Upregulation of EphA2 during in vivo and in vitro renal ischemia-reperfusion injury: role of Src kinases

Cindy Baldwin,1 Zhongchuan Will Chen,1 Arda Bedirian,1 Naoko Yokota,2 Samih H. Nasr,3 Hamid Rabb,2 and Serge Lemay1

Departments of 1Medicine and 3Pathology, McGill University, Montreal, Quebec, Canada; and 2Division of Nephrology, Johns Hopkins University School of Medicine, Baltimore, Maryland

Submitted 19 January 2006; accepted in final form 25 May 2006

Baldwin, Cindy, Zhongchuan Will Chen, Arda Bedirian, Naoko Yokota, Samih H. Nasr, Hamid Rabb, and Serge Lemay. Upregulation of EphA2 during in vivo and in vitro renal ischemia-reperfusion injury: role of Src kinases. Am J Physiol Renal Physiol 291: F960–F971, 2006. First published May 30, 2006; doi:10.1152/ajprenal.00020.2006.—Ischemia-reperfusion injury (IRI) is a major cause of acute kidney injury in both native kidneys and renal allografts. Disruption of the actin cytoskeleton is a key event with multiple repercussions on cell adhesion and function during IRI. However, receptors involved in regulating cytoskeletal repair following injury have not been identified. In an in vivo model of renal IRI, we used multiprobe RNase protection assay to examine the expression of Eph receptor tyrosine kinases, key regulators of actin dynamics in embryonic development. We found that one receptor in particular, EphA2, was strongly upregulated in the kidney following IRI. Ephrins, the cell-bound ligands of Eph receptors, were also strongly expressed. In cultured renal tubular cells, diverse injurious stimuli mimicking IRI also resulted in upregulation of EphA2 protein expression. Upregulation of EphA2 was inhibited by the Src kinase inhibitor PP2. Conversely, overexpression of Src kinases strongly enhanced the expression of endogenous EphA2 as well as the activity of a human EphA2 promoter construct. Activation of the Erk pathway was necessary, but not sufficient for full induction of EphA2 upregulation by Src kinases. Stimulation of renal tubular epithelial cells with the EphA2 ligand ephrin-A1 caused tyrosine phosphorylation of endogenous EphA2, paxillin, and an unidentified ~65-kDa protein and resulted in increased cortical F-actin staining. In summary, under in vitro conditions mimicking IRI, EphA2 expression is strongly upregulated through a Src kinase-dependent pathway. Interactions between upregulated EphA2 and its ephrin ligands may provide critical cell contact-dependent, bidirectional cues for cytoskeletal repair in renal IRI.

epithelial injury; tyrosine kinases; MDCK; IMCD-3

ISCHEMIA-REPERFUSION INJURY (IRI) is a major cause of acute kidney injury in both native kidneys and renal allografts. Despite the frequency and clinical importance of IRI, the key pathophysiological events underlying injury and repair remain poorly understood.

Experimentally, IRI can be divided into two distinct phases: an initial “passive” phase (ischemia) during which blood flow is reduced or cut off and where depletion of oxygen, ATP, and other nutrients predominates; and an “active” (reperfusion) phase that follows restoration of blood flow and is characterized by multiple deleterious reactive changes, including the generation of free radicals and inflammatory mediators with consequent leukocyte-endothelial adhesion. This, in turn, leads to microvascular stasis (37) and possibly interstitial infiltration of leukocytes (4), resulting in further tissue damage.

Studies in several models suggest that, at the cellular level, one of the earliest and most critical events in the pathogenesis of IRI is the disruption of the tubular epithelial actin cytoskeleton (24, 32–34). Disruption of the actin cytoskeleton is associated with several other important functional alterations such as loss of polarity (2, 34, 36) and weakening of both cell-cell junctions (5, 6) and cell-matrix adhesion (43, 57). These disturbances, in turn, result in loss of epithelial barrier function and vectorial transport as well as shedding of tubular cells, cast formation, and tubular obstruction.

Following reperfusion, a complex and incompletely defined repair process takes place where cytoskeletal reorganization likely plays a key role. However, very little is known about this process, particularly the identity and contribution of surface receptors that orchestrate cytoskeletal repair. Given that reexpression of developmental gene products (e.g., Wnt-4, vimentin) had been described in IRI (52, 55), we hypothesized that additional developmental ligand/receptor systems might be upregulated and activated to provide cues for rapid and orderly repair of the actin cytoskeleton following IRI. We also postulated that an ideal configuration for receptors involved in this coordinated repair would involve cell contact-dependent and bidirectional signaling. Members of the Eph receptor family seemed most likely to fulfill these requirements.

Eph receptors are a developmentally regulated group of molecules that constitute the largest family of receptor tyrosine kinases (38). Structurally, they are divided into EphA and EphB subfamilies based on their ligand specificity. Eph receptors are triggered by membrane-bound proteins called ephrins. A-type ephrins are GPI-linked molecules and bind EphA receptors, whereas B-type ephrins are transmembrane proteins that bind EphB receptors. Notably, in addition to stimulating their corresponding Eph receptor (forward signaling), both types of ephrins can carry reverse signals (10, 18). Signaling through Eph receptors typically impacts actin cytoskeletal organization by coupling to unique regulators of Rho GTPases (39), the critical molecular switches for actin dynamics control (13).

Functionally, Eph receptors are known for their critical roles in embryonic development, particularly with respect to the vascular and central nervous systems (14, 15). Because of their developmental and cytoskeletal functions, we hypothesized...
that Eph receptors could play a novel and important role in cellular and tissue repair in the context of renal IRI. However, the role of Eph receptors in IRI has never been explored previously and their expression and function in the adult kidney have been largely ignored to date.

Therefore, in the current report, we examined the expression profile and regulation of Eph receptors in vivo and in vitro models of IRI. Our data indicated that one member of the Eph family in particular, EphA2, was strongly upregulated in a Src-kinase-dependent manner in renal tubular cells subjected to IRI. Ligand-induced activation of EphA receptors resulted in phosphorylation of the focal adhesion molecule paxillin and increased cytosolic and cortical F-actin staining, confirming a likely role for Eph/ephrin signaling in regulating how cell-matrix and cell-cell adhesion are linked to the actin cytoskeleton during tissue remodeling of the ischemic kidney.

METHODS

In Vivo Model of Renal IRI

A previously described model of murine renal IRI was used (45). CD-1 mice (Jackson Laboratories) weighing 20–30 g were anesthetized with pentobarbital sodium (60 mg/kg ip). Bilateral flank incisions were made to expose the kidneys and renal pedicles were bluntly dissected. A nontraumatic vascular clamp (Roboz microaneurysm clamp, Roboz Surgical Instruments, Washington, DC) was applied across the left pedicle for 30 min. The right kidney served as sham control. After clamp removal, the flanks were closed and the animals received 100 ml/kg of warm saline instilled into the peritoneal cavity. After 2 h, 24 h, or 7 days of reperfusion, the animals were reanesthetized, the flank incisions were reopened, and the kidneys were harvested and placed in liquid nitrogen. All animal studies were approved by the institutional animal review committee.

RNase Protection Assay

RNA was extracted from frozen kidney by homogenization in TRIzol reagent (Invitrogen). The integrity of ribosomal RNA was verified by agarose gel electrophoresis. Assays were performed as previously described (27). Radiolabeled antisense RNA probes were prepared by T7 polymerase-directed in vitro transcription of appropriate RiboQuant murine DNA templates (from BD-Pharmingen) in the presence of [α-32P]UTP (New England Nuclear, Boston, MA). The mEPH-1 and mEFN-1 template sets were used, respectively, for mEPH-1 and mEFN-1 template sets were used, respectively, for IRI. Ligand-induced activation of EphA receptors resulted in phosphorylation of the focal adhesion molecule paxillin and increased cytosolic and cortical F-actin staining, confirming a likely role for Eph/ephrin signaling in regulating how cell-matrix and cell-cell adhesion are linked to the actin cytoskeleton during tissue remodeling of the ischemic kidney.

Cells

COS-1 cells, 293 embryonic kidney cells, Madin-Darby canine kidney (MDCK) cells (from ATCC), and IMCD-3 cells [from Dr. M.-J. Hébert (47)] were cultured in DMEM-high glucose containing pyridoxine-HCl and sodium pyruvate (GIBCO-Invitrogen) with 10% FBS (GIBCO-Invitrogen). Rat glomerular epithelial cells (GEC; from Dr. Andrey Cybulsky) were grown in K1 medium as described previously (51).

Stimulation of Cells with Ephrin-A1-Fc

For stimulation of EphA receptors with ligand, 0.5 μg of soluble recombinant mouse ephrin-A1-Fc (R&D Systems) was cross-linked with 250 ng of goat anti-human Fcγ-specific F(ab′)2 fragments (Jackson ImmunoResearch) for 30 min at room temperature in a 10-μl total volume. This cross-linked ligand was added to 1 ml of cell suspension obtained by careful scraping and centrifugation from a 100-mm culture dish.

Multiwound Assay

Confluent monolayers of IMCD-3 cells grown on 100-mm dishes were extensively wounded (~50% of surface area) with sterile tips fitted on an 8-channel pipetter. Lysates were obtained at various time points thereafter using a standard NP-40-containing immunoprecipitation buffer.

Chemical ATP Depletion

Cell culture model of IRI. Ischemia-reperfusion was produced in cultured cells following a protocol established by Molitoris et al. (35). Cells were washed in PBS and growth medium was replaced with glucose-free buffer (145 mM NaCl, 1 mM Na2HPO4, 20 mM HEPES, 4 mM KCl, 0.5 mM MgSO4, 0.5 mM CaCl2 adjusted to pH 7.40). To simulate ischemia, antimycin A (5 μg/ml) and 2-deoxyglucose (10 mM) were added. As a negative control, only D-glucose (10 mM) was added to the buffer. Following incubation periods ranging from 1 to 4 h, glucose-free buffer was replaced with fresh culture medium.

Immunohistochemistry

For immunohistochemistry, pieces of freshly harvested kidney were snap-frozen in OCT compound and stored at −80°C until use. Four-micrometer sections were prepared in a Microm cryostat and laid onto gelatin-coated slides. These were then fixed in cold 4% paraformaldehyde for 30 s. To block endogenous peroxidase activity, sections were incubated with 0.1% NaNO3 and 0.3% H2O2 in PBS. To block nonspecific antibody binding, each section was incubated with 50 μl of 5% donkey serum in 3% BSA/PBS for 1 h. The primary antibody was diluted 1:100 in blocking solution and applied overnight at room temperature in a wet chamber. For peptide adsorption controls, primary antibody was preabsorbed with a 5:1 ratio of corresponding blocking peptide (Santa Cruz Biotechnology) for 2 h before application. On the following day, sections were washed in PBS for 10 min and then incubated with 50 μl of horseradish peroxidase-coupled anti-rabbit F(ab′)2 fragments from donkey (Amersham Biosciences) diluted 1:100 in blocking solution for 1 h. Slides were then washed in 0.1% Tween/PBS for 10 min and incubated with 50 μl of Metal Enhanced DAB Substrate (Pierce) for 10 min. Finally, sections were counterstained with Mayer’s hematoxylin (Sigma) and mounted with coverslips using GelTel (Fisher Scientific).

Plasmids and cDNAs

The human EphA2 cDNA was cloned by RT-PCR from Caco-2 cells, cloned in vector pCDNA3.1 (Invitrogen) and verified by full sequencing. The murine EphB4 cDNA was obtained from Dr. Axel Ullrich (8). The human platelet-derived growth factor receptor (PDGFR)-β cDNA cloned in the pCDNA3 vector was from Dr. Sylvain Meloche. The human Ret cDNA (from Dr. Lois Mulligan) was subcloned in the pCDNA3.1 vector. The pME18S vectors containing cDNAs for mouse Fyn and Src were obtained from Dr. James Ihle. The pME18S vector containing cDNA (from Dr. Lois Mulligan) was subcloned in the pCDNA3 vector. The human EphA2 cDNA was cloned by RT-PCR from Caco-2 cells, cloned in vector pCDNA3.1 (Invitrogen) and verified by full sequencing.
RESULTS

Eph Receptors and Ephrins in the Normal and Reperfused Kidney: Prominent Expression of EphA2

To test the possible involvement of Eph receptors in renal IRI, we examined the mRNA expression of multiple Eph family members in the normal mouse kidney as well as in kidneys subjected to unilateral IRI. Contralateral sham kidneys were used as controls. As a positive control, we used RNA extracted from different areas of the central nervous system, where it is known that multiple members of the family serve important functions. Gene expression for 12 different Eph family members was assessed by multiprobe RNase protection assay. As shown in Fig. 1A, the cerebral cortex, brain stem/midbrain, and cerebellum expressed high levels of multiple Eph receptors of both A and B subtypes in a largely overlapping pattern. In contrast, normal mouse kidney contained lower but nonetheless easily detectable levels of a more limited subset of distinct Eph receptors. In particular, three transcripts very weakly expressed in the brain, EphA1, A2, and B4 were highly represented in the kidney. Following unilateral renal IRI, marked upexpression of EphA2 mRNA was observed beginning within 2 h of reperfusion and continuing as long as 7 days after reperfusion (Fig. 1B). A more modest and/or delayed upregulation of other family members also occurred, particularly for EphA3, B2, and B4. In parallel to these changes in Eph receptor expression, significant expression and upregulation of both A and B type ephrin ligands were also detected (Fig. 1C). Expression of Eph receptors and ephrins was comparable in both sham-operated kidneys and kidneys harvested from normal animals (data not shown).

Detection of EphA2 Protein Expression in Epithelial Cells

To detect EphA2 protein expression, we first screened epithelial cells by immunoblotting. As seen in Fig. 2A, EphA2 was detected in multiple epithelial cell lines derived from colon (Caco-2), breast (SKBR3), kidney tubule (MDCK), cervix (HeLa), and skin (A431) as well as in GEC. Because the cytoplasmic domain of Eph receptors contains many conserved sequences, we tested the specificity of the anti-EphA2 antibody used for these studies in transiently transfected COS cells transfected with vector alone, EphA2, or EphB4 cDNA. Although these cells expressed substantial levels of endogenous EphA2, only overexpression of EphA2 and not EphB4 resulted in increased signal (Fig. 2B).

Localization of EphA2 Protein in the Kidney

To confirm upregulation of EphA2 protein during renal IRI and to localize its main sites of expression, we performed immunohistochemistry on frozen mouse kidney sections. By this method, we were able to localize basal and upregulated EphA2 protein expression to renal tubules (Fig. 3). Because of their low cuboidal epithelium and lack of brush border, these EphA2-positive tubules were most consistent with a distal rather than proximal phenotype, at least under basal conditions. Following IRI, increased intensity and increased numbers of EphA2-staining tubules occurred exclusively in the ischemia-sensitive corticomedullary area (not shown).
H₂O₂ Induces EphA2 Upregulation in Renal Epithelial Cells

We then attempted to create a cell culture model mimicking IRI-induced EphA2 upregulation. We found that, in IMCD-3 renal tubular epithelial cells, treatment with hydrogen peroxide (H₂O₂) in concentrations ranging from 0.25 to 2 mM caused an intense, specific and sustained upregulation of EphA2 in a dose-dependent manner (Fig. 4A). Upregulation was evident within 6 h of stimulation (Fig. 4B) and persisted for at least 72 h (Fig. 4C). In contrasts, expression...
of the tyrosine phosphatase Shp-2 remained unchanged following treatment with H2O2. Using immunofluorescence, we confirmed that both basal and H2O2-induced EphA2 upregulation was mostly associated with the cell surface (Fig. 4, D and E). Interestingly, in cells treated with H2O2, EphA2 upregulation was concentrated in cells at the edge of patches (Fig. 4E, arrowheads).

**EphA2 Upregulation Is Mediated by Src Kinases**

In the course of parallel experiments, we identified a putative mechanism for in vivo upregulation of EphA2. Microarray analysis of 293 cells engineered to overexpress the Src family tyrosine kinase Fyn in an ec Dysone-inducible manner revealed EphA2 mRNA as one of several prominently upregulated transcripts (Lemay S et al., unpublished results). No other Eph receptor was identified as a likely transcriptional target of Fyn in this assay (data not shown). Within hours of treatment with the ec Dysone analog ponasterone A, this inducible cell line expressed high levels of Fyn protein (Fig. 5A, bottom), which resulted in autoactivation and phosphorylation of multiple endogenous proteins (top). Ponasterone-induced EphA2 upregulation was confirmed by immunoblotting (Fig. 5B, top). The immediate early gene product Egr-1 was also upregulated in response to induction of Fyn (bottom), consistent with its previously established properties as a transcriptional target of Src kinase activity (44). Fyn-induced upregulation of both EphA2 and Egr-1 was inhibited by the MEK inhibitor U0126, suggesting a requirement for the Erk MAP kinase pathway in this process.

Because both IRI (49) and treatment with H2O2 (1) are known to activate Src kinases, we examined whether Src kinases might be involved in H2O2-induced upregulation of EphA2 in IMCD-3 cells. Indeed, the pharmacological Src kinase inhibitor PP2 inhibited both basal and H2O2-induced expression of EphA2 in these cells (Fig. 6).

**ATP Depletion, Mechanical Wounding, and Hypoxia-Reoxygenation Also Induce Src-Mediated Upregulation of EphA2**

To determine whether other injurious stimuli might also result in upregulation of EphA2 in epithelial cells, we subjected IMCD-3 cells to mechanical wounding. As shown in
Fig. 6. Mechanical wounding resulted in upregulation of EphA2 protein expression. Upregulation of EphA2 by mechanical wounding was prevented by treatment with the Src inhibitor PP2 (Fig. 6D). In MDCK cells, neither treatment with H2O2 nor mechanical wounding caused significant upregulation of EphA2 and treatment with PP2 did not inhibit basal expression of EphA2 (data not shown). However, transient, chemically induced ATP depletion in these cells resulted in a...
strong upregulation of EphA2 expression, which was again prevented by treatment with PP2 (Fig. 6E). In both MDCK and IMCD-3 cells, upregulation of EphA2 was observed following hypoxia-reoxygenation produced in a hypoxic chamber (Fig. 7). PP2 inhibited the hypoxic induction of EphA2 in both cell lines (Fig. 7, B and C). However, in IMCD-3 cells, the effect of PP2 was very potent on the sustained upregulation of EphA2 (at 24 h), but negligible on the early phase of induction, suggesting that both Src-dependent and -independent mechanisms might be involved at different stages of hypoxic induction.

Src Kinases Regulate EphA2 Transcription

To further explore the mechanisms of Src kinase-induced upregulation of EphA2, we performed reporter gene assays with an EphA2 promoter-luciferase construct (12). As shown in Fig. 8A, cotransfection of EphA2-luc with wild-type but not kinase-negative Fyn caused transcriptional activation of the promoter. Similar results were obtained with Src, whereas the unrelated tyrosine kinase Jak-2 was neutral or even inhibitory. More dramatic transcriptional activation of the EphA2 promoter was observed with constitutively activated (Y528F) Fyn (Fig. 8B). In contrast, ligand-induced stimulation of the receptor tyrosine kinases Ret, PDGFR-β, and EGFR did not significantly alter EphA2 promoter activity (Fig. 8, B and C), although it strongly induced Elk-1-luciferase activity (Fig. 8C and data not shown). Because activation of the Erk MAP kinase pathway appeared necessary for Fyn-induced upregulation of EphA2 (Fig. 5B), we examined whether strong activation of this pathway through constitutively active Ras (RasV12) might be sufficient for activation of the EphA2 promoter. As shown in Fig. 8D, expression of both Fyn Y528F and RasV12 resulted in strong activation of an Elk-1 reporter (an indirect measure of Erk activation), but only Fyn Y528F was able to activate the EphA2 promoter. Activated Fyn was also able to upregulate EphA2 promoter activity in MDCK renal tubular cells (Fig. 8E).

Ephrin-A Stimulation Induces EphA2 Activation and Downstream Signaling in Renal Epithelial Cells

To determine whether endogenous EphA2 was functional in renal epithelial cells, we examined its capacity to undergo tyrosine phosphorylation following stimulation with a soluble form of one of its ligands, ephrin-A1. Treatment with cross-linked, soluble ephrin A1-Fc resulted in tyrosine phosphorylation of EphA2 in IMCD-3 cells (Fig. 9A). Ligand-induced
tyrosine phosphorylation of EphA2 was also demonstrated in canine MDCK renal tubular cells (Fig. 9, B and C) and in rat GEC (Fig. 9D). In addition, we observed increased tyrosine phosphorylation of the focal adhesion protein paxillin (Fig. 9C) and of an unidentified ~65-kDa protein (Fig. 9, B and C) after treatment with ephrin-A1 in MDCK cells. The unidentified ~65-kDa protein did not correspond to p62Dok (data not shown), a protein previously reported to undergo phosphorylation on activation of EphB2 in neuronal cells (17).

Stimulation by A type ephrins has previously been shown to inhibit growth factor-induced depolymerization of cortical actin in MDCK cells (31). Consistent with this observation, we found that in MDCK cells transiently transfected with EphA2, treatment with ephrin-A1-Fc strongly increased cytosolic and cortical F-actin staining (Fig. 9, E and F).

**DISCUSSION**

Increasing evidence exists for the role of actin cytoskeletal damage in the pathogenesis of IRI. However, there has been limited study of the pathophysiological roles of actin-modulating receptors in renal IRI. The purpose of the current study was to identify novel mechanisms potentially involved in the regeneration of tubular renal epithelium after ischemic injury. We hypothesized that Eph family receptor tyrosine kinases, which are critically implicated in cytoskeletal remodeling during embryonic development, might be important for tissue repair following renal IRI. We found that at least one Eph family member, EphA2, was rapidly, intensely, and persistently upregulated in a mouse model of renal IRI (Fig. 1). Basal expression of EphA2 protein seemed concentrated in distal tubular segments, but IRI resulted in more intense and generalized expression of EphA2 in tubules of the ischemia-sensitive corticomedullary junction (Fig. 3). A dramatic and sustained upregulation of EphA2 was also observed in cultured renal tubular epithelial cells following stimulation with hydrogen peroxide (Fig. 4) and, less prominently, after mechanical wounding (Fig. 4, C and D) and ATP depletion/repletion (Fig. 6E). Hypoxia-reoxygenation was a strong inducer of EphA2 upregulation in both MDCK and IMCD-3 cells (Fig. 7). Notably, overexpression of Fyn induced upregulation of endogenous EphA2 (Fig. 5) and treatment with the pharmacological Src kinase inhibitor PP2 attenuated EphA2 upregulation induced by H2O2, mechanical wounding, ATP depletion/repletion (Fig. 6) and hypoxia-reoxygenation (Fig. 7). Src kinases also strongly activated the human EphA2 promoter (Fig. 8), suggesting that they upregulated EphA2 expression through a transcriptional mechanism. Although activation of the Erk MAP kinase pathway contributed to upregulation of EphA2 by Src kinases (Figs. 5B and 6), strong activation of this pathway through growth factor stimulation or overexpression of activated Ras was not sufficient to induce EphA2 promoter activity (Fig. 8, B-D). Therefore, activation of the Erk pathway was necessary but not sufficient for induction of EphA2 transcription, suggesting that additional intracellular signals triggered specifically by Src kinases were required for EphA2 upregulation. EphA2 was functional in renal tubular cells as stimulation with ephrin-A1 led to tyrosine phosphorylation of EphA2, paxillin, and an unidentified ~65-kDa protein (Fig. 9, A-D), as well as increased cortical actin polymerization (Fig. 9, E and F).
Until now, overexpression of EphA2 appeared to be a phenomenon restricted to cancer cells. Numerous groups had reported upregulation of EphA2 mRNA or protein in a variety of tumor types, usually in correlation with metastatic spread and poor prognosis (25), perhaps because EphA2 is capable of inducing VEGF production, thus promoting neovascularization (11).

Two very recent studies reported the prominent upregulation of EphA2 expression under distinct stress conditions using gene-profiling approaches. Specifically, EphA2 upregulation has been observed in the liver of rats exposed to bacterial lipopolysaccharides (21) and in the renal medulla and in cultured IMCD-3 cells in response to hypertonicity and urea stress (40, 56). Upregulation of EphA2 has also been noted in epithelial cells treated with the detergent deoxycholic acid (28). Taken together with the data reported herein, it appears that upregulation of EphA2 is a major and previously unrecognized feature of cellular responses to diverse injuries.

Interestingly, Src kinases have been implicated in the intracellular signaling responses to both lipopolysaccharide (29, 50) and osmotic stress (9, 23). Therefore, in view of the results reported herein, Src kinase activation may provide a common link between diverse noxious stimuli and EphA2 upregulation. However, proof that Src kinases are responsible for the observed in vivo upregulation of EphA2 in our model of renal IRI will require additional studies.

One previously identified mechanism of EphA2 promoter activation is through p53 (12). Regulation of EphA2 expression has also been known to occur through p53-independent pathways (28, 56), including the Erk MAP kinase pathway (28, 40). Consistent with this, we observed that treatment with the MEK inhibitor U0126 partially inhibited Fyn-induced EphA2 upregulation. Further studies will be needed to determine whether p53 might also contribute to Src-induced EphA2 upregulation.

Fig. 8. EphA2 promoter responds to Src kinase activity. A luciferase reporter construct containing the ∼2-kb human EphA2 promoter region was transiently transfected in triplicate in 293 (A–D) and MDCK cells (E). Dual luciferase assays were performed 48 h after transfection and normalized promoter activity was reported as the ratio of firefly to Renilla luciferase activity, as described in METHODS. A: transfection of 293 cells with of 50 ng of EphA2-luciferase together with 400 ng of either empty vector, wild-type (WT) Fyn, kinase-negative (KN) Fyn, Src, or Jak2. B: EphA2-luciferase (50 ng) was cotransfected in 293 cells with 100 ng of either activated (Y528F) Fyn or wild-type Ret or PDGFR-β, followed by stimulation with the corresponding ligands (GDNF and GFRα1 for Ret; PDGF-bb for PDGFR-β) during the last 24 h of culture. C: parallel luciferase assays were carried out in 293 cells to compare EphA2 promoter activity (filled bars) and activation of the transcription factor Elk-1 (open bars) induced by Fyn Y528F vs. the ligand-stimulated receptor tyrosine kinases EGFR and PDGFR-β. D: similar comparison of Eph-luciferase and Elk-1-luciferase activities induced by Fyn Y528F vs. activated Ras (Ras V12). E: activation of EphA2 promoter activity by Fyn Y528F in MDCK cells. A–E: representative of at least 3 independent experiments.
a cell contact-dependent structural cue for organized tissue repair (42, 48). Similarly, EphA2 might regulate cell extracellular matrix adhesion, either positively or negatively (19, 20, 30). In the current study, we found that stimulation of MDCK cells with ephrin-A1 increased the tyrosine phosphorylation of the focal adhesion protein paxillin (Fig. 9C). However, the impact of this event on cell adhesion and movement has not yet been defined.

Another potential function of Eph receptors in ischemic epithelial cells is the regulation of cell-cell junctions. Indeed, stimulation of intestinal epithelial cells with ephrin-A1 has been reported to improve recovery of barrier function follow-
ing injury with bile salts (46). This observation suggests a potentially important role for EphA receptors in the context of renal IRI where alterations of cell-cell junctions are a prominent feature (5, 6, 26). Our studies showed that treatment with the EphA2 ligand ephrin-A1 enhanced cortical F-actin staining in EphA2-transfected cells (Fig. 9, E and F). This suggests that stimulation through upregulated EphA2 could stabilize cell-cell junctions in regenerating epithelia by increasing anchorage of adherens junction complexes to the actin cytoskeleton. Further studies will be required to determine whether the observed changes in cortical F-actin induced by ephrin-A1 indeed translate into increased stability of cell-cell adhesion structures or increased transepithelial resistance.

In summary, we found that, under a variety of injurious conditions, including those mimicking IRI, EphA2 expression is strongly upregulated in epithelial cells through a transcriptional mechanism involving Src kinase activation.

Ephrin-A1-induced activation of EphA receptors resulted in phosphorylation of the focal adhesion molecule paxillin and increased cytosolic and cortical F-actin staining, suggesting a possible role for Eph/ephrin signaling in the regulation of membrane-cytoskeletal anchoring at sites of cell-matrix and cell-cell adhesion. Therefore, in the context of IRI, EphA2 upregulation provides an opportunity for cell contact-dependent, bidirectional signaling events that directly impact actin cytoskeletal reorganization and epithelial function during recovery.

Finally, the emerging evidence that ephA2 behaves as an injury- and stress-responsive gene suggests that its main functions are carried out during responses to cellular challenges. This could help explain the lack of an obvious phenotype in unchallenged EphA2-deficient mice (7, 41). Studies of EphA2 deficiency in disease models such as IRI will be needed to examine this possibility and to more directly determine the role of EphA2 signaling in tissue repair processes. In addition, it will be necessary to more definitively establish which tubular segment(s) is(are) involved in EphA2 expression and upregulation and to correlate EphA2 expression with functional markers of injury, such as tubular apoptosis.

ACKNOWLEDGMENTS

We thank G. Zaaour and V. Savransky for technical assistance, Dr. X. Chen for the gift of the EphA2-luciferase construct, Dr. S. Hussain for generous assistance with hypoxia-reoxygenation experiments, Dr. J. Cooper for the RasV12 cDNA, Dr. A. Ullrich for the EphB4 cDNA, Drs. K. Murai and A. Cybulsky for helpful discussions, and Dr. J.-J. Lebrun for support in the generation of edcysone-inducible cell lines.

Present address of S. H. Nasr: Dept. of Pathology, Columbia University College of Physicians and Surgeons, New York, NY 10032.

GRANTS

This work was supported by grants to S. Lemay from the Canadian Institutes of Health Research (CIHR) and Kidney Foundation of Canada. S. Lemay is the recipient of a CIHR New Investigator Award.

REFERENCES


