We have previously reported that 14,15-epoxyeicosatrienoic acid (14,15-EET) is a potent mitogen for the renal epithelial cell line, LLC-PK1. This mitogenic effect is dependent upon activation of a protein-tyrosine kinase cascade that results in activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Because of suggestive evidence that 14,15-EET also activated Src in these cells, we stably transfected LLC-PK1 with an expression construct of the C-terminal Src kinase (CSK), which inhibits Src family kinase activity. In vitro Src kinase activity assays confirmed that in empty vector-transfected cells (Vector cells), 14,15-EET increased Src kinase activity, while in clones overexpressing CSK mRNA and immunoreactive protein (CSK cells), 14,15-EET-induced activation of Src was almost completely blocked (94% inhibition). Of interest, epidermal growth factor (EGF) and fetal bovine serum (FBS) also increased Src activity in Vector cells, but not in CSK cells, further confirming the ability of CSK overexpression to prevent Src activation. CSK cells failed to increase [3H]thymidine incorporation in response to exogenous 14,15-EET. In contrast, both EGF and FBS significantly increased [3H]thymidine incorporation in CSK cells. Immunoprecipitation with anti-phosphotyrosine antibodies and immunoblotting with an antibody against extracellular signal-regulated kinase (ERK) indicated that in CSK cells, 14,15-EET failed to activate ERK1 and ERK2; however, EGF- and FBS-induced activation of ERKs was not different from that seen in Vector cells. In Vector cells, the 14,15-EET-stimulated tyrosine phosphorylation of ERKs was blocked by pretreatment with 1 μM PP2, a selective inhibitor of Src kinases. The present study demonstrates that 14,15-EET exerts its mitogenic effects predominantly through a Src kinase-mediated pathway, which is the most upstream signaling step determined to date in the 14,15-EET-activated tyrosine kinase cascade in renal epithelial cells.

In addition to cyclooxygenase and lipoxygenase pathways, the cytochrome P450-dependent monoxygenase pathway also catalyzes the in vivo metabolism of arachidonic acid to biologically active compounds. This pathway metabolizes arachidonic acid in two ways: epoxidation, producing 5,6-, 8,9-, 11,12-, and 15,16-epoxyeicosatrienoic acids and ω-ω-1 hydroxylation, resulting in the formation of 19- and 20-hydroxyeicosatetraenoic acids (1, 2). Epoxyeicosatrienoic acids (EETs) have been demonstrated to play important roles in regulating vascular tone, mitogenesis, platelet aggregation, tissue and body homoeostasis, and Ca2+ signaling (1–7). The EETs are produced predominantly by epoxygenases of the 2C family of cP450s, which have been localized to the mammalian proximal tubule cells of the kidney. In this segment of the nephron, cyclooxygenase and lipoxygenase are expressed at nearly undetectable levels (8, 9).

pp60c-src is the prototype of a family of nine cytoplasmic protein-tyrosine kinases that is activated by a number of receptor protein-tyrosine kinases, such as epidermal growth factor receptor and platelet derived-growth factor receptor (10, 11), and a variety of extracellular stimuli-induced cellular responses, including DNA synthesis, mitosis, proliferation, hypertrophy, differentiation, adhesion and cytokine production (10, 12–17). Another cytoplasmic protein-tyrosine kinase, the C-terminal Src kinase (CSK), is a mediator responsible for negative regulation of the Src family kinase activity (18, 19). CSK phosphorylates Tyr-527 in the C-terminal tail of c-Src and thus creates a binding site for the Src homology 2 domain, locking the molecule in an inactive state. Dephosphorylation of Src Tyr-527, followed by autophosphorylation on Tyr-416 by the c-Src kinase activity, increases Src kinase activity up to 10–20-fold (20–24).

Our previous studies demonstrated that 14,15-EET is a potent mitogen for the renal epithelial cell line, LLC-PK1c4, and that these effects are mediated by a tyrosine kinase phosphorylation cascade that activates the mitogen-activated protein kinases, p44/p42 extracellular signal-regulated kinases (ERKs), and phosphatidylinositol 3-kinase (4). We also found evidence for possible Src kinase involvement in the mitogenic signaling pathways of 14,15-EET (4). In certain systems, Src family members are mediators of cell division at multiple points in the cell cycle (25–27). The demonstration of an important role Src in cell division and the illustration of a negative regulatory role of the C-terminal Src kinase (CSK) on c-Src (23, 25, 28) stimulated us to investigate the potential role of Src kinases in the mitogenic signaling pathways of 14,15-EET.

**EXPERIMENTAL PROCEDURES**

**Materials**—Polyclonal rabbit anti-CSK antibodies were from Transduction Laboratories (Lexington, KY). Polyclonal and monoclonal anti-phosphotyrosine antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA). Polyclonal anti-ERK antibodies and protein A-agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA). Arachidonic acid was obtained from NuCheck-Prep, Inc. (Elysian, MN).

(+)14,15-Epoxyeicosatrienoic acid sulfonamide analog was synthesized as described previously (4). EGF (receptor grade) was purchased from Collaborative Research (Bedford, MA). PP2 was from Calbiochem (San Diego, CA). All other chemicals were from Sigma.

**Cell Culture**—LLCPKc4, an established renal proximal tubule epithelial cell line derived from pig kidney (29), was routinely cultured as described previously (4, 30).
cDNA Manipulation and Stable Transfection—In order to stably transfact CSK cDNA into LLCPK14 cells, the cDNAs containing the entire coding region of CSK were cut out from pCDNA-I vector (Invitrogen, San Diego, CA) by EcoRI digestion and then ligated into the EcoRI site of the neomycin resistant gene-containing vector pIRE1neo (CLONTECH Laboratories Inc., Palo Alto, CA). The sense orientation to the human cytomegalovirus (CMV) promoter was identified by restriction enzyme digestion analysis and further confirmed by sequencing analysis. One µg/ml CSK cDNA in pIRE1neo or empty pIRE1neo vector alone was used for stable transfection into LLCPK14 cells using LipofectAMINE (Life Technologies, Inc.) as described previously (30). After 7 passages in medium containing 600 µg/ml G418, G418-resistant clones were then isolated and screened by slot blot hybridization. Nine clones that expressed the highest levels of CSK mRNA by slot blotting, which were used here to select further the highest levels of the immunoreactive CSK by immunoblotting with polyclonal antibodies against CSK. NT, nontransfected; V, empty vector-transfected LLCPK14 cells; P, mouse macrophase cell lysate, used as a positive control from Transduction Laboratories (Lexington, KY).

Immunoprecipitation and Immunoblotting—pIRE1neo vector-transfected cells and CSK-transfected individual clones were grown in 6-mm dishes and made quiescent for 3 days, and then total cellular RNA was isolated as described previously (4). 10 µg of total RNA, as determined by absorbance at 260 nm, was slot blotted onto Nytran nylon membranes (Schleicher & Schuell) and immobilized with a UV cross-linker. [3H]cDNA probe was labeled to a specific activity of >2 × 10^6 dpm/µg by random priming (Megaprime DNA labeling system; Amersham Pharmacia Biotech). After overnight hybridization, membranes were washed twice with 2 × SSC (0.3 M NaCl and 0.03 M sodium citrate), 0.1% SDS at room temperature for 15 min, and 0.2 × SSC, 0.1% SDS at 65 °C for 30 min. Autoradiography was performed at −70 °C by exposing the washed membranes to Hyperfilm (Amersham Pharmacia Biotech) with intensifying screens.

Immunoprecipitation and Immunoblotting—pIRE1neo vector-transfected and CSK-transfected cells were made quiescent and treated with indicated agents, washed twice with ice-cold Ca^2+/-Mg^2+/-free phosphate-buffered saline, and lysed on ice for 30 min in RIPA buffer (4). Cell lysates were clarified at 10,000 × g for 15 min at 4 °C, and protein concentrations were determined by the bicinchoninic acid assay (Pierce). Target proteins were immunoprecipitated at 4 °C for 2 h with appropriate antibodies. Immune complexes were captured with 50 µl of protein A-agarose beads and washed four times with wash buffer (20 mM HEPES, pH 7.2, 100 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 100 µM Na_3VO_4). Phosphorylated complexes were resuspended and boiled in sample buffer before separation on a 7.5% or 12% SDS-PAGE, and protein bands were visualized by autoradiography. As described under "Experimental Procedures," Src kinase activity was determined by measuring incorporation of γ-32P into enolase.
Role of CSK in the Mitogenic Signaling Pathways of 14,15-EET

We stably transfected CSK cDNA, cloned in the mammalian expression vector pIREs1neo, into the LLCPKcl4 cell line, which was cloned from the parent LLC-PK1 cell line and selected for its expression of high levels of proximal tubule characteristics (31). G418-resistant clones were then isolated and screened by for mRNA expression levels (data not shown), and selected for immunoreactive CSK expression with polyclonal antibodies against CSK (Fig. 1). Two of the clones that overexpressed immunoreactive CSK, clones 13 and 26, along with CSK 26, were rendered quiescent and exposed to 14,15-EET for subsequent signaling studies.

Cells transfected with empty vector alone and CSK-overexpressing cells were rendered quiescent and exposed to the indicated agents. Cell lysates were prepared and subjected to immunoprecipitation with a polyclonal antibody to phosphorylated proteins in the cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies (anti-PY) and immunoblots probed with an anti-ERK antibody, which recognizes the two isoforms of ERK (p44 ERK1 and p42 ERK2) proteins. B, immunoblots cell lysates were probed with antibodies to phosphorylated ERKs.

A

**Vector**  
I.P.: anti PY  
L.B.: anti ERK

**CSK**  
I.P.: anti PY  
L.B.: anti ERK

**Fig. 4.** CSK overexpression blocked 14,15-EET-induced ERK activation. Empty vector-transfected cells (Vector) and the CSK-overexpressing cells (CSK), clone 26, were rendered quiescent and treated with or without FBS (2.5%), EGF (30 nM), or 14,15-EET (20 μM) for 15 min. A, tyrosine-phosphorylated proteins in the cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies (anti-PY) and immunoblots probed with an anti-ERK antibody, which recognizes the two isoforms of ERK (p44 ERK1 and p42 ERK2) proteins. B, immunoblots cell lysates were probed with antibodies to phosphorylated ERKs.

RESULTS AND DISCUSSION

We stably transfected CSK cDNA, cloned in the mammalian expression vector pIREs1neo, into the LLCPKc14 cell line, which was cloned from the parent LLC-PK1 cell line and selected for its expression of high levels of proximal tubule characteristics (31). G418-resistant clones were then isolated and screened by for mRNA expression levels (data not shown), and selected for immunoreactive CSK expression with polyclonal antibodies against CSK (Fig. 1). Two of the clones that overexpressed immunoreactive CSK, clones 13 and 26, along with LLCPKc14 cells transfected with empty vector alone, were used for subsequent signaling studies.

Cells transfected with empty vector alone and CSK-overexpressing cells were rendered quiescent and exposed to the indicated agents. Cell lysates were prepared and subjected to immunoprecipitation with a monoclonal antibody specifically against pp60sca, and the immunoprecipitates were used for in vitro Src kinase activity assays. As shown in Fig. 2, 14,15-EET increased Src kinase activity 3.1-fold in empty vector-transfected cells, whereas in the CSK-overexpressing cells, 14,15-EET-induced activation of Src kinase activity was almost completely blocked (94% inhibition). Of interest, EGF and FBS also significantly increased Src kinase activity in empty vector-transfected cells, but not in CSK-overexpressing cells. This inhibition of EGFR-, FBS- and 14,15-EET-induced activation of Src kinase confirmed the ability of CSK overexpression to prevent Src activation.

14,15-EET has previously been demonstrated to be a potent mitogen in the nontransfected LLCPKc14 cells, and Src has been suggested to involved in the signal transduction (4). To test the effects of CSK overexpression on the mitogenic properties of 14,15-EET, nontransfected cells, empty vector-transfected LLCPKc14 cells, and CSK-overexpressing clones CSK and CSK 26, were made quiescent and exposed to 14,15-EET (20 μM), EGF (30 nM), FBS (2.5%), or vehicle prior to determination of [3H]thymidine incorporation. As indicated in Fig. 3, 14,15-EET significantly stimulated [3H]thymidine incorporation in both nontransfected and empty vector-transfected cells. The EET effect was almost completely abolished by overexpression of CSK in these cells. These data suggest that pp60sca and/or other Src family kinases (Yes, Fyn, Lyn) were required for the 14,15-EET-induced increase in [3H]thymidine incorporation. Interestingly, both EGF and FBS significantly stimulated [3H]thymidine incorporation in the CSK-overexpressing clones as well as in nontransfected and empty vector-transfected cells, indicating that they can exert mitogenic effects through Src-independent signaling pathways while 14,15-EET’s mitogenic effect is Src-dependent.

We have reported that 14,15-EET activates both extracellular signal-regulated kinases (ERKs) and phosphatidylinositol 3-kinase in this cell line, and both mitogen-activated protein kinase pathway and phosphatidylinositol 3-kinase pathway are required for EET-induced mitogenesis (4). In order to ex-

**Fig. 5.** Effect of Src kinase inhibition on 14,15-EET-induced ERK tyrosine phosphorylation in LLCPKcl4 cells. Empty vector-transfected cells were made quiescent and pretreated with or without 1 μM PP2, a selective inhibitor of Src kinase, for 30 min. Cell lysates were subjected to immunoprecipitation with a polyclonal antibody to phosphotyrosine (anti-PY) and immunoblotting with an anti-ERK antibody, which recognizes the two isoforms of ERK (p44 ERK1 and p42 ERK2) proteins.

**Fig. 6.** Effect of the Src kinase inhibitor, PP2, on arachidonic acid- or EGF-induced ERK tyrosine phosphorylation in empty vector-transfected cells and the F87V BM3-transfected cells. Quiescent cells were pretreated with or without PP2 (1 μM), then treated with or without arachidonic acid (AA, 30 μM) or EGF (30 nM) as indicated, and then immunoprecipitated and immunoblotted as described in Fig. 4.
amino whether CSK overexpression affected the early signals activated by 14,15-EET, cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine antibodies and immunoblotted with anti-ERK antibody (Fig. 4A). In CSK-overexpressing cells, 14,15-EET failed to activate either 44-kDa ERK1 or 42-kDa ERK2, whereas EGF- and FBS-induced activation of both ERK1 and ERK2 were not different from that observed in the cells transfected with empty vector alone. Similar results were obtained when cell lysates were probed with antibodies specific for phosphorylated ERKs (Fig. 4B).

In empty vector-transfected cells, pretreatment of the cells for 30 min with PP2, a selective inhibitor of kinase activity of pp60c-src and other Src family kinases, at a concentration (1 μM) that did not affect basal ERK expression, markedly inhibited 14,15-EET-increased tyrosine phosphorylation of both ERK1 and ERK2 (Fig. 5).

Synthetic eicosanoids have been widely utilized for the experimental analysis of their cellular and organ functions. However, in many cases, this approach provides only limited information with regard to their mechanisms of action and the enzymatic steps responsible for their biosynthesis from endogenous precursors, activation, and disposition. This is of special relevance regarding P450-derived eicosanoids, since in most cultured cells, there is a rapid and progressive decrease in the expression of the P450 isoforms found in vivo. LLCPKcI4 cells have no detectable endogenous P450 expression (30). In order to study further the involvement of Src kinase in endogenous EET signaling, we utilized stable LLCPKcI4 transfectants expressing a regio- and stereoselective 14S,15R-epoxygenase (14S,15R-EET, 99% of total products, 98% optical purity), the enantiomer that predominates in vivo in the kidney (30–32).

We previously showed that CSK overexpression inhibited the EGF-induced increases in 14,15-EET biosynthesis from endogenous arachidonic acid pools.

In summary, the present studies indicate that in renal epithelial cells, the epoxygenase metabolites of arachidonic acid, epoxygenoicosteriogenic acids, exert their mitogenic effects predominantly through a pathway mediated by pp60c-src and/or other Src family kinases and that this Src kinase activity is the most upstream signaling protein determined to date in the 14,15-EET-activated tyrosine kinase cascade. EETs have also been shown to activate tyrosine kinase cascades and induce mitogenesis in smooth muscle, mesangial, and endothelial cells (33–35), but whether these effects are also mediated through Src kinase dependent pathways will require further study.

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