Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II Stimulates c-fos Transcription and DNA Synthesis by a Src-Based Mechanism in Glomerular Mesangial Cells

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Abstract. Mesangial cell growth factors elevate intracellular free [Ca\textsuperscript{2+}], but mechanisms linking [Ca\textsuperscript{2+}] to gene expression and DNA synthesis are unclear. This study investigated the hypothesis that Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMK II), which is activated by elevated [Ca\textsuperscript{2+}], increases c-fos transcription and DNA synthesis via a Src-based mechanism. In cultured rat mesangial cells, dominant negative Src (SrcK\textsuperscript{H11002}) blocked activation of the c-fos gene promoter by CaMK II 290, a constitutively active form of CaMK II\textalpha. Activation of the c-fos promoter by CaMK II 290 was also blocked by COOH-terminal Src kinase, which phosphorylates and inactivates c-Src. A pharmacologic CaMK inhibitor, KN-93, did not block activation of the c-fos promoter by ectopically expressed v-Src. Stimulation of c-Src by endothelin-1 required CaMK II activity, further supporting the notion that CaMK II acts upstream of Src in a signaling cassette. Activation of the c-fos promoter by CaMKII 290 and Src required the c-fos serum response element. Dominant negative SrcK\textsuperscript{H11001} also blocked induction of DNA synthesis in mesangial cells by CaMK II 290. Collectively, these results suggest that in mesangial cells Src protein tyrosine kinases act downstream of CaMKII in a signaling pathway in which [Ca\textsuperscript{2+}] induces the c-fos promoter and increases DNA synthesis. mss5@po.cwru.edu

Mesangial cells and the associated mesangial matrix are essential for normal structure and function of the glomerular capillaries (1–5). The mesangial phenotype is highly plastic, and control of mesangial cell growth is critical for normal development of the glomerular tuft in metanephrogenesis and in the response of adult kidneys to glomerular injury (6). In the adult kidney, mesangial cells are largely quiescent, with a renewal rate of less than 1% (2–4). Glomerular injury can alter the phenotype of mesangial cells, resulting in hypertrophy, hyperplasia, and/or expansion of the mesangial matrix. This injury-dependent phenotypic switch of mesangial cells contributes to glomerulosclerosis and is mediated in part by growth factors and inflammatory mediators that evoke immediate early gene expression and DNA synthesis. The signal transduction pathways that control mesangial cell growth are complex but seem to share as an early event a rapid and transient increase in intracellular free [Ca\textsuperscript{2+}] (7,8). A variety of experiments in cultured mesangial cells and in experimental models of glomerular injury with mesangial expansion demonstrate that inhibiting the rise in intracellular free [Ca\textsuperscript{2+}], attenuates mesangial cell proliferation (2,4,9–11).

The molecular mechanisms linking [Ca\textsuperscript{2+}], to gene expression and DNA synthesis in mesangial cells are unclear. In renal and nonrenal cells, recent evidence suggests a possible role in Ca\textsuperscript{2+} signaling for non-receptor protein-tyrosine kinases of the Src family. Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+}-permeable ion channels activates c-Src tyrosine kinase activity in epithelial cells (12,13), in neurons (14), and in mesangial cells (15). The ability of dominant negative c-Src to block c-fos immediate early gene induction and neurite outgrowth by Ca\textsuperscript{2+} influx in neurons suggests an important role for Src in cell signaling by Ca\textsuperscript{2+} (14). In mesangial cells, Ca\textsuperscript{2+} influx and subsequent activation of Src are necessary for c-fos immediate early gene induction by the endothelin-1 G protein-coupled receptor (15,16), which has been suggested to stimulate mesangial cell growth in glomerular injury (17,18). These observations suggest c-Src as a candidate in signal transduction pathways that link elevation of cytosolic free [Ca\textsuperscript{2+}], to c-fos induction in mesangial cells, but a functional role for c-Src in stimulating DNA synthesis by specific effectors of Ca\textsuperscript{2+} signaling has not been demonstrated.

One of the primary mechanisms by which intracellular [Ca\textsuperscript{2+}] regulates signal transduction is through activation of the Ca\textsuperscript{2+}/calmodulin-dependent protein kinases (CaMK) (19). In this study, we asked whether a CaMK II signaling pathway activates c-fos transcription and mesangial cell growth by a Src-based mechanism. We report here that dominant negative mutants of Src and the Src-inactivating kinase Csk block activation of the c-fos promoter by CaMKII. Dominant negative Src mutants also inhibit mesangial cell DNA synthesis stimulated by an active CaMKII 290 mutant. We conclude, therefore, that CaMKII signals through Src and suggest that...
this pathway regulates genes that control cell growth in the mesangium.

Materials and Methods

Cell Culture

Mesangial cell strains from male Sprague-Dawley rats were isolated and characterized as previously reported (20). Cells were maintained in RPMI 1640 medium supplemented with 17% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml each of insulin and transferrin, and 5 mg/ml of sodium selenite at 37°C in 5% CO2 incubator. HeLa cells, A7r5 vascular smooth muscle cells, and bovine pulmonary artery endothelial cells were obtained from the American Type Culture Collection and were cultured in DMEM supplemented with 10% fetal bovine serum, 24 mM HEPES (pH 7.4), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmids, Transient Transfections, and Reporter Gene Assays

The following plasmids were as described: p-356wt/fosLUC (16); point mutants (pm) of the c-fos promoter, pmxfosLUC (16,21); pSrcK−/K+ (22,23); pCMVCsk (24); pCMVCaMKIIα 290 (21); pMv-src (25). Cells were transfected using the calcium phosphate method as previously reported (16,26). Cells in 6-well plates (35 mm; 2 × 104 cells/well) were transfected with 0.5 μg of the p-356wt/fosLUC, 0.1 μg pRSVβGal internal control, and 2 μg of pCMV or pCMVCaMKII290. When indicated, 2 μg pSrcK+/K− or pCMVCsk was also added. Total DNA was adjusted to 6 μg of DNA/well with pUC19. Total promoter strength in control transfections was held equivalent by inclusion of expression plasmids lacking a cDNA insert. After incubation with precipitates for 16 to 18 h at 37°C, cells were then washed three times with DMEM and incubated with DMEM/0.5% FBS for an additional 24 h before cell lysis (RLB Buffer, Promega). Luciferase activity was assessed as described previously (26), and relative light units were measured in a Berthold Lumat Luminometer (Wallac, Gaithersburg, MD) for two 10-s intervals.

Transfection efficiency was determined using a pRSVβGal construct that directed β-galactosidase expression from the viral LTR. β-Galactosidase activity was measured using the Galacto-Light protocol as described by the manufacturer (Tropix, Bedford, MA). All reporter gene assays were in the linear range. In experiments where KN-93 (Calbiochem, San Diego, CA) was used to inhibit endogenous CaMK activity, KN-93 at 10 μM was added 8 h after DNA addition and was present throughout the remaining transfection period.

Western Blotting of Epitope-Tagged CaMKII290

To determine if SrcK− repressed expression of CaMKII290, mesangial cells were transiently transfected with pCMV CaMK II 290 (containing an HA epitope tag (21)) and with increasing amounts of the plasmid expressing dominant negative SrcK−. Forty-eight hours after transfection, cells in 100-mm dishes were washed once in DPBS and scraped into 1 ml of sample lysis buffer (0.5 M Tris-HCl [pH 6.8], 2.5 ml; 10% [wt/vol] SDS, 4.0 ml; glycerol, 2 ml; 0.1% bromphenol blue, 0.5 ml; β-mercaptoethanol, 0.5 ml; distilled water, to 10 ml). The lysate was vortexed, boiled for 5 min, and aliquots were resolved on 8 to 16% SDS-PAGE gradient gels and proteins transferred to 0.2-μm nitrocellulose filters. Transferred proteins were stained with Ponceau S, and the filter was blocked in blocking buffer (1.0% BSA, 10 mM Tris [pH 7.5], 100 mM NaCl, 0.1% Tween 20) with shaking overnight at 4°C. To detect the HA epitope-tagged CaMKII290 in transfected cells, the blots were incubated with a monoclonal anti-HA antibody (clone 12CA5; Boehringer Mannheim, Indianapolis, IN). After extensive washing, the appropriate peroxidase-labeled secondary antibodies in blocking buffer (1:10,000) were added, and the proteins were detected by chemiluminescence (ECL; Amersham, Arlington Heights, IL). Typical exposure times were 30 to 60 s. The same blot was reprobed with an antibody that recognizes rat β-actin (Sigma, mAb A5316) to confirm equal protein loading.

Inhibition of Endothelin-1-Activated c-Src by a CaMKII Inhibitor

Serum-starved cells (24 h; 0.5% FBS) in 60-mm plates were stimulated with 100 nM endothelin-1 (ET-1), and the cells were prepared for Western blotting as described above. To block CaMK II activity, cells were preincubated for 1 h with 20 μM of a myristoylated autocomamide-2-inhibitory peptide (myr-AIP), a nonphosphorylatable analog of autocomamide-2 that corresponds to the autophosphorylation site of CaMK II (Calbiochem). This protocol with myr-AIP has previously been shown to inhibit CaMK II activation in cultured cells (27,28). The membranes were probed with 1:2000 of an affinity-purified polyclonal phosphospecific antibody that recognizes the active form (P-Tyr 416) of c-Src exactly as described by the manufacturer (Cell Signaling Technology, Beverly, MA). To confirm amounts of total c-Src, the blot was reprobed with mAb clone 327 (Calbiochem) as described by Lipsich et al. (29).

Measurements of DNA Synthesis in Transiently Transfected Cells

Mesangial cells in 6-well plates were transfected as described above, except that the amount of RSVβGal was increased to 1 μg per well, and the fos luciferase reporter was omitted. Incorporation of 5-bromo-2-deoxyuridine (BrdU; Sigma, St. Louis, MO) into DNA in transiently transfected cells (i.e., β-galactosidase–positive cells) was measured exactly as described previously (30). Briefly, transfection cells were labeled for 24 h with 20 μM BrdU; Sigma, St. Louis, MO). Monolayers were then washed twice with ice-cold DPBS and fixed for 5 min on ice with 2.0% formaldehyde/0.2% glutaraldehyde in DPBS (Dulbecco phosphate-buffered saline). Cells expressing β-galactosidase were detected by histochemical analysis (30), and under these conditions 1 to 3% of mesangial cells were transfected (i.e., β-galactosidase–positive). BrdU incorporation into DNA was then identified by immunocytochemistry using 6 μg/ml IgG of a specific monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) as described (30). Cells were visualized under brightfield microscopy (Nikon Diophot) with a neutral filter. Red cells (i.e., expressing β-galactosidase-positive) with dark nuclei (BrdU-positive) and light nuclei (BrdU-negative) were counted, and percent inhibition of DNA synthesis was calculated as follows:

\[ \frac{(N_c - N_v)}{N_c} \]

where \( N_v = \% \) BrdU-positive nuclei in cells transfected with vector only and \( N_c = \% \) BrdU-positive nuclei in cells transfected with expression plasmid. Statistical significance was analyzed by the χ² test using InStat for Macintosh (GraphPad, SanDiego, CA).

Results

A Dominant Negative Src Mutant (SrcK−) Blocks Activation of the c-fos Promoter by CaMK II

Our first approach was to ask if dominant negative mutants of Src would interfere with activation of the c-fos promoter by CaMK II. Kinase-inactive forms of Src (SrcK−) function as dominant negative mutants and inhibit DNA synthesis stimulated
by colony stimulating factor or platelet-derived growth factor in fibroblasts (23,31) and block stimulation of the c-fos promoter by the G protein-coupled ET-1 receptor in mesangial cells (16). We therefore tested whether dominant negative SrcK− (Lys 295 to Met) would block stimulation of the c-fos promoter by a constitutively active mutant of CaMK II, CaMK II 290 (Figure 1A). Mesangial cells were transiently transfected with a plasmid containing a genomic DNA fragment of the c-fos promoter (−356 to +109) that drives transcription of a luciferase reporter gene (Figure 1A) (16). Transfection with a plasmid vector expressing CaMK II 290 stimulated a 3.9-fold increase in c-fos promoter activity (Figure 1B). Cotransfection with a vector expressing SrcK− completely blocked activation of the c-fos promoter by CaMK II 290 (Figure 1B). Inhibition by SrcK− was apparently specific as transfection with wild-type SrcK+ did not block CaMK II 290-stimulated c-fos promoter activity (Figure 1B). SrcK+ did not potentiate the effect of CaMK II 290. These results suggest that CaMK II acts upstream of Src in a signaling cascade that activates the c-fos promoter.

One potential interpretation of the inhibitory effects of SrcK− is that SrcK− might block expression of CaMKII290. Therefore, in mesangial cells transfected with CaMKII290 and SrcK−, we measured CaMKII290 levels by Western blotting using the HA epitope tag fused to the NH₂ terminus (Figure 1A). As shown in Figure 2, SrcK− did not inhibit CaMKII290 expression at any concentration used in these experiments (lane 2 versus lanes 3 and 4 with SrcK−, Figure 2). Coexpression of SrcK+ (lanes 5 and 6, Figure 2) did not significantly alter CaMKII 290 levels, which suggests that the Src expression vector was without any apparent effect on CaMKII 290 expression under control of the CMV promoter/enhancer. We conclude that SrcK− does not inhibit expression of CaMKII290 and that CaMKII does indeed act upstream of Src in the pathway to c-fos.

Figure 1. SrcK− dominant negative mutant blocks Ca²⁺/calmodulin-dependent protein kinase II (CaMK II) signaling to c-fos. (A) Schematic diagram of plasmids used in transient transfections. p-356wt/fos LUC is a reporter construct containing −356 to +109 of the mouse c-fos promoter driving transcription of a luciferase reporter gene. SIE, sis-inducible element; SRE, serum response element; Ca/CRE, Ca²⁺-cAMP response element. pCMV CaMK II 290 and pSrcK− are expression plasmids encoding a truncated, constitutively activated form of CaMK II and a dominant negative Src mutant, respectively. (B) Glomerular mesangial cells were co-transfected with p-356wt/fos LUC and either pCMV or pCMV CaMK II 290 as described below. As indicated in the legend, plasmids encoding SrcK− or wild-type Src K+ were also added. Forty-eight hours after DNA addition, the cells were lysed and processed for luciferase and β galactosidase expression. Data are mean ± SEM from four independent experiments in duplicate. ** P < 0.01 by t test.
COOH-Terminal Src Kinase Also Inhibits Activation of the c-fos Promoter by CaMK II

An independent approach to inactivate c-Src is to express Csk, which phosphorylates the COOH-terminal tyrosine (Y527) in c-Src and maintains the kinase in an inactive conformation (24,32). When a vector expressing Csk (Figure 3A) was transiently co-transfected into cells with the c-fos luciferase reporter, Csk inhibited activation of the c-fos promoter by CaMK II 290 (Figure 3B). As shown previously (16), Csk does not nonspecifically inhibit the c-fos promoter because Csk does not block activation of the c-fos promoter by fetal bovine serum or a constitutively active mutant of Raf-1 kinase (16). Also, Csk had no effect on c-fos luciferase activity in the absence of CaMK II 290, (i.e., pCMV; Figure 3B). Collectively, the results with SrcK− and Csk strongly suggest that CaMK II functions upstream of Src in a signaling pathway to the c-fos promoter.

Activation of Src by ET-1 Requires CaMK II

To determine whether CaMK II participates in Src activation in mesangial cells by physiologic ligands, we treated serum-starved cells with 100 nM ET-1 and measured Tyr-416 phospho-Src in the presence and absence of myr-AIP to block CaMK II activity. ET-1 increased phospho-Src levels at 10 min, and Src remained activated at 60 min (Figure 4A). Ca²⁺ ionophore A23187, which increases CaMK II activity, also increased phospho-Src levels but with a more rapid time course. Preincubation of cells with myr-AIP before addition of ET-1 greatly attenuated Src activation (Figure 4B); reprobing of the blot for total Src protein confirmed the presence of unactivated Src. These results suggest that CaMK II is upstream of Src and participates in a pathway of Src activation by ET-1.

CaMK II Acts Upstream of Src in Diverse Cell Types

We next asked if the ability of CaMK II to function upstream of Src was cell type–specific or was instead a common feature of diverse cell types. We conducted the same cotransfection experiments described above in Figure 1 with CaMK II 290 and SrcK− in A7r5 vascular smooth muscle cells, porcine aortic endothelial cells, and HeLa cells. SrcK−, but not SrcK+, blocked activation of the c-fos promoter by CaMK II 290 in A7r5 vascular smooth muscle and endothelial cells (Figure 5). SrcK− also blocked activation of the c-fos promoter by CaMK II 290 in HeLa cells, but SrcK+ potentiated activation by CaMK II 290 (Figure 5). It is not clear why SrcK+ potentiated stimulation by CaMK II 290 in HeLa cells and not in the other cell types we studied, but this probably reflects cell type-specific differences in cross-talk between...
CaMK II or Src. However, the key finding is that dominant negative SrcK/H11002 blocked activation of the c-fos promoter by CaMK II 290 in four distinct cell types and suggests that a CaMK II-Src signaling cassette is conserved.

A Pharmacologic Antagonist of CaMK Fails to Block Activation of the c-fos Promoter by v-Src

Additional evidence placing CaMKII upstream of Src came from experiments in which a selective pharmacologic antagonist of CaMK, KN-93 (33), did not block stimulation of the c-fos promoter by constitutively active v-Src (Figure 6). Eight hours after co-transfecting mesangial cells with v-Src and the fos-luciferase reporter, KN-93 at 10 μM was added and maintained throughout the remainder of the transfection. KN-93 did not significantly reduce activation of the c-fos promoter by v-Src and did not affect basal promoter activity in cells transfected with the expression vector alone (i.e., pM-MuLV, Figure 6). We have previously shown that 10 μM KN-93 blocks stimulation of the c-fos promoter by ET-1 (21), a finding that was confirmed here in concurrent experiments to demonstrate that KN-93 was indeed effective (Figure 6). The inability of a CaMK antagonist to block c-fos promoter activation by Src is further evidence that Src functions downstream of CaMKII on a signaling pathway to c-fos.

CaMKII/Src Signaling to c-fos Requires the c-fos Serum Response Element (SRE)

We next sought to determine which cis-element(s) of the c-fos promoter is targeted by CaMKII/Src signaling. Mesangial cells were transfected with c-fos reporter constructs in which point mutations have been introduced to inactivate specific cis-elements as shown in Figure 7A. Inactivation of the SIE and FAP cis-elements had no significant effect on activation of the c-fos promoter by either constitutively active CaMKII290 or by v-Src (pm6 and pm9, Figure 7A). Point mutations in the Ca/CRE, which responds to Ca2+/cAMP-based signaling pathways (34), slightly inhibited the response to CaMKII290 (i.e., 6.4-fold versus 4.3-fold with pm3) but did not alter the response to v-Src. In contrast, point mutations in the SRE completely inhibited stimulation of the c-fos promoter by either

Figure 4. Blockade of CaMK II inhibits Src activation by endothelin-1 (ET-1). (A) Serum-starved cells were stimulated with 100 nM ET-1 or 10 μM Ca2+ ionophore A23187 and levels of phospho-Tyr-416 Src were measured by Western blotting with an activation state-specific antibody. The blot was reprobed with mAb against total Src to confirm equal protein loading (bottom panel). (B) Cells were preincubated with myr-AIP (20 μM for 1 h), a cell-permeable peptide that blocks CaMKII activation, before addition of ET-1 and measurement of phospho-Tyr-416 Src levels as in panel A. Identical results were observed in two independent experiments.

Figure 5. Dominant negative SrcK− blocks activation of the c-fos promoter by CaMK II 290 in different cell types. Embryonic rat A7r5 vascular smooth muscle cells, porcine aortic endothelial cells, and human HeLa cells were transfected with p-356wt/fos LUC, pCMV CaMK II 290, and SrcK− or SrcK+ exactly as described in Figure 1. Data are mean ± SEM from three experiments in duplicate. Inhibition by SrcK− was significant (P < 0.01) in all cell types.

Figure 6. A CaMK antagonist (KN-93) fails to block c-fos promoter activation by v-Src. Mesangial cells were co-transfected with p-356wt/fos LUC and p-v-Src (2 μg) or the parent expression vector pM-MuLV. Eight hours later, KN-93 (10 μM) was added and maintained throughout the transfection. In experiments to demonstrate that KN-93 was effective, mesangial cells transfected with p-356wt/fos LUC were treated with KN-93 followed by 100 nM endothelin-1 (ET-1). Results (mean ± SEM) of 4 independent experiments in duplicate. ** P < 0.01.
CaMKII290 or v-Src (pm12, Figure 7A). Thus the SRE appears to be the critical \textit{cis}-element required for the CaMKII/Src signaling pathway. It is important to note, however, that full activation of the \textit{c-fos} promoter by CaMKII290 requires both the SRE and Ca/CRE \textit{cis}-elements.

To further investigate the role of the SRE in CaMKII/Src signaling, we used a minimal promoter construct (p56LUC, 2 μg) or the same vector with a single copy of the \textit{c-fos} SRE upstream of TATA (pSRELUC, 2 μg). Cotransfections were with pCMV alone (no addition), CaMKII290, or CaMKII290 plus dominant negative SrcK−. Data are mean ± SEM from four independent experiments.

Figure 7. The \textit{c-fos} SRE is necessary for activation of the \textit{c-fos} promoter by CaMKII/Src signaling. (A) Mesangial cells were transfected with \textit{c-fos} promoter LUC constructs with inactivating point mutations (pm) as indicated schematically at left. Cells were cotransfected with pCMV (open bars), pCMVCaMKII290 (solid bars), or pSrcK− (hatched bars). Data are means from three independent experiments; error bars are omitted for clarity but were never more than 20% of the mean. (B) Cells were transfected with a minimal promoter construct (p56LUC, 2 μg) or the same vector with a single copy of the \textit{c-fos} SRE upstream of TATA (pSRELUC, 2 μg). Cotransfections were with pCMV alone (no addition), CaMKII290, or CaMKII290 plus dominant negative SrcK−. Data are mean ± SEM from four independent experiments.
signaling, we transfected mesangial cells with a luciferase reporter construct in which transcription is controlled by a single copy of the SRE upstream of TATA, pSRELUC (26). Cotransfection of CaMKII290 or CaMKII290 plus SrcK− failed to alter luciferase expression from the parent vector, p56LUC (Figure 7B). However, in cells transfected with pSRELUC, CaMKII290 stimulated a 4.7-fold increase in SRE cis-element activity that was markedly inhibited by SrcK− (i.e., 1.6-fold, Figure 7B). Taken together, these results suggest that CaMKII/Src signaling targets the SRE of the c-fos promoter.

**Dominant Negative Src K− Inhibits DNA Synthesis Stimulated by CaMK II 290**

To assess the biologic importance of Src in signaling by CaMK II, we asked whether Src is a downstream effector in pathways whereby CaMK II controls mesangial cell growth. To this end, we measured bromodeoxyuridine uptake into DNA in cells transiently transfected with CaMK II 290. Transfected cells were identified by cotransfection with a lacZ gene and histochemical detection of expressed β-galactosidase (Figure 8, A and B). BrdU was incorporated into DNA in 71% of cells transfected with CaMK II 290 (Figure 8, A and C) versus 22% of cells with the control vector (Figure 8C). Expression of Src K− attenuated the increase in DNA synthesis in cells expressing CaMK II 290 (Figure 8, B and C; 35% versus 71%, P < 0.01 by χ² analysis). In contrast, SrcK+ had no effect on CaMK II 290-stimulated DNA synthesis. These results support the concept that Src acts downstream of CaMK II in a signaling cassette that controls gene expression and cell growth.

**Discussion**

Mesangial hypertrophy and hyperplasia are adaptive responses to glomerular injury and are mediated by a variety of paracrine and autocrine growth factors that increase [Ca²⁺]. Mesangial cell growth requires coupling of [Ca²⁺]i to transcription factors that activate and maintain hypertrophy and hyperplasia, but elucidation of the intracellular signals involved represents an important challenge. The results presented here demonstrate a novel mechanism whereby the Ca²⁺-dependent effector CaMKII functions upstream of Src in a signaling pathway that activates the c-fos promoter and stimulates DNA synthesis in cultured mesangial cells.

Several lines of evidence suggest that CaMK II lies upstream of Src in mesangial cells. We blocked CaMK II-stimulated c-fos induction and DNA synthesis by expressing dominant negative SrcK− and Csk to inactivate Src. SrcK− and Csk interfere with Src activity by distinct molecular mechanisms, thereby providing an important internal control for possible nonspecific effects of either protein. Transfection with the empty expression vector likewise did not reduce luciferase activity. That inactivation by SrcK− was specific was further supported by the finding that overexpression of wild-type SrcK+ did not alter activation of the c-fos promoter by CaMK II 290. SrcK− did not block expression of CaMKII290 as verified by Western blotting of HA epitope-tagged CaMKII290 in mesangial cells transfected with SrcK−. Moreover, we have previously shown that SrcK− and Csk do not inhibit activation of the c-fos promoter by Raf-1, which functions downstream of Src and Ras (16). A pharmacologic CaMK inhibitor (KN-93) did not significantly block activation of the c-fos promoter by v-Src, providing additional evidence that Src lies downstream of CaMKII in this signaling pathway. We also demonstrated that CaMKII acts upstream of Src in several different cell types. We had previously shown that Src activation by ET-1 requires Ca²⁺ influx and elevation of cytosolic free [Ca²⁺] (15), and the present studies in mesangial cells demonstrated that Src activation by ET-1 was inhibited in the presence of myr-AIP to block CaMKII. Taken together, these results suggest that CaMK II functions upstream of Src in a Ca²⁺-dependent signaling pathway.

An important caveat is that SrcK− and Csk might also inactivate closely related Src PTK family members (23,24,31,32), so we cannot formally rule out participation of Src-related kinases in CaMK II signaling. In addition, our experiments have not addressed the question of how Ca²⁺ and CaMKII might activate Src. We were unable to demonstrate a direct protein-protein interaction between CaMK II and c-Src (Wang and Simonson, unpublished results). Src contains a consensus phosphorylation site for CaMKs (R-X-X-S/T) in the unique NH2 terminal sequence, raising the possibility of phosphorylation and control of Src activity by CaMK II. An indirect mechanism by which CaMK II activates Src might depend on some as yet unidentified cofactor or protein as part of an heterooligomeric protein complex. Indeed, Zhao et al. (12)
propose that Ca\(^{2+}\) ionophore A23187 increases Src activity in keratinocytes by an indirect mechanism possibly involving a Ca\(^{2+}\)-stimulated tyrosine phosphatase activity or the Ca\(^{2+}\)-dependent association of three unidentified proteins with c-Src. Another possibility is that CaMKII activates Ca\(^{2+}\) channels and/or ion pumps (19), thereby elevating [Ca\(^{2+}\)]\(_i\), and stimulating Src PTK activity.

Our results with point mutants of the c-fos promoter suggest that the SRE is required for CaMKII/Src signaling to the c-fos immediate early gene. Point mutations in the SRE blocked activation of the c-fos promoter by CaMKII290 and Src. Mutation of the Ca/CRE only modestly blocked the response to CaMKII290 and did not alter activation of the c-fos promoter by Src. The c-fos SRE is activated by the Ras/MAPK pathway, and it seems possible that CaMKII/Src signaling also activates the Ras/MAPK pathway to c-fos. Indeed, Rosen et al. (35) have previously shown in PC12 cells that Ca\(^{2+}\) influx activates the Ras/MAPK pathway, and Rusanescu et al. (14) have further demonstrated that Src is required for Ca\(^{2+}\) activation of Ras/MAPK. It is not entirely clear how Ca\(^{2+}\) influx and Src activate Ras, but the mechanisms probably involve Src-mediated phosphorylation of the Shc adapter protein and subsequent recruitment of Grb2-Sos complexes (14). The new finding of the present experiments concerns the role of CaMKII contributing to Src-based signaling to the c-fos SRE.

An important aspect of our study was to test a possible role for the CaMKII-Src signaling cassette in mesangial cell growth, which is a pathologic hallmark of many forms of glomerular disease. Previous experiments in cultured mesangial cells and in experimental models of glomerular injury with mesangial growth demonstrate that inhibiting a rise in intracellular free [Ca\(^{2+}\)]\(_i\) attenuates mesangial cell proliferation (2,4,9–11), but the molecular mechanisms and effectors of Ca\(^{2+}\) signaling that underlie these results remain unclear. We showed that ectopic expression of CaMKII290 stimulated DNA synthesis in quiescent mesangial cells that was effectively blocked by SrcK–. The control vector lacking SrcK– and the vector expressing SrcK+ had no effect on DNA synthesis driven by CaMKII290. These results are the first direct demonstration in mesangial cells that activated CaMKII drives DNA synthesis, and the results also support a role for Src in this signal transduction pathway. Many mesangial cell growth factors stimulate a rapid and transient increase in intracellular free [Ca\(^{2+}\)]\(_i\) (7,8), and our experiments suggest that a CaMKII-Src signaling cassette is one mechanism coupling the rise in [Ca\(^{2+}\)]\(_i\) to DNA synthesis.

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