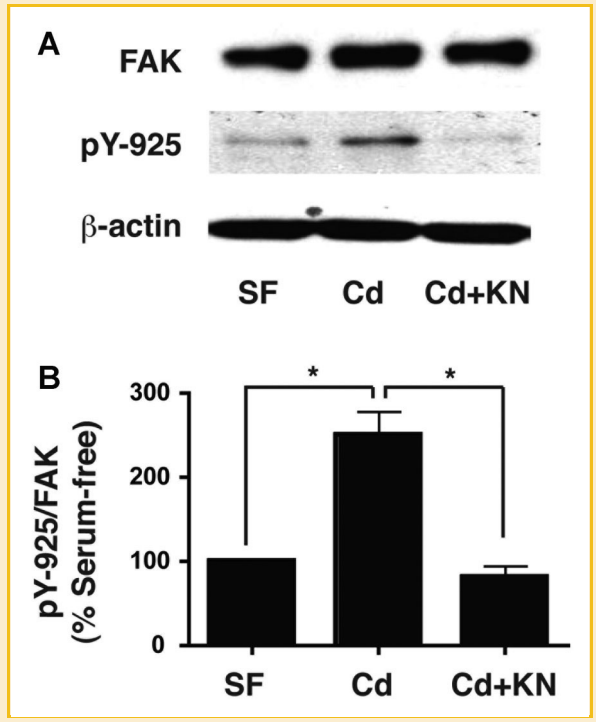

Fig. 8. Cell membrane localization of FAK-phosphoTyr925 in Cd-treated cells. Cell membrane fractions were prepared as described in Materials and Methods Section. Western blots of the membrane fraction were performed with antibodies to FAK and FAK-phosphoTyr925, as well as β-actin as a protein loading control. Cells were held under serum-free condition (SF), or treated with 10 μM $CdCl_2$ in the absence (Cd) or presence (Cd + KN) of 10 μM KN93 for 6 h. Representative blots are depicted (A) and the histogram (B) shows the results of the ratio of FAK-phosphoTyr925 to total FAK from densitometric scans (mean ± SD, n = 3) from independent experiments, normalized to the SF control taken as 100%. Significant differences determined by ANOVA and Tukey's post hoc test are indicated by * at $P < 0.001$.

[Deramaudt et al., 2011]. Therefore, it is likely that the loss of the balance between phosphorylated and unphosphorylated FAK in mesangial cells treated with Cd contributes to the observed focal adhesion disruption.

FAK is found primarily in the cytosol. However, it must translocate to the plasma membrane to mediate focal contact assembly and disassembly in response to changes in extracellular signaling [Bongiorno-Borbone et al., 2002]. Phosphorylation of FAK at Tyr925 has been shown to be enhanced in a membrane-targeted FAK fusion protein which is primarily excluded from focal adhesions [Katz et al., 2003]. In agreement with this, we found that Cd increases the presence of FAK-phosphoTyr925 in the membrane fraction, while not affecting total FAK levels (Fig. 8). Interestingly, FAK-phosphoTyr925 in the membrane fraction was decreased by inhibition of CaMK-II, although CaMK-II inhibition has no effect on total FAK phosphorylation. This may be an effect of a CaMK-II-dependent influence on actin dynamics by Cd that facilitates release of FAK phosphorylated at Tyr295 from focal adhesions, resulting in its localization to other membrane domains.

Other toxic metals, such as arsenic and lead, have also been shown to cause changes in focal contacts. The toxic mechanisms of arsenic are somewhat similar to those of Cd, in that both poison thiol groups and impose an oxidative stress on cells. However, whereas sodium arsenite caused decreased cell migration with focal adhesion and cytoskeletal disruption in myoblasts, it actually decreased FAK tyrosine phosphorylation [Yancy et al., 2005]. Similarly, lead caused

a decrease in tyrosine phosphorylation of FAK, while total FAK levels were unchanged [Giuliani et al., 2005]. This apparent difference between the effects on FAK phosphorylation of Cd versus arsenic and lead may occur due to an effect of Cd^{2+} on Ca^{2+} signaling. The ionic radius of Cd^{2+} is similar to that of Ca^{2+}, and activation of CaMK-II by Cd^{2+} is calmodulin-dependent, as demonstrated by the effects of TFP. Furthermore, a local rise in intracellular Ca^{2+} has been implicated in increased focal adhesion disassembly [Giannone et al., 2004]. Shinohara et al. [2001] have shown that calmodulin, but not CaMK-II is required for tyrosine phosphorylation of FAK. As our study did not investigate the effects of TFP on tyrosine phosphorylation, it is possible calmodulin activation is responsible for increased FAK tyrosine phosphorylation by Cd, independent of CaMK-II.

In conclusion, this study has shown for the first time that disruption of the integrity of focal adhesions is a potential target for Cd toxicity in mesangial cells. Cd causes a loss of vinculin and FAK from focal adhesions. Actin dynamics are important in maintaining focal adhesion integrity, as both disruption and stabilization of actin filaments disrupt the adhesions, and Cd appears to act in part by favoring actin depolymerization by a mechanism dependent upon CaMK-II. Cd also increases the translocation of FAK to the cytoskeletal fraction and increases the association of FAK-phosphoTyr925 associated with a membrane fraction, and both these effects are dependent upon activation of CaMK-II. CaMK-II-dependent disruption of the actin cytoskeleton may allow diffusion of FAK out of focal adhesions, and this may be facilitated by a Cd-dependent phosphorylation of FAK at Tyr925 that results in sequestration of the phospho-FAK in another membrane domain.

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