Involvement of Calcineurin in Transforming Growth Factor-β-mediated Regulation of Extracellular Matrix Accumulation*

Received for publication, August 7, 2003, and in revised form, January 23, 2004
Published, JBC Papers in Press, January 23, 2004, DOI 10.1074/jbc.M308759200

Jennifer L. Gooch‡§, Yves Gorin†, Bin-Xian Zhang§, and Hanna E. Abboud‡§

From the ‡Department of Medicine, Division of Nephrology, University of Texas Health Science Center, San Antonio, Texas 78284 and the §South Texas Veterans Administration, Audie Murphy Memorial Hospital, San Antonio, Texas 78284

Calcineurin is a calcium-dependent, serine/threonine phosphatase that functions as a signaling intermediate. In this study, we investigated the role of calcineurin in transforming growth factor-β (TGF-β)-mediated cellular effects and examined the signaling pathway involved in activation of calcineurin. Calcineurin is activated by TGF-β in a time- and dose-dependent manner. Consistent with increased phosphatase activity, the calcineurin substrate, NFATc1, is dephosphorylated and transported to the nucleus. Inhibition of calcineurin prior to the addition of TGF-β revealed that calcineurin is required for TGF-β-mediated accumulation of extracellular matrix (ECM) proteins but not cell hypertrophy. Conversely, overexpression of constitutively active calcineurin was sufficient to induce ECM protein expression. The mechanism of calcineurin activation by TGF-β was found to be induction of a low, sustained increase of intracellular calcium. Chelation of extracellular calcium blocked both TGF-β-mediated calcium influx and calcineurin activity. Finally, calcium entry was found to be dependent upon generation of reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide. Accordingly, inhibition of ROS generation also blocked TGF-β-mediated calcineurin phosphatase activity and decreased ECM accumulation. In conclusion, this study describes a new pathway for TGF-β-mediated regulation of ECM via generation of ROS, calcium influx, and activation of calcineurin.

TGF-β is a pleiotropic cytokine that functions in multiple cellular processes. Although many of the effects of TGF-β may be attributed to signaling via the Smad pathway, recent findings indicate that cell-specific action of TGF-β may be due to activation of other signaling pathways that are parallel to Smads or perhaps participate in cross-talk with Smad pathways. As such, there remains a great deal to be learned about mediators of TGF-β action. Specifically, Smads 2, 3, and 4 have been shown to play vital roles in TGF-β-mediated proliferation, apoptosis, and differentiation (review in Ref. 1). However, the signaling mechanisms of TGF-β-mediated regulation of hypertrophy and accumulation of ECM proteins are unclear. For example, fibroblasts from Smad3 knockout mice (2) and cells that lack Smad1 (3) still produce fibronectin in response to TGF-β. Thus it is reasonable to conclude that there are multiple pathways that participate in TGF-β regulation of ECM accumulation, possibly contributing to the tissue-specific effects of TGF-β.

One potential signaling mechanism that may be critical for TGF-β action is activation of calcium-dependent molecules such as calcineurin. Calcineurin has been shown to be required for activation of transcription and induction of cell hypertrophy in a number of cell types including cardiac myocytes (4, 5), smooth muscle cells (6), and mesangial cells (7). In addition, calcineurin is implicated in the regulation of collagen transcription factor-1 (8). Moreover, inhibition of TGF-β blocks increased ECM protein accumulation in the kidney caused by calcineurin inhibitors such as cyclosporin A (CsA) and FK506 (9). Because two important functions of TGF-β are induction of cell hypertrophy and regulation of ECM accumulation, these findings support a potential role for calcineurin in TGF-β signal transduction. However, a direct role for calcineurin in TGF-β action has not been demonstrated.

Calcineurin is a calcium-dependent signaling protein, but there is not yet a thorough understanding of how calcium contributes to TGF-β signaling and action or the mechanism of calcium modulation. TGF-β has been reported both to increase intracellular calcium (10, 11) as well as to inhibit calcium mobilization stimulated by other growth factors (12). Similarly, TGF-β has been shown to induce generation of ROS such as superoxide anion and hydrogen peroxide in a variety of cell types (13–19), and ROS generation is linked to TGF-β induction of interleukin-6 gene expression in a signaling pathway that also requires mobilization of calcium (20). However, in some cells, TGF-β is shown to have no effect (21) or even to decrease ROS generation (22). Interestingly, generation of ROS has been linked to increased fibronectin expression and collagen mRNA accumulation in a TGF-β-dependent manner (23, 24), suggesting that regulation of ECM may involve ROS generation upstream and/or downstream of TGF-β action.

In this study, we investigated the role of calcineurin in TGF-β effects in cultured renal cells. Upstream of calcineurin activation, we examined the effect of TGF-β on calcium mobilization and investigated the contribution of generation of ROS to TGF-β-mediated changes in calcium and activation of calcineurin. Finally, we placed generation of ROS, modulation of calcium, and activation of calcineurin together in a pathway that functions to regulate ECM proteins in renal cells.

---

* This work was supported by funds from the South Texas Veterans Health Care Administration Research Enhancement Award Program (to J. L. G., Y. G., and H. E. A.), the American Diabetes Association (to J. L. G.), the American Heart Association, National (to Y. G.), the National Institutes of Health (to H. E. A.), and the George O’Brien Kidney Research Center (to J. L. G. and H. E. A.) and by United States Public Health Service Award DK 43988. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Renal Division, UTHSCSA, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900. E-mail: Gooch@uthscsa.edu.

‡ The abbreviations used are: TGF, transforming growth factor; ECM, extracellular matrix; ROS, reactive oxygen species; CsA, cyclosporin A; NAC, N-acetylcysteine; DPI, diphenyleneiodonium; SFM, serum-free medium; AM, acetoxymethyl ester; IGF-I, insulin-like growth factor I; IP₃, inositol 1,4,5-triphosphate; IP₃R, IP₃ receptor.

This paper is available on line at http://www.jbc.org
**EXPERIMENTAL PROCEDURES**

**Materials**

Recombinant TGF-β was purchased from R & D Systems, Inc. (Minneapolis, MN). Cyclosporin A, anti-fibronectin antibody, thapsigargin, nifedipine, N-acetylcysteine (NAC), diphenyleneiodonium (DPI), and ferricytochrome c were from Sigma; anti-collagen type IV antibody was purchased from Chemicon (Temecula, CA); anti-NFATc1 and total Erk1/Erk2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); and lanthanum chloride was obtained from Aldrich. wtCnA and CA-CnA constructs were a gift from P. Camacho (University of Texas Health Science Center, San Antonio, TX).

**Cell Culture**

Rat mesangial cells were cultured from glomeruli isolated by differential sieving as previously described (25). For these experiments, rat mesangial cells that have been maintained in our laboratory (University of Texas Health Science Center, San Antonio, TX) were used. Normal renal fibroblasts and LLCKP-1 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in our laboratory in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. The cells were transiently transfected by the addition of 5 μg of DNA premixed with LipofectAMINE™/10-cm cell culture plate according to the manufacturer’s instructions (Invitrogen).

**Western Blots**

Mesangial cells were plated in 60-mm dishes and allowed to grow to 80–90% confluence. The medium was changed to SFM for 24 h, and the cells were treated as indicated. The cells were then harvested with trypsin-EDTA, pelleted, and washed with 1× phosphate-buffered saline. The protein was extracted using TNESV lysis buffer (50 mM Tris-HCl pH 7.4, 2 mM EDTA, 1% Nonidet P-40, 100 mM NaCl, 100 mM sodium orthovanadate, 100 μg/ml leupeptin, 20 μg/ml aprotinin, and 10–7 M phenylmethylsulfonyl), and 25 μg of protein was analyzed by 7.5% SDS-PAGE. Following transfer of the proteins to nitrocellulose, the membrane was incubated in 5% milk-TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and then immunoblotted with appropriate dilutions of primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies, and the proteins were visualized by enhanced chemiluminescence (Pierce).

**Immunofluorescence**

Mesangial cells were plated in 8-well chamber slides and allowed to grow to 80–90% confluence. The medium was replaced with SFM for 24 h, and the cells were treated as indicated. Following treatment, the cells were fixed and permeabilized in 100% methanol for 5 min and then rehydrated in phosphate-buffered saline with 0.1% bovine serum albumin for 15 min. The fixed cells were then blocked with donkey IgG for 15 min prior to the addition of anti-NFATc1 (15 μg/ml), an antibody that was generated against NFATc1 but has broad cross-reactivity with all four calcium-regulated NFATc proteins, for 30 min. The cells were then washed three times for 5 min with phosphate-buffered saline with 0.1% bovine serum albumin before the addition of Texas Red-conjugated anti-rabbit secondary antibody (1:100). Finally, the cells were washed three times for 5 min with phosphate-buffered saline with 0.1% bovine serum albumin, the chambers were removed from the slides, and the coverslips were mounted with Crystal Mount (Biomeda, Foster City, CA) for viewing by fluorescent microscopy.

**Calcineurin Assay**

Calcineurin activity was determined following the protocol published by Fruman et al. (26) and previously described in mesangial cells (7). Mesangial cells were grown to 80–90% confluence in 6-well plates, and then, three medium was replaced with SFM for 24 h. The cells were treated as indicated in triplicate and then collected for determination of calcineurin activity.

**ROS Measurements**

Detection of Intracellular H$_2$O$_2$—Mesangial cells were grown to near confluence in coverglass chambers and were made quiescent by serum deprivation for 48 h prior to treatment. The peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) was used to assess the generation of intracellular H$_2$O$_2$ as described previously (27). H$_2$O$_2$ production was also determined by Amplex Red Assay (Molecular Probes) according to the instructions provided by the manufacturer.

Measurement of Superoxide Anion Production in Intact Cells—Approximately 2 × 10$^6$ mesangial cells/well were grown in six-well plates until 80–90% confluent. The cells were serum-starved for 24 h and then treated as indicated. The measurement of superoxide anion (O$_2^-$) released into the medium by mesangial cells was performed by detection of ferricytochrome c reduction as described (28).

**Measurement of Intracellular Calcium**

Changes of [Ca$^{2+}$], in mesangial cells were determined in cell suspensions using the fluorescent Ca$^{2+}$ indicator fura-2/AM as previously described (29). Briefly, mesangial cells grown to 80–90% confluence were harvested by trypsin-EDTA and loaded in PSS buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 10 mM glucose, 5 mM HEPES, pH 7.4) with 1 μM fura-2/AM (Molecular Probes) in the dark at 37°C with gentle agitation for 30 min. Loaded cells were washed and resuspended in fresh PSS solution supplemented with high calcium (1.2 mM). The [Ca$^{2+}$], signals were measured in suspension of fura-2/AM-loaded cells at a density of 2.5–5 × 10$^5$ cells/ml at 37°C. The excitation ratio of 340/380 was analyzed with a Delta Scan spectrofluorometer (Photon Technology International Inc., South Brunswick, NJ) using 340- and 380-nm wavelengths for excitation and 510 nm for emission. Changes in [Ca$^{2+}$], were measured and indexed by the alterations in the fluorescence ratio of 340/380.

**RESULTS**

Calcineurin Is Activated by TGF-β and Is Required for ECM Accumulation—Calcineurin is a calcium-dependent, serine/threonine phosphatase that may be involved in TGF-β effects in the kidney. Therefore, we examined calcineurin activity using a previously described in vitro phosphatase assay (see “Experimental Procedures”) in response to TGF-β in cultured rat mesangial cells. Fig. 1 shows that treatment of cells with TGF-β increases calcineurin activity in a time- and dose-dependent manner. Maximum activity is seen between 2 and 5 min, with basal levels of activity restored by 20 min. Pretreatment of cells with CsA, a specific inhibitor of calcineurin, lowers the basal levels of activity and completely abolishes TGF-β-mediated activation of calcineurin. Increased activity was seen with as little as 0.1 ng/ml, and maximal stimulation was achieved with ~2 ng/ml. In addition to short term activation of phosphatase activity, increased expression of calcineurin was observed 24 and 48 h after the addition of TGF-β (Fig. 1C). The mechanism by which long term exposure to TGF-β increases calcineurin expression may be due to enhanced transcription or protein stabilization. Increased expression of calcineurin is dependent upon early induction of calcineurin activity because pretreatment with CsA blocked TGF-β-mediated increase of the protein (Fig. 1D).

NFATc proteins, which are basally phosphorylated and localized to the cytoplasm, are substrates of calcineurin (30). Following dephosphorylation by calcineurin, NFATc proteins translocate to the nucleus and interact with other transcription factors to alter gene transcription. Therefore, NFATc phosphorylation and cellular localization was determined following TGF-β treatment to demonstrate calcineurin activity in vivo. Consistent with early activation of calcineurin, 15 min following the addition of TGF-β, there is a marked increase in dephosphorylated calcineurin, evident as a faster migrating band on an SDS-PAGE gel (Fig. 1E). In addition, there is a marked increase in the nuclear localization of NFATc, which is completely blocked in cells pretreated with CsA (Fig. 1F). It is of interest that cyclosporin treatment also reduced the abundance of NFATc protein both in control and TGF-β-treated cells.

TGF-β is a potent mediator of cell hypertrophy (7, 31) and accumulation of extracellular matrix proteins (32, 33). Therefore, the role of calcineurin in TGF-β-mediated hypertrophy and ECM accumulation was determined in cultured mesangial cells. First, mesangial cell protein synthesis was determined by incorporation of $^3$H-leucine as previously described (34) as a marker of cell hypertrophy. Fig. 2A shows that TGF-β treat-
ment results in increased cell hypertrophy, which is unaffected by inhibition of calcineurin with CsA. As a control, cells were also treated with IGF-I, which has previously been shown to induce cell hypertrophy via calcineurin (5, 7, 35, 36). CsA blocks IGF-I-mediated hypertrophy, as expected, but had no effect on TGF-β-mediated cell hypertrophy. The combination of IGF-I and TGF-β produced an additive hypertrophic effect. Consistent with activation of independent pathways, CsA treatment only resulted in a small decrease in the combined effect of IGF-I plus TGF-β. Next, the role of calcineurin in TGF-β-mediated accumulation of ECM proteins was determined. Fig. 2B shows that the addition of TGF-β markedly increases expression of both fibronectin and collagen type IV. CsA alone has no effect on levels of fibronectin or collagen type IV, but pretreatment of cells with CsA is sufficient to reduce accumulation of ECM proteins. Finally, transient expression of a constitutively active calcineurin construct (CA-CnA), and to a lesser extent wild type calcineurin (wtCnA), induces fibronectin expression (Fig. 2C) compared with vector alone. Furthermore, pretreatment with CsA inhibited ECM expression resulting from overexpression of CA-CnA and wtCnA.

Calcineurin Is Activated by Extracellular Calcium—Calcineurin is a calcium-dependent phosphatase, but it is not clear what effect TGF-β has on intracellular calcium in mesangial cells. Calcium influx ([Ca\(^{2+}\)]\(_i\)) was measured in primary rat mesangial cells loaded with the fluorescent calcium indicator fura-2/AM as the ratio of excitation at 340- and 380-nm wavelengths. Fig. 3A shows that the addition of TGF-β at 2 and 10 ng/ml stimulates a slow, sustained increase in calcium that persists up to 10 min. Furthermore, TGF-β-mediated alterations in calcium are completely blocked by chelation of extracellular calcium with EGTA. Fig. 3B confirms that calcineurin

---

**Fig. 1. Calcineurin is activated in response to TGF-β.** A, mesangial cells were pretreated with SFM (control) or CsA (2.5 μM) for 10 min to inhibit calcineurin and then stimulated with 2 ng/ml TGF-β for various lengths of time up to 40 min. The cells were lysed, and calcineurin activity was determined by an in vitro phosphatase assay (see “Experimental Procedures”). The data shown are the means of triplicate assays ± S.E. and are representative of three independent experiments. *, p < 0.05; **, p < 0.01 compared with control. B, mesangial cells were treated with increasing doses of TGF-β for 2.5 min. The cells were lysed, and calcineurin activity was determined. Each point represents the mean of triplicate assays ± S.E. The data are representative of three independent experiments. C, mesangial cells were treated with TGF-β (2 ng/ml) for up to 48 h, and then whole cell lysates were collected. The expression of calcineurin was determined by direct immunoblotting. Erk1/2 expression was examined to verify equal loading. D, mesangial cells were pretreated with increasing concentrations of CsA (up to 3 μg/ml) and then stimulated with TGF-β (2 ng/ml) for 24 h. Whole cell lysates were collected, and the expression of calcineurin was examined by direct immunoblotting. Erk1/2 expression was also examined to verify equal loading. E, mesangial cells were pretreated with SFM (control) or CsA for 30 min prior to the addition of TGF-β (2 ng/ml) for 15 min. The cells were lysed, total protein was harvested, and NFATc was visualized by direct immunoblotting. Phosphorylated NFATc is the higher molecular weight band, and dephosphorylated NFATc is the more quickly migrating band. F, subcellular localization of NFATc was determined by immunofluorescence in mesangial cells treated as follows: SFM alone (panel i), TGF-β (2 ng/ml) (panel ii), CsA (2.5 μM) (panel iii), and CsA followed by TGF-β (panel iv). NFATc protein was visualized using a Cy3-conjugated secondary antibody. The data shown are representative of three independent experiments.
activation by TGF-β is dependent upon entry of extracellular calcium because EGTA also blocks calcineurin activity. As a control, the cells were treated with calcium ionophore, which increased TGF-β-mediated calcineurin activity as expected, and CsA, which abolished TGF-β-mediated activation of calcineurin. Finally, Fig. 3C demonstrates that TGF-β-mediated NFATc1 nuclear localization is also dependent upon influx of extracellular calcium. Chelation of calcium with the addition of EGTA blocked NFATc1 translocation following TGF-β treatment. As expected, the addition of ionophore alone was sufficient to induce nuclear translocation, and CsA caused cytoplasmic retention of NFATc1.

To further investigate the mechanism of TGF-β-mediated [Ca\(^{2+}\)], the cells were stimulated with ATP after 5 min of TGF-β treatment. ATP induces a rapid increase of [Ca\(^{2+}\)], indicating that intracellular calcium stores remain intact following TGF-β treatment (Fig. 4A). Next, the cells were pre-treated with thapsagargin for 10 min to deplete intracellular stores prior to the addition of TGF-β (Fig. 4B). TGF-β treatment stimulates increased [Ca\(^{2+}\)] even after depletion of intracellular stores, further indicating that TGF-β-mediated calcium influx is independent of intracellular calcium stores. Finally, the role of two possible calcium channels in TGF-β-mediated [Ca\(^{2+}\)] were investigated. First, the cells were pre-treated with nifedipine, a specific inhibitor of L-type calcium channels prior to the addition of TGF-β (Fig. 4C). Second, the cells were pretreated with an inhibitor of store-operated calcium channels, lanthanum chloride, before stimulation with TGF-β (Fig. 4D). TGF-β induces a consistent [Ca\(^{2+}\)] in the presence of both inhibitors, suggesting that neither L-type calcium channels or store-operated calcium channels are involved in TGF-β-mediated [Ca\(^{2+}\)].

TGF-β Increases Cellular Calcium via Generation of ROS—TGF-β has been shown to induce generation of ROS including...
superoxide anion (O$_2^-$) and H$_2$O$_2$, which may participate in TGF-β signal transduction as signaling second messengers (15, 16, 18–20). Furthermore, generation of ROS has been demonstrated to be a potential mechanism for alteration of intracellular calcium (20, 37) and may be involved in regulation of ECM protein accumulation (23, 24). Therefore, we examined generation of ROS and the role of ROS in TGF-β-mediated changes in calcium and calcineurin activation. First, production of O$_2^-$ was measured over time in mesangial cells treated with SFM (control) or TGF-β (2 ng/ml). Fig. 5A shows that O$_2^-$ is rapidly generated in response to TGF-β in a similar time frame as increased calcineurin activity. O$_2^-$ production by TGF-β was blocked by inhibition of flavoprotein-containing enzymes using the anti-oxidant DPI and by a scavenger of oxygen radicals, NAC (Fig. 5B). In contrast, inhibition of calcineurin with CsA had no effect on O$_2^-$ production, consistent with a role for O$_2^-$ upstream of calcium and calcineurin.

Next, TGF-β-mediated generation of H$_2$O$_2$ was examined. Mesangial cells were incubated with 10 μM 2′,7′-dichlorofluorescein diacetate prior to the addition of TGF-β. This compound is converted by intracellular esterases to the 2′,7′-dichlorofluorescin and then oxidized by H$_2$O$_2$ to the highly fluorescent 2′,7′-dichlorofluorescin. As a positive control, H$_2$O$_2$ alone was added to cells (Fig. 5C). Relative fluorescence was observed for up to 40 min and semi-quantitated to generate a time course of H$_2$O$_2$ production in response to TGF-β. Similar to generation of O$_2^-$, production of H$_2$O$_2$ is an early event following TGF-β stimulation (Fig. 5D). Further experiments to detect H$_2$O$_2$ activity demonstrated that TGF-β treatment results in levels of H$_2$O$_2$ ~7-fold over basal. Similar to results with O$_2^-$, H$_2$O$_2$ production is also blocked by DPI and NAC but not by CsA or EGTA (Fig. 5E). When compared with absorbance of a standard curve of H$_2$O$_2$ (Fig. 5E, inset), control cells contain ~3.3 nmol/10$^4$ cells, and TGF-β-treated cells contain 23.2 nmol/10$^4$ cells of H$_2$O$_2$.

To place generation of ROS in the TGF-β signaling cascade, the contribution of ROS generation to calcium mobilization and calcineurin activation was assessed. Fig. 6A shows that TGF-β-mediated alterations in calcium are inhibited by DPI and NAC to the same extent as calcium chelation with EGTA. Accordingly, calcineurin activation by TGF-β was also inhibited by pretreatment with DPI and NAC (Fig. 6B). Furthermore, TGF-β-mediated ECM accumulation is reduced by pretreatment of cells with DPI (Fig. 6C). Calcineurin activity and generation of ROS are very early signaling events, whereas accumulation of ECM proteins is a relatively long term outcome of TGF-β treatment. Therefore, the importance of early signaling events including generation of ROS and activation of calcineurin to the delayed accumulation of ECM proteins was investigated. Mesangial cells were treated with CsA to inhibit calcineurin and DPI to inhibit generation of ROS either 30 min before the addition of TGF-β, at the same time as addition of TGF-β, or 30 min after the addition of TGF-β. Fig. 6D shows that when inhibition of either calcineurin or ROS generation is delayed 30 min after the addition of TGF-β, the accumulation of ECM proteins is no longer inhibited.

Finally, the prevalence of TGF-β-mediated calcineurin activity via generation of ROS was measured in two other renal cell types: fibroblasts (Fig. 7A) and tubular epithelial cells (Fig. 7B). In both cell types, calcineurin activity is significantly increased in response to TGF-β and is blocked by inhibition of ROS generation.

**DISCUSSION**

In this study, we show for the first time that TGF-β directly activates calcineurin. Calcineurin is a calcium-dependent phos-
phatase; accordingly, we also show that TGF-β/H9252 increases influx of extracellular calcium, which is required for calcineurin activation. In addition, we show that changes in cytosolic calcium are downstream of generation of ROS, which is also a novel observation for TGF-β/H9252 signaling in renal cells. We confirmed the importance of early signaling events to regulation of ECM proteins as delay of inhibition of either calcineurin activity or ROS generation failed to reduce the accumulation of ECM. Together, these experiments delineate a new pathway for TGF-β action: generation of ROS, modulation of calcium entry, and activation of calcineurin (see schematic in Fig. 8). Furthermore, we demonstrate that this pathway is significant for TGF-β-mediated regulation of ECM protein accumulation in mesangial cells, one important function of TGF-β in many cell types. Decreased phosphorylation and nuclear translocation of NFATc demonstrate additional biological relevance of TGF-β-mediated calcineurin activation and suggest one possibility for a transcriptional mechanism of calcineurin-mediated regulation of ECM accumulation. Cyclosporin treatment not only reverses the nuclear translocation of NFATc but also induces a marked decrease in protein expression, suggesting that the phosphorylated state of NFATc protein influences its stability. The significance of cyclosporin-mediated alterations in the NFATc protein level and the mechanism of such regulation are areas that warrant further investigation.

There are several important conclusions to be drawn from this work. First, this study demonstrates roles for ROS in the modulation of calcium and in the activation of calcineurin in renal cells. Interestingly, we show that ROS act upstream of calcineurin activation, despite evidence that H$_2$O$_2$ can inhibit calcineurin activity in some cells (38, 39). One explanation for this is that the amount of H$_2$O$_2$ generated in response to TGF-β is several orders of magnitude lower than those used to demonstrate calcineurin inhibition by H$_2$O$_2$. In addition, H$_2$O$_2$ production in mesangial cells appears to be an early event, whereas in the study reported by Bogumil et al. (39) 30–40 min were required to see maximal calcineurin inhibition, and occurs in conjunction with activation of multiple signaling events as opposed to the addition of H$_2$O$_2$ alone. Nonetheless, it is interesting to speculate that the generation of ROS may contribute to TGF-β cell effects by multiple mechanisms. ROS may act quickly to modify cell membrane proteins such as calcium channels and may also serve to negatively regulate redox-sensitive intracellular enzymes. Indeed, Musci et al. (40) report that TGF-β activation of ROS-dependent gene transactivation is dependent upon the small G protein, Rac. Recent reports suggest that Rac may be a mechanism for intracellular signaling specificity because it can function to couple the NAD(P)H oxidase system to redox-sensitive intracellular targets (41). In addition, this study provides more evidence that generation of ROS can participate in the regulation of ECM proteins. Previous work demonstrated a link between ROS and ECM production in mesangial cells (23, 24). Our work provides one mechanism for this effect: modulation of calcium and activation of calcineurin. However, it is also possible that ROS contribute to the activation of additional signaling pathways parallel to calcium-dependent activation of calcineurin.

Next, these data add to our knowledge of TGF-β modulation
of intracellular calcium and identify one calcium-dependent signaling target: calcineurin. To date, regulation of calcium by TGF-β is still a complicated area because there is evidence that TGF-β both inhibits calcium mobilization induced by growth factors and increases intracellular calcium levels, likely dependent upon cell type. First, there is evidence that TGF-β may negatively regulate calcium signaling in the kidney. Sharma et al. (12) showed that pretreatment of mesangial cells with TGF-β inhibits epidermal growth factor- or platelet-derived growth factor-induced increases in intracellular calcium levels. It was postulated that TGF-β-mediated inhibition of calcium mobilization was due to regulation of the main calcium release channel on the endoplasmic reticulum, the type I IP₃ receptor (IP₃R). Further research showed that TGF-β induces phosphorylation of the IP₃R and down-regulates IP₃R protein levels over time (42, 43). It is not known whether the phosphorylation of calcium-binding protein levels are responsible for TGF-β-mediated inhibition of calcium transients induced by epidermal growth factor or platelet-derived growth factor in mesangial cells. In vivo, similar findings have supported a model for TGF-β-mediated inhibition of type I IP₃R-mediated calcium modulation. For example, Sharma et al. (43) reported that renal IP₃R expression is lower in diabetic animals compared with control animals. Interestingly, calcineurin has been shown to bind the type I IP₃ receptor and may function to inhibit its activity by dephosphorylation (44).

However, there is also evidence to suggest that TGF-β directly modulates intracellular calcium levels by stimulating entry of calcium through plasma membrane channels. Alevizopoulos et al. (8) showed that TGF-β stimulates calcium
Calcineurin is activated by TGF-β/H9252 in an ROS-dependent manner in tubule epithelial cells and renal fibroblasts. 

A. calcineurin activity was determined in LLCPK-1 renal tubule epithelial cells as described for Fig. 6B. The bars shown are the means of triplicate assays ± S.E. **, p < 0.01 compared with control.

B. calcineurin activity was determined in renal fibroblasts as described for Fig. 6B. The bars shown are the means of triplicate assays ± S.E. *, p < 0.05 compared with control.

Fig. 6. TGF-β-mediated calcium modulation and calcineurin activation require generation of ROS. A. mesangial cells were loaded with the fluorescent calcium indicator fura-2/AM and then pretreated with SFM, DPI (10 μM), NAC (20 mM), or EGTA for 10 min prior to the addition of TGF-β. [Ca²⁺], was measured by the ratio of excitation and emission at 335-nm/505-nm wavelengths, respectively. B. mesangial cells were pretreated with SFM, DPI (10 μM), NAC (20 mM), or CsA (2.5 μM) for 30 min and then stimulated with 2 ng/ml TGF-β for 2.5 min. The cells were lysed, and calcineurin activity was determined by an in vitro phosphatase assay (see "Experimental Procedures"). The bars are the means of triplicate assays ± S.E. The data are representative of three independent experiments. *, p < 0.05 compared with control.

Fig. 7. Calcineurin is activated by TGF-β in an ROS-dependent manner in tubule epithelial cells and renal fibroblasts. A. calcineurin activity was determined in LLCPK-1 renal tubule epithelial cells as described for Fig. 6B. The bars shown are the means of triplicate assays ± S.E. **, p < 0.01 compared with control. B. calcineurin activity was determined in renal fibroblasts as described for Fig. 6B. The bars shown are the means of triplicate assays ± S.E. *, p < 0.05 compared with control.
mobilization in NIH3T3 cells. In contrast to the effects of other growth factors, TGF-β-mediated changes first involve a slow, sustained increase in calcium over a period of 6–8 min followed by a large, transient spike consistent with release of intracellular calcium stores. McGowan et al. (11) also recently reported a slow, sustained increase in intracellular calcium following TGF-β treatment, although no spike of intracellular store release was observed up to 10 min following the addition of TGF-β. In this study, we show that TGF-β treatment results in a slow increase in calcium that is completely inhibited by chelation of extracellular calcium. There is no evidence of release of intracellular calcium stores up to 10 min following the addition of TGF-β. Interestingly, calcineurin is reported to be activated by low, sustained increases in intracellular calcium (45) consistent with both our observations and those of McGowan et al. (11) in mesangial cells. Neither L-type calcium channels nor store-operated calcium channels are implicated in TGF-β-mediated calcium modulation by our data. The work reported by McGowan et al. (11) point to the type III IP$_3$R as a mediator of extracellular calcium entry by TGF-β. Our data are consistent with this finding because the type III IP$_3$R receptor is insensitive to calcium feedback inhibition and mediates a sustained increase in intracellular calcium (46). Furthermore, the type III IP$_3$R can be localized within or adjacent to the plasma membrane in some cell types and could therefore be the source of extracellular calcium entry mediated by TGF-β (47).

Finally, we describe an additional role for calcineurin in mesangial cell function. The dose of TGF-β required for activation of calcineurin demonstrated in Fig. 1 is consistent with the amount of TGF-β reported to induce a variety of effects such as ECM accumulation, hypertrophy, and Smad activation. Our data suggest that calcineurin may function as a common signaling mediator in mesangial and possibly other renal cells. However, the effects of calcineurin are likely to be cell and/or tissue-specific. The data presented in this work are consistent with the previous observation that inhibition of calcineurin in a rat model of type I diabetes reduces glomerular ECM accumulation (48). Interestingly, in the same study CsA administration did not have the same effect in the tubulointerstitium. Indeed, it has been well established that the calcineurin inhibitor CsA increases ECM protein production as well as TGF-β expression in some renal cells in vitro and in vivo (9, 49–51). In fact, increased ECM associated with CsA can be inhibited by neutralization of TGF-β (9, 36), suggesting that one mechanism of CsA-mediated ECM production is via TGF-β. However, we show in Fig. 7 that both renal fibroblasts and tubule epithelial cells activate calcineurin in a ROS-dependent manner in response to TGF-β. This suggests that the pathway of ROS generation, calcium modulation, and calcineurin activation is intact in these cells, although the biological outcome of this signal may be different than that observed in mesangial cells.

In conclusion, our work places calcineurin in the TGF-β signaling pathway in renal cells, and we show that generation of ROS and calcium modulation are also important signaling second messengers. How calcineurin interacts with other pathways activated by TGF-β such as Smad proteins and the mitogen-activated protein kinase pathway remain to be determined. Similarly, mechanisms of TGF-β signaling specificity are important areas for further study, and much more work is needed to understand the consequences of calcineurin activation or inhibition in the kidney.

Acknowledgments—We acknowledge Jian-Hua Zhang and Rebecca Guler for technical assistance and Dr. Patricia Camacho for reagents and helpful discussion.

REFERENCES

1. Massague, J. (1998) Annu. Rev. Biochem. 67, 753–791
2. Pek, E. J., Ju, W. J., Heyer, J., Escalante-Alcaide, D., Stewart, C. L., Weinstein, M., Deng, C., Kucherlapati, R., Bottonier, E., P., and Roberts, A. B. (2001) J. Biol. Chem. 276, 19945–19953
3. Hocevar, B., Brown, T., and Howe, P. (1999) EMBO J. 18, 1345–1356
4. Palmer, S., Groves, N., Schindler, A., Yeeh, T., Biben, C., Wang, C.-C., Koentgen, F., Mohun, T., and Harvey, R. P. (2001) J. Cell Biol. 153, 985–997
5. Miyashita, T., Takahashi, Y., Takahashi, H., Kato, S., Kubota, I., and Tomoiike, H. (2001) Jpn. Circ. J. 65, 815–819
6. Stevenson, A. S., Gomez, M. F., Hill-Rubanks, D. C., and Nelson, M. T. (2001) J. Biol. Chem. 276, 15118–15124
7. Goesch, J. L., Tang, Y., Ricono, J. M., and Abboud, H. E. (2001) J. Biol. Chem. 276, 42492–42500
8. Alevizopoulos, A., Dussorde, Y., Rues, E., and Mermod, N. (1997) J. Biol. Chem. 272, 23597–23605
9. Islam, M., Burke, J. F., McGowan, T. A., Zhu, Y., Dun, S. R., McCue, P., Canadas, J., and Sharma, K. (2001) Kidney Int. 59, 498–506
10. Ishiyama, N., Shiba, H., Kanzaki, M., Shiozaki, S., Miyazaki, J.-I., Kobayashi, I., and Kojima, I. (1996) Mol. Cell Endocrinol. 117, 1–6
11. McGowan, T. A., Madesh, M., Zhu, Y., Wang, L., Ruso, M., Deelman, L., Henning, R., Joseph, S., Hajnoczky, G., and Sharma, K. (2002) Am. J. Physiol. 282, P910–P920
12. Sharma, K., McGowan, T. A., Wang, L., Madesh, M., Kaspar, V., Szalai, G., Thomas, A. P., and Hajnoczky, G. (2000) Am. J. Physiol. 278, F1022–F1029
13. Herrera, B., Alvarez, A. M., Sanchez, A., Fernandez, M., Roncero, C., Benito, M., and Fabregat, I. (2001) FASEB J. 15, 741–751
14. Cao, Q., Mak, R., and Lieber, C. (2002) Am. J. Physiol. 283, G1051–G1061
15. Thannickal, V. J., Hassoun, P. M., White, A. C., and Fanburg, B. L. (1993) Am. J. Physiol. 265, L622–L626
16. Ohba, M., Shibanuma, M., Kuroki, T., and Nose, K. (1994) J. Cell Biol. 126, 1079–1088
17. Chia, C., Maddock, D., Zhang, Q., Souza, K., Townsend, A., and Wan, Y. (2001) Int. J. Mol. Med. 8, 251–255
18. Thannickal, V. J., Aldweh, K. D., and Fanburg, B. L. (1998) J. Biol. Chem. 273, 23611–23615
19. Thannickal, V. and Fanburg, B. (1995) J. Biol. Chem. 270, 30334–30338
20. Junt, E., Lee, K. N., Ju, H. R., Han, S. H., Iml, J. Y., Kang, H. S., Lee, T. H., Bae, Y. S., Ha, K. S., Lee, Z. W., Bhee, S. G., and Choi, I. (2000) J. Immunol. 162, 2190–2197
21. Tudor, K., Hess, K., and Cook-Mills, J. (2001) Cytokine 15, 196–211
22. Delorme, N., Remond, C., Sartelet, H., Petitfrere, E., Clement, C., Schneider, C., Bellon, G., Virion, A., Haye, B., and Martiny, L. (2002) *J. Endocrinol.* 173, 345–355
23. Ha, H., and Lee, H. (2000) *Kidney Int.* 77, (suppl.) S19–S25
24. Iglesias-De La Cruz, M., Ruiz-Torres, P., Alcami, J., Diez-Marques, L., Ortega-Velazquez, R., Chen, S., Rodriguez-Puyol, M., Ziyadeh, F., and Rodriguez-Puyol, D. (2001) *Kidney Int.* 59, 87–96
25. Shultz, P., DeCorleto, P., Silver, B., and Abboud, H. (1988) *Am. J. Physiol.* 255, F674–F684
26. Fruman, D. A., Pai, S.-Y., Klee, C. N., Burakoff, S. J., and Bierer, B. E. (1996) *Methods Enzymol.* 25, 146–154
27. Gorin, Y., Kim, N.-H., Feliers, D., Bhandari, B., Ghosh-Choudhury, G., and Abboud, H. (2001) *FASEB J.* 15, 1909–1920
28. Johnston, R. J. (1984) *Methods Enzymol.* 105, 365–368
29. Zhang, B.-X., Ma, X., Yeh, C.-K., Lifschitz, M., Zhu, M., and Katz, M. (2002) *J. Biol. Chem.* 277, 12710–12715
30. Lewis, R. S. (2001) *Annu. Rev. Immunol.* 19, 497–521
31. Chui, M., Eung-Gook, K., and Ballerman, B. (1993) *Kidney Int.* 44, 948–958
32. Nakamura, T., Okuda, S., Rueslahti, E., and Border, W. A. (1992) *Kidney Int.* 41, 1213–1221
33. Ziyadeh, F. N., Sharma, K., Erickson, M., and Wolf, G. (1994) *J. Clin. Invest.* 93, 536–542
34. Jaime, F. A., Galceran, J. M., and Raji, L. (1998) *Kidney Int.* 54, 775–784
35. Musaro, A., McCallagh, K. J., Naya, F. J., Olsen, E. N., and Rosenthal, N. (1999) *Nature* 400, 581–585
36. Sharma, K., Yin, Y., Guo, J., and Ziyadeh, F. (1996) *Diabetes* 45, 522–530
37. Lee, Z., Kweon, S., Kim, B., Leem, S., Shin, I., Kim, J., and Ha, K. (1998) *J. Biol. Chem.* 273, 12710–12715
38. Reiter, T. A., and Busnaka, F. (2002) *J. Biol. Inorg. Chem.* 7, 823–834
39. Bogumil, R., Nangaladze, D., Schaaarschmidt, D., Schmachtel, T., Hellstern, S., Mutzel, R., and Ulirsch, V. (2000) *Eur. J. Biochem.* 267, 1407–1415
40. Mucci, I., Skorecki, K. L., and Goldberg, H. J. (1996) *J. Biol. Chem.* 271, 16567–16572
41. Nimmul, A. S., Taylor, L. J., and Bar-Sagi, D. (2003) *Nat. Cell Biol.* 5, 236–241
42. Sharma, K., Wang, L., Zhu, Y., Bokkala, S., and Joseph, S. (1997) *J. Biol. Chem.* 272, 14617–14623
43. Sharma, K., Wang, L., Zhu, Y., DeGuzman, A., Coa, G.-Y., Lynn, R. B., and Joseph, S. K. (1999) *Am. J. Physiol.* 276, F54–F61
44. Cameron, A. W., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V., and Snyder, S. H. (1995) *Cell 83*, 463–472
45. Dolmetsch, R., Lewis, R., Goodnow, C., and Healy, J. (1997) *Nature* 6627, 855–858
46. Hagar, R. E., Burgstahler, A. D., Nathanson, M. H., and Ehrlich, B. E. (1998) *Nature* 396, 81–84
47. Tanimura, A., Tojyo, Y., and Turner, R. J. (2000) *J. Biol. Chem.* 275, 27488–27493
48. Geoch, J. L., Barnes, J. L., Garcia, S., and Abboud, H. E. (2003)*Am. J. Physiol.* 284, F144–F154
49. Johnson, D. W., Saunders, H. J., Johnson, F. J., Huq, S. O., Field, M. J., and Pollock, C. A. (1999) *J. Pharm. Exp. Ther.* 289, 535–542
50. Wolf, G., Zahner, G., Ziyadeh, F. N., and Stahl, R. A. (1996) *Exp. Nephrol.* 5, 304–308
51. Wolf, G., Killen, P. D., and Neilson, E. G. (1990) *J. Am. Soc. Nephrol.* 6, 918–922
Involvement of Calcineurin in Transforming Growth Factor-β-mediated Regulation of Extracellular Matrix Accumulation
Jennifer L. Gooch, Yves Gorin, Bin-Xian Zhang and Hanna E. Abboud

*J. Biol. Chem.* 2004, 279:15561-15570.
doi: 10.1074/jbc.M308759200 originally published online January 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M308759200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 51 references, 17 of which can be accessed free at http://www.jbc.org/content/279/15/15561.full.html#ref-list-1