Basal and Src Kinase–Mediated Activation of the EphA2 Promoter Requires a cAMP-Responsive Element but is CREB-Independent

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ABSTRACT

We have previously identified the EphA2 receptor tyrosine kinase as a potentially important injury-responsive gene and a transcriptional target of Src kinase activity in renal ischemia-reperfusion injury (IRI). In the present study, we confirmed, using EphA2 gene trap mice that the endogenous EphA2 promoter is strongly activated following renal IRI. We also examined in more detail the mechanisms responsible for Src kinase-induced activation of the −2 kb human EphA2 promoter and found that the minimal Src-responsive elements were contained in the −145 to +137 region of the human EphA2 gene. This region contains a canonical cAMP-responsive element (CRE) that we found to be critical for both basal and Src kinase-induced transcriptional activity. However, despite activation of the prototypical CRE-binding factor CREB by the Src kinase Fyn, siRNA-mediated knockdown of CREB had no significant impact on either basal or Fyn-induced EphA2 promoter activity. Similarly, activation of CREB by the adenylate cyclase agonist forskolin failed to induce EphA2 promoter activation. Thus, Src kinase-induced activation of the EphA2 promoter is CRE-dependent but CREB-independent. J. Cell. Biochem. 112: 1268–1276, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: Eph A2; Src KINASES; Fyn; CREB; CYCLIC AMP-RESPONSIVE ELEMENT; TRANSCRIPTIONAL REGULATION; MDCK CELLS; ACUTE KIDNEY INJURY; ISCHEMIA/REPERFUSION INJURY

Eph receptors are a developmentally regulated group of molecules that constitute the largest family of receptor tyrosine kinases and that perform well-defined roles as chemorepulsive signals in neuronal and vascular development [Flanagan and Vanderhaeghen, 1998; Gale and Yancopoulos, 1999; Murai and Pasquale, 2003]. Eph receptors engage membrane-bound ligands called ephrins and therefore signal in a cell–cell contact-dependent manner. Intracellular signaling by Eph receptors is characterized by the recruitment and activation of an expanding repertoire of guanine nucleotide exchange factors (GEFs) that modulate Rho GTPase activity and thus cytoskeletal functions [Murai and Pasquale, 2005]. Beyond their role in development, Eph receptors are increasingly being ascribed diverse roles in adult organisms, ranging from the sorting of intestinal epithelial cells [Batlle et al., 2002] to the control of insulin release in pancreatic islet cells [Konstantinova et al., 2007], and their importance in epithelial physiology is becoming more widely recognized [Miao and Wang, 2009].

In contrast to most other Eph family members, EphA2 is primarily expressed outside of neuronal tissues. Moreover, EphA2 is frequently overexpressed in metastatic cancer [Kinch and Carles-Kinch, 2003], where it is thought to promote angiogenesis [Dobrzanski et al., 2004] and loosening of cell–cell junctions [Fang et al., 2008]. We have recently reported that EphA2 is upregulated in a rapid and sustained manner in renal tubular cells following in vivo or in vitro renal ischemia-reperfusion injury (IRI) [Baldwin et al., 2006]. Indeed, EphA2 upregulation may be an event common to many organs under a wide range of stress and injury conditions [Li et al., 2003; Ivanov et al., 2005; Xu et al., 2005; Zhang et al., 2008], and Src kinase activation may be a common link between diverse noxious stimuli and EphA2 upregulation.

Surprisingly little is known about the transcriptional mechanisms responsible for EphA2 upregulation. The tumor suppressor p53 has been shown to activate the human EphA2 promoter through a canonical p53 binding site located 1.5 kb upstream of the transcriptional start site [Dohn et al., 2001]. More recently, the

existence of a putative novel p53-binding element has been reported in the proximal promoter region of EphA2 [Jin et al., 2006]. Upregulation of EphA2 has also been reported to occur through p53-independent pathways [Xu et al., 2005], including through the Erk MAP kinase pathway [Nahm et al., 2002; Li et al., 2003], although the promoter elements responsible for these responses have not been examined.

Vihanto et al. [2005] have also reported on the upregulation of several ephrins and Eph receptors, including EphA2, in hypoxic skin through the action of HIF-1α. However, it was not determined whether this represented a direct or indirect effect of HIF-1α on the EphA2 promoter.

Wnt signaling regulates reciprocal gradients of ephrin-B and EphB receptor expression along the intestinal crypt-villus axis [Batlle et al., 2002]. Putative binding sites for the Wnt-responsive TCF/LEF transcription factors have been found in the promoter regions of several Eph and ephrin family members, including one in EphA2 [Katoh, 2006]. Furthermore, in mice with an Apc mutation causing exaggerated Wnt signlaing and intestinal polyps, increased EphA2 expression has been reported in polyps, but this appeared to represent an indirect effect mediated through upregulated Myc [Yekkala and Baudino, 2007].

The identity of transcription factors responsible for Src kinase-mediated upregulation of EphA2 is unknown. The direct involvement of p53, TCF/LEF, Myc, or HIF-1α has yet to be tested. In addition to these transcription factors, number of other transcription factors could be considered as candidate mediators of IRI and Src kinase-induced transcriptional responses. Among them is CREB, which has recently been shown to undergo activation in response to oxidant stress and to exert protective actions in acute kidney injury [Arany et al., 2005, 2008]. CREB binds primarily cAMP-responsive elements (CREs), which consist of eight-nucleotide palindromes initially described in the somatostatin promoter [Montminy et al., 1986].

In the present study we attempted to define the importance of promoter activation in IRI-induced upregulation of EphA2 in vivo and to define the regulatory elements required for Src kinase-mediated activation of the promoter in cultured cells. We identified a conserved canonical CRE as a key determinant of both basal and Src kinase-induced EphA2 promoter activation. We also found that the Src kinase Fyn strongly activated CREB. However, surprisingly, CREB was completely dispensable for Fyn-induced activation of EphA2 promoter activity.

METHODS

IN VIVO RENAL ISCHEMIA–REPERFUSION INJURY (IRI)

EphA2$^{+/-}$ gene trap mice [Mitchell et al., 2001] were obtained from Dr. Marc Tessier-Lavigne (University of California and Howard Hughes Medical Institute). In vivo renal IRI was performed by clamping both renal pedicles for 30 min with a microvascular clamp (Roboz Surgical Instruments, Washington, DC) under isoflurane anesthesia. After 24 h of reperfusion, animals, were re-anesthetized for cardiac puncture and then killed by cervical dislocation. The left kidney was harvested and 1/3 was frozen in OCT compound for storage at $-80°C$ and subsequent preparation of tissue sections.

Acute renal failure was confirmed by serum creatinine measurement and histology (data not shown). Animal studies were approved by the institutional animal review committee.

IN SITU β-GALACTOSIDASE ASSAYS

For in situ detection of β-galactosidase activity, 7 μm cryosections of mouse kidney were prepared in a Microm cryostat and laid onto gelatin-coated slides. These were then fixed in cold formalin for 10 min, washed three times, and incubated overnight in a wet chamber at room temperature with X-gal (1 mg/ml) in PBS containing 2 mM MgCl$_2$, 5 mM potassium ferricyanide, and 5 mM ferrocyanide (from Sigma). On the following day, sections were washed in PBS for 10 min, counterstained with Nuclear Fast Red (Sigma), and mounted with coverslips using GelTol (Fisher Scientific).

ALIGNMENT AND ANALYSIS OF EphA2 PROMOTER SEQUENCES

Genomic sequences located within 1 kb of the atg of EphA2 genes from human (NW_00183864), mouse (NW_001030763), rat (AC_000073), dog (NC_006584), and cow (NC_007300) were retrieved from NCBI and compared two-by-two using the blastn program to define conserved regions. The highly conserved proximal sequences of all five genes (-120 to $+33$ in human) were then aligned together using the Megalign program (DNA Star). A search for transcription factor binding sites within the human promoter sequence was performed with MatInspector and Transfac.

CELLS

Parental 293 HEK cells, 293 IND-Fyn cells [Baldwin et al., 2006], and MDCK-TetOFF cells (from BD-Clonetech) were cultured in DMEM-high glucose containing pyridoxine–HCl and sodium pyruvate (Gibco-Invitrogen) with 10% FBS (Gibco-Invitrogen). Caco-2 cells were grown in alpha-MEM (Gibco-Invitrogen) containing 10% FBS.

PLASMIDS AND cDNAs

The pME18S vector containing the cDNA for activated mouse Fyn (Y528F) were from Dr. T. Yamamoto as described previously [Bedirian et al., 2004]. The $-1880/+137$ human EphA2-luciferase promoter construct (pGL2-EphA2) was obtained from Dr. X. Chen [Dohn et al., 2001]. The $-145/+137$ truncated construct was obtained by restriction enzyme digestion with Sma I. The $-1519/+137$ truncated constructs as well as point mutants of the p53RE and CRE sites were generated by PCR. The p53RE mutation changed four critical residues (underlined) in the -1530 sequence CACCATGTTGGCCAGGCATGTCT to CACAATTTTGGCCAGGAATT TCT. The CRE mutation changed the -47 palindrome TGACGTCA to TCAACAGA (mutations underlined). The cDNA for the catalytic subunit of PKA, the pFA2-CREB plasmid (encoding a Gal4-CREB transactivation domain), and the pFR-Luc plasmid (encoding Gal4-binding DNA sequences placed upstream of the firefly luciferase cDNA) were obtained from Stratagene/Agilent.

RNAi

Non-targeting siRNA #1 (Dharmacon) was used as negative control; a dog Csk-specific siRNA targeting the sense sequence GGAA-

CAAGGTTGCTGTCAA was designed using the on-line siRNA design tool from Dharmacon and was obtained from the same company. The siRNA was delivered into MDCK-TetOFF cells using Dharmafect #1 reagent. Cells were lysed after 48 h.

CHEMICALS
X-gal was from Invitrogen and was dissolved in dimethylformamide at a stock concentration of 20 mg/ml. Ponasterone A was from A.G. Scientific and was dissolved in methanol at a stock concentration of 10 mM. Forskolin was from Sigma and was dissolved in DMSO at a stock concentration of 10 mM.

ANTIBODIES AND IMMUNOBLOTTING
Antibodies for EphA2, c-Abl, c-Src, Fyn, Csk, and CREB were from Santa Cruz. Anti-p53 antibody was from BD Biosciences and anti-α-tubulin antibody was from Sigma. Anti-phospho-Src (Y416) antibody was from Cell Signaling. Horseradish peroxidase-coupled secondary antibodies were from Jackson Immunoresearch. Cell lysis and immunoblotting were performed according to standard protocol as detailed previously [Bedirian et al., 2004]. Protein concentration was estimated using a standard Bradford assay (BioRad) so that equal amounts of lysate (30 or 40 μg/lane) could be loaded for each experimental condition. Blots were revealed using ECL or ECL Plus reagents (GE Healthcare).

LUCIFERASE ASSAYS
Parental 293 HEK cells were transiently transfected in triplicates on 24-well plates using Fugene 6 and Lipofectamine 2000 reagents, respectively. In addition to EphA2-luciferase construct, the pRL-null *Renilla* luciferase vector (Promega) was included for normalization. Empty vector was added where necessary to equalize the amounts of transfected DNA. Cells were lysed 48 h after transfection and luciferase activity was determined using the Dual-Luciferase Reporter system (Promega) on a Lumat LB 9507 luminometer (Berthold). Reporter gene activity was expressed as the ratio of firefly to *Renilla* luciferase activity+/− standard deviation. The signal ratio obtained with the empty pGL2 vector was used as the zero and was always several-fold lower than the signal ratio obtained with Eph-luciferase construct (data not shown). For experiments in 293 IND-Fyn cells, simultaneous delivery of siRNA and plasmids was performed with the Extreme Gene reagent (Roche). After 24 h, Fyn expression was induced with ponasterone A (5 μM) and cells were lysed after 48 h.

RESULTS

EphA2 PROMOTER ACTIVITY IS STRONGLY INDUCED IN RENAL TUBULES FOLLOWING ISCHEMIA–REPERFUSION INJURY
In a previous study of renal IRI, we had observed a striking overexpression of EphA2 protein in renal tubular cells, particularly within the ischemia-sensitive cortico-medullary area [Baldwin et al., 2006]. To determine whether this IRI-induced upregulation could be attributed to activation of the EphA2 promoter, we subjected heterozygous EphA2 gene trap mice [Skarnes et al., 1995; Mitchell et al., 2001] to renal IRI. In these mice, one epha2 allele is disrupted by insertion, between exons V and VI, of a βgeo cassette containing

a splice acceptor. As a result, the mice express intracellular β-galactosidase under the control of the endogenous EphA2 promoter. EphA2 promoter activity was detected in situ using X-gal staining in kidneys harvested from mice subjected to unilateral left kidney IRI. As shown in Figure 1, both basal and upregulated β-galactosidase activities were observed primarily in renal tubules. In the sham operated kidney, β-galactosidase signal was strong and homogeneous in the papilla but weak and dispersed in the cortex and medulla (Fig. 1A,B). In contrast, in reperfused kidneys, β-galactosidase activity increased dramatically outside of the papilla and was most intense in the ischemia-sensitive outer medulla (Fig. 1C,D), consistent with the pattern of upregulated EphA2 protein we had described previously by immunhistochemistry [Baldwin et al., 2006]. In both sham and IRI kidneys, the X-gal signal appeared confined to tubular structures.

To confirm EphA2 protein upregulation in renal IRI, we obtained kidneys from mice subjected to unilateral IRI, dissected the cortico-medullary region (removing cortex, papilla, and inner medulla) and examined EphA2 expression by immunoblotting. As shown in Figure 1E, EphA2 protein was indeed highly overexpressed in IRI compared to sham kidney.

THE MINIMAL ELEMENTS REQUIRED FOR Src–RESPONSIVENESS ARE CONTAINED IN THE −145 TO +137 REGION OF THE HUMAN EphA2 GENE
We had previously demonstrated that expression of an activated Src kinase was sufficient to activate the −1880/+137 human EphA2-luciferase construct in both 293 HEK cells and in MDCK renal tubular cells [Baldwin et al., 2006]. To better delineate the minimal promoter region necessary for responsiveness to Src kinases, we performed 5′ truncations of this construct (Fig. 2A) and measured the transcriptional activity of the resulting constructs in response to activated Fyn (Y528F) or empty vector. As shown in Figure 2B, the −1519/+137 construct, where an upstream region including a canonical p53 responsive element centered around −1530 of the TSS was removed [Dohn et al., 2001] displayed decreased basal promoter activity (−28%) and Fyn-induced promoter activity (−62%). A much larger truncation preserving only the −145/+137 region caused a more substantial decrease in basal promoter activity (47%) but did not further reduce Fyn-induced activation. This suggested that the key elements necessary for responsiveness to Src kinases are contained in the −145/+137 region of the human EphA2 promoter. Interestingly, this region of the EphA2 promoter contained neither a hypoxia-responsive element nor a candidate binding sites for Myc. A putative TCF/LEF binding site was present in the −2 kb EphA2 promoter [Katoh, 2006], but it was located well outside of the −145/+137 region.

THE MINIMAL EphA2 PROMOTER CONTAINS A cAMP–RESPONSIVE ELEMENT (CRE) THAT IS CRITICAL FOR BASAL AND Src–INDUCED ACTIVITY OF THE EphA2 PROMOTER
The above result was consistent with alignment of the human, mouse, rat, dog, and cow EphA2 promoter regions, which defined a highly conserved sequence located between base pairs −120 and +33 of the human gene to be by far the most highly conserved (Fig. 3A). In contrast, neither the upstream p53RE nor the region
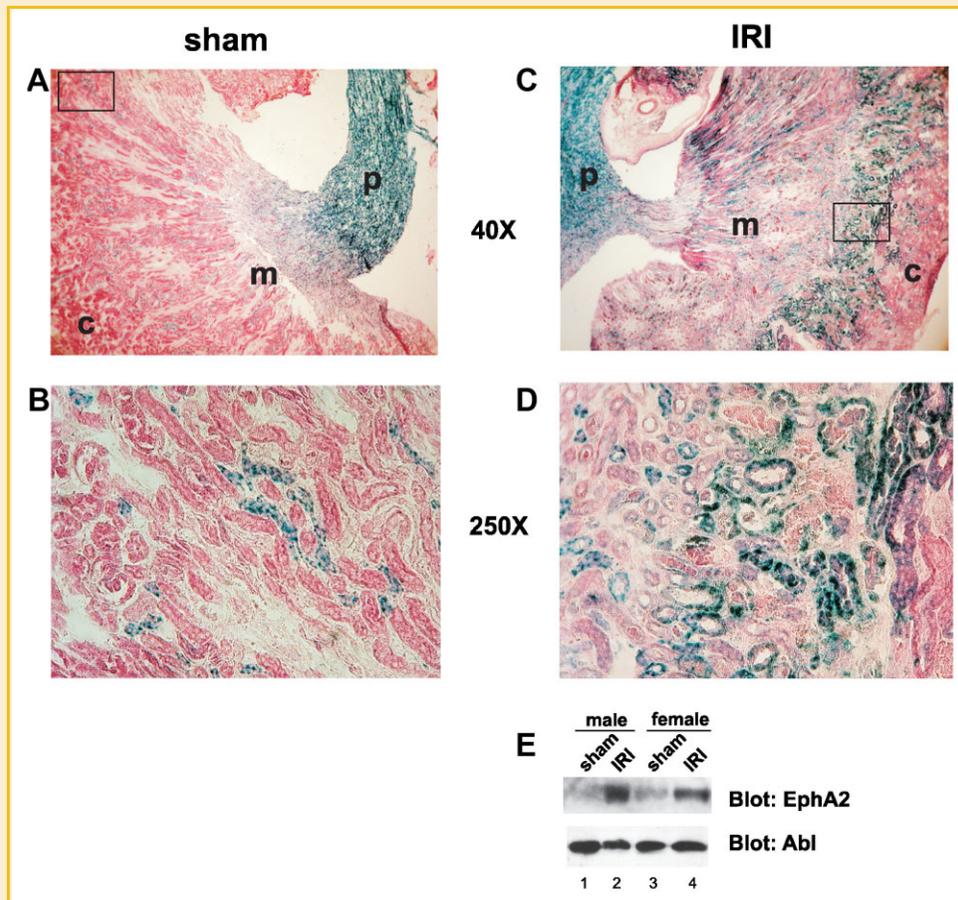
Fig. 1. Upregulation of EphA2 promoter activity and protein expression in ischemic kidney. An heterozygous EphA2 gene trap mouse was subjected to unilateral left renal ischemia/reperfusion injury and both kidneys were harvested 24 h after reperfusion. Frozen kidney sections were stained with X–gal to reveal β–galactosidase activity. Photographs were taken at magnifications of 40X (A, C) and 250X (B, D) using a Nikon Coolpix camera mounted on a Leica Laborlux K microscope. A,B: In the sham operated right kidney, β–galactosidase was highest and most homogeneous in the papilla (p) with more scattered signal in the medulla (m) and cortex (c). C,D: In the kidney subjected to IRI, EphA2 promoter activity increased dramatically in renal tubules of the cortico–medullary junction. The rectangles in (A,C) represent the areas shown at high magnification in (B,D), respectively. Photos are representative of more than five independent experiments. E: Kidneys were obtained from wild type C57BL/6 mice subjected to unilateral IRI and the cortico–medullary region was dissected out and lysed to detect EphA2 and c–Abl by immunoblotting. Anti–c–Abl immunoblotting was used here as a loading control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

surrounding it is conserved in the -2 kb upstream of the mouse transcriptional start site (data not shown). Sequence analysis of the highly conserved proximal region with MatInspector (Genomatix) and JASPAR revealed the existence of several putative binding sites for transcription factors. However, only a few very short sequences were fully conserved across five species (Fig. 3A). The most highly conserved putative binding site was a canonical CRE (TGACGTCA) located at around position -47 relative to the human transcriptional start site. Additional putative binding sites displaying relative conservation across species were those for KLF4, TEAD1/4, Sp1, HBP1, and Stat1 (Fig. 3A and S1 of Supporting Information). Interestingly, the CRE sequence was contained within a 10-nucleotide palindrome (GTGACGTCAC) that had recently been proposed to function as a novel p53 binding site [Jin et al., 2006], but sequence alignment showed that only the first nine nucleotides of the palindrome (including the eight nucleotide CRE) were conserved across species (C in human and cow becomes G in mouse, rat, and dog). To investigate the role of this conserved CRE, we mutated it

within the -2 kb construct. This mutation resulted in a substantially reduced basal promoter activity (-61%) and in an even more dramatically reduced Fyn-induced transcriptional activity (-88%; Fig. 3B). In contrast, mutation of the -1530 canonical p53RE resulted in a consistent but more modest reduction in basal and Fyn-induced promoter activity (-18% and -42%, respectively).

When the CRE was mutated within the context of the $-145/+137$ promoter construct, the reduction of basal and Fyn-induced promoter activity was equally important (-40% and -82%, respectively; Fig. 3C). Taken together these results suggest that the major Src kinase-responsive elements are contained in the $-145/+137$ region of the EphA2 gene and that a conserved canonical CRE is a critical component for both basal and Src kinase-induced activity.

We had previously shown that activated Fyn was sufficient to cause activation of the EphA2 promoter in MDCK renal tubular cells, as is the case in 293 cells [Baldwin et al., 2006]. Similarly, we found that the CRE mutation abolished the activation of the EphA2
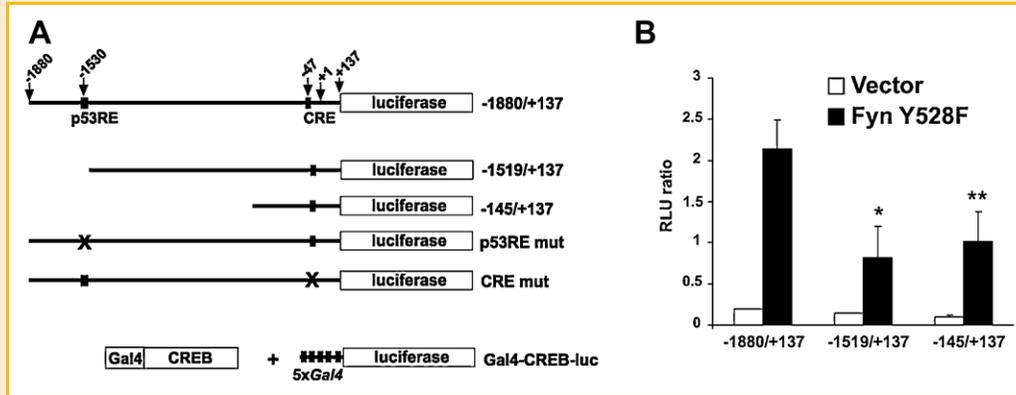
Fig. 2. Structure of the EphA2 promoter. A: Reporter gene constructs used in the current study. A luciferase reporter comprising the −1880/+137 bp of the human EphA2 gene was used as the basic EphA2 reporter construct. It contains a p53RE at −1530 bp and a CRE at postion −47 bp. Two 5′truncations were obtained. −1519/+137 and −145/+137, which both lacked the p53RE. Full–length constructs containing a point mutation of either the p53RE (p53RE mut) or CRE (CRE mut) were also obtained. In some experiments, CREB activation was assessed by co–transfection of a gal4–luciferase reporter and a chimera comprising the DNA–binding domain of Gal4 and the transactivation domain of CREB (Gal-4–CREB–luc). B: Transfection with activated Fyn increased EphA2 luciferase activity. Activation was preserved, albeit at attenuated levels, even in the −145/+137 deletion construct (n = 3 independent experiments; $^*P = 0.02$; $^{**}P = 0.03$).

promoter in response to Fyn overexpression in MDCK cells (data not shown). To show that Src kinases could also regulate the endogenous EphA2 promoter in these cells, we activated endogenous Src kinases using dog Csk-specific siRNA and performed immunoblotting studies. As shown in Figure 3D, Csk siRNA appropriately reduced Csk expression and caused an activation of Src kinases as determined by anti-phospho-Src (Y416) immunoblotting. This was accompanied by a striking increase in EphA2 expression as well.

CRE's are present in multiple promoters and often, though not always confer responsiveness to cAMP. We therefore examined the impact of the adenylate cyclase activator forskolin on expression EphA2 promoter activity. As shown in Figure 4A, compared to activated Fyn, forskolin did not alter EphA2 promoter activity. Similarly, over a range of concentrations from 5 to 100 μM, forskolin failed to enhance expression of endogenous EphA2 protein in 293 cells (data not shown). Failure of forskolin to activate the EphA2 promoter was not due to a failure to activate CREB, as both forskolin and even more so Fyn caused increased transactivation by a Gal4-CREB chimera (Fig. 4B).

CREB IS DISPENSABLE FOR EphA2 PROMOTER ACTIVITY

The prototypical transcription factor binding to CRE is a homodimer or heterodimer comprised of CREB or related basic leucine zipper (bZIP)-containing transcription factors [Montminy, 1997; Mayr and Montminy, 2001; Vinson et al., 2006]. CREB is activated by a number of stimuli, including elevation of cAMP. Failure of the EphA2 promoter to respond to the adenylate cyclase agonist forskolin suggested that CREB was not sufficient to activate this promoter. To determine whether CREB was necessary for EphA2 responsiveness to Fyn, we used siRNA-mediated knockdown. As shown in Figure 5A, compared to control siRNA, CREB-specific siRNA resulted in an approximately 75% reduction in CREB protein expression (as assessed by densitometry analysis with the ImageJ software). However, this resulted in a small increase in basal EphA2

promoter activity and had no significant impact on responsiveness to Fyn. Similarly, siRNA-mediated knockdown of p53 had no significant impact on Src-induced activation of the −1880/+137 EphA2 promoter.

Our initial knockdown strategy involved two successive transfections (siRNA followed 24 h later by plasmids), and we wondered whether the sequential transfection strategy might have altered Src kinase-mediated responses in our cells. We therefore repeated similar experiments in 293 cells expressing ecdysone-inducible Fyn, which permitted simultaneous delivery of siRNA and plasmids in a single transfection step and subsequent induction of Fyn overexpression with ponasterone A. In these experiments, luciferase-specific siRNA was included as a positive control of siRNA-mediated knockdown. Consistent with results obtained in sequentially transfected cells, neither CREB-specific nor p53-specific siRNA had any impact on Fyn-mediated activation of the EphA2 promoter in this system (Figure 5B). Similarly, CREB-specific siRNA had no consistent impact on ponasterone-induced overexpression of endogenous EphA2 in these cells (data not shown).

DISCUSSION

EphA2 is strongly upregulated at the mRNA and protein level in both in vivo and in vitro models of renal IRI. In vitro, Src kinase activity is both necessary and sufficient for activation of the EphA2 promoter, but the mechanism of this transcriptional event remains incompletely defined [Baldwin et al., 2006]. In the current study we have demonstrated that in vivo renal IRI causes strong activation of the endogenous EphA2 promoter in tubules of the ischemia-sensitive outer medulla. This confirms that promoter activation is a key determinant of EphA2 upregulation in this model. Our studies also demonstrated that a −47 bp CRE was critical for both basal and Src kinase-induced activity of the EphA2 promoter in cultured cells. This was not entirely surprising given recent evidence that the major
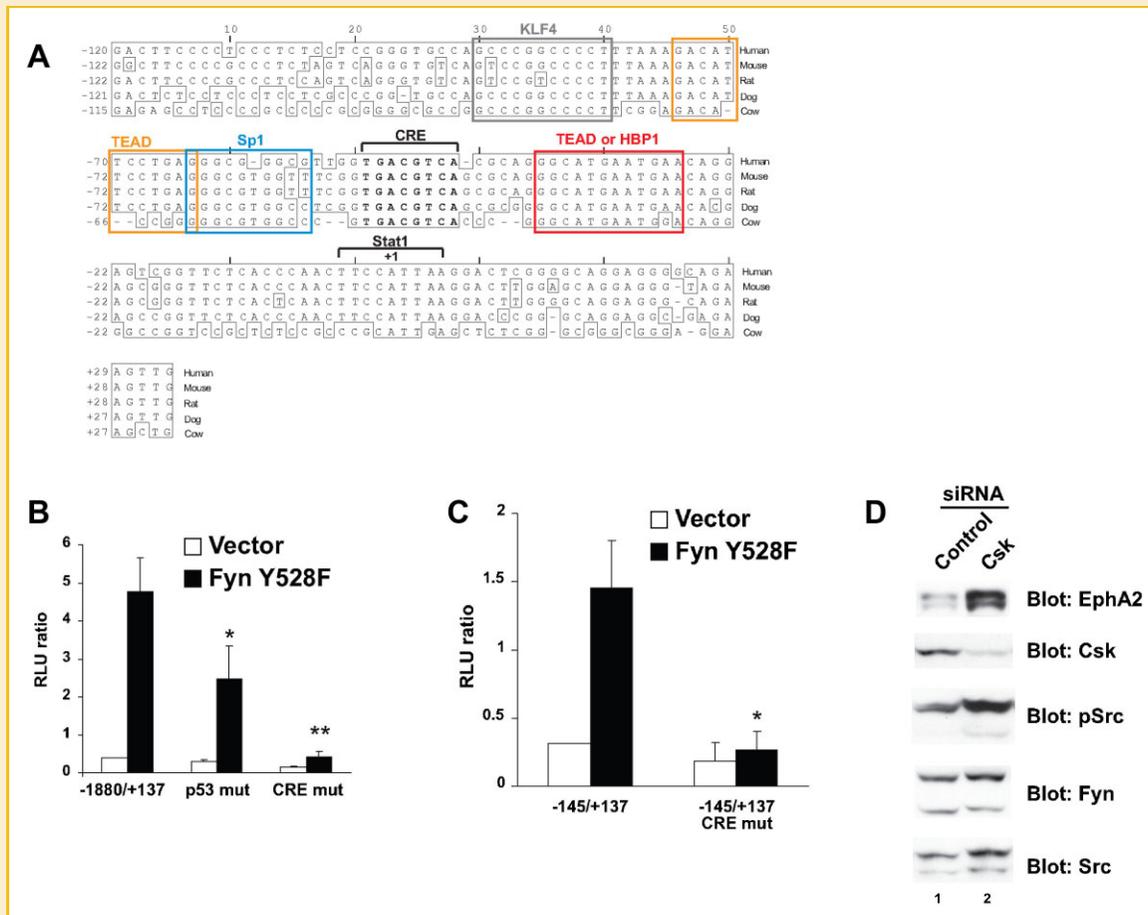
Fig. 3. Role of the −145/+137 proximal promoter region and −47 bp CRE in activation of the EphA2 promoter by Fyn. Luciferase assays were performed in 293 cells transiently co-transfected with the indicated reporters and either empty vector (white bars) or activated Fyn (black bars), as described in Methods. A: Sequence of a highly conserved region surrounding the transcription initiation site of EphA2. Two-by-two alignment of human, mouse, rat, dog, and cow genomic sequences located up to 1 kb upstream of the translation initiation codon was performed and identified the following highly conserved region. Alignment of the five conserved sequences was then performed. +1 refers to the transcription start site. A fully conserved CRE centered at −47 is highlighted in bold. In addition to this CRE, detailed analysis with JASPAR and Genomatix MatInspector reveal additional putative binding sites for the transcription factors KLF4 (gray), TEAD1/4 (orange), Sp1 (blue) and HBP1, or TEAD1/4 (red). B: Mutation of the CRE dramatically reduced Fyn-induced promoter activity, while mutation of the p53RE only modestly decreased basal and Fyn inducible activity (n = 4; *$P = 0.05$; **$P = 0.004$). C: mutation of the CRE within the truncated −145/+137 construct also inhibited Fyn-induced promoter activation (n = 3; *$P = 0.005$). D: Activation of endogenous Src kinases by siRNA-mediated knock-down of Csk results in upregulation of EphA2 protein in MDCK cells. Csk was knocked-down by RNAi in MDCK cells and lysates were obtained for immunoblotting after 48 h. This result is representative of more than five experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
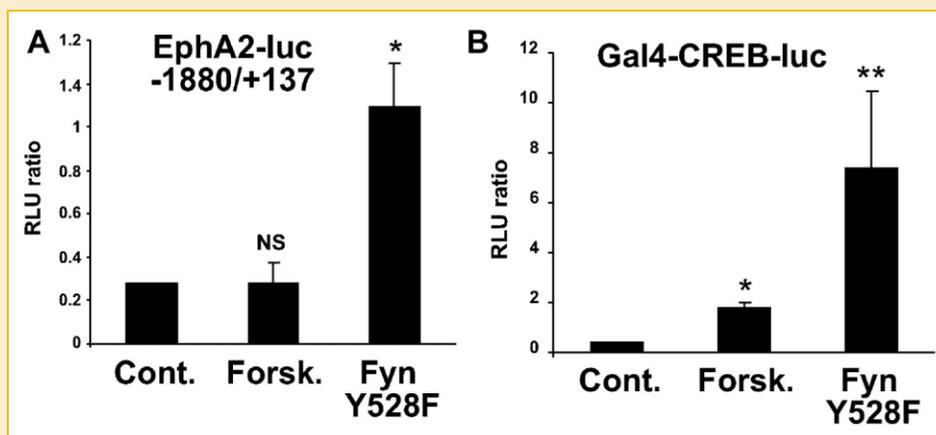
Fig. 4. The EphA2 promoter is unresponsive to cyclic AMP. A: Stimulation with the adenylate cyclase agonist forskolin (50 μM) failed to activate the −1880/+137 EphA2 promoter construct (n = 5; $P = 0.004$). B: Stimulation with forskolin activated CREB, as determined with a Gal4–CREB chimera-driven reporter.
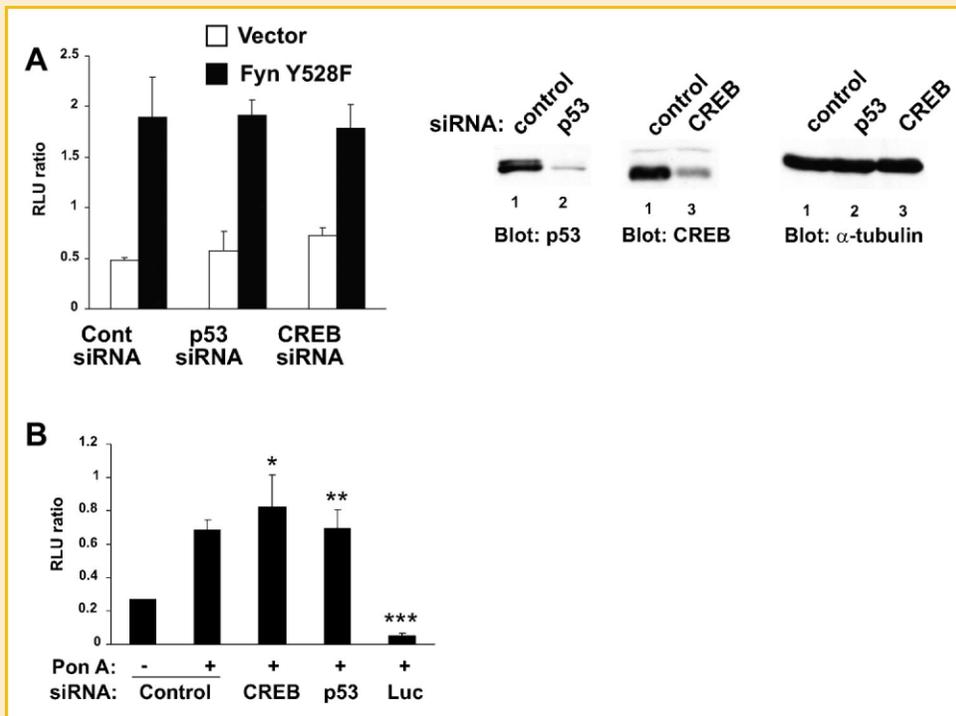
Fig. 5. CREB is dispensable for EphA2 promoter activity. A: Dual luciferase assays were performed in 293 cells sequentially transfected first with CREB-specific, p53-specific, or control siRNA followed by reporter vectors together with either empty vector (white bars) or Fyn Y528F (black bars). Control samples from each experiment were probed for knock-down efficiency by immunoblotting (left panel). One representative experiment is shown (n = 2). B: Simultaneous transfection of siRNA and expression and reporter plasmids was performed in 293 cells expressing a ponasterone-inducible Fyn. Dual luciferase assays were performed after treatment with vehicle alone or with ponasterone A (5 μM). Neither CREB-specific or p53-specific siRNA had a significant impact on luciferase activity. Luciferase-specific siRNA was used as control for knock-down efficiency (n = 5; *P = 0.25; **P = 0.46; ***P = 0.001).

known CRE-binding transcription factor, CREB, is activated in response to oxidant stress exerts protective actions in acute kidney injury [Arany et al., 2005, 2008]. Indeed, in reporter gene assays, Fyn strongly activated CREB. However, surprisingly, CREB was completely dispensable for basal and Fyn-induced activation of EphA2 promoter activity.

Canonical CRE sequences (TGACGTCA) and half sites (CGTCA or TGACG) with various degrees of cross-species conservation have been identified in the promoter regions of more than 4000 human genes, mostly within 200 bp of transcriptional start sites and approximately 5000 promoters are occupied by CREB [Zhang et al., 2005]. However, canonical CREs confer cAMP-responsiveness in only a very small minority of such promoter [Impey et al., 2004; Zhang et al., 2005]. Basal occupancy by CREB or phospho-CREB is not only gene-specific, but also tissue specific [Cha-Molstad et al., 2004], and it is likely restricted in part by DNA methylation [Zhang et al., 2005]. More importantly, even CRE occupancy by phosphorylated CREB is not sufficient to activate transcription in response to cAMP. Instead, transcriptional activation requires the coordinated recruitment of at least two additional components: the promiscuous co-activator p300/CBP and a cAMP-responsive co-activator of the TORC family [Conkright et al., 2003; Screaton et al., 2004]. Thus, in our studies, Fyn strongly activated CREB, but it likely did so in a manner that was not sufficient for CREB to participate in canonical CRE-mediated transactivation.

The unresponsiveness of the EphA2 promoter construct to forskolin observed in the current study is consistent with the apparent lack of occupancy by CREB and pCREB on the endogenous EphA2 promoter, as determined by genome-wide ChIP-on-chip analysis [Zhang et al., 2005] (see also http://natural.salk.edu/CREB/).

However, because CREB can be activated in response to signals other than cAMP, CREs cannot be categorized simply on the basis of their response to cAMP. Indeed, a recent genome-wide study has clearly defined at least two distinct sets of CREB-regulated promoters: those that respond to cAMP and those that respond to mitogenic or stress signals [Impey et al., 2004]. The unique context of each CRE appears to impose or restrict the binding of CREB co-factors, thus determining the sensitivity to specific upstream signals [Xu et al., 2007]. For instance, the presence of nearby Srf/Elk-1 binding sites appears to render certain CREs responsive to mitogenic signals [Ravnskjaer et al., 2007].

Despite these advances in understanding the mechanisms of cAMP-regulated transcription, the function of the large majority of cAMP-unresponsive CREs and the range of transcription factors capable of binding them in the absence of CREB occupancy remain unclear. The current study shows that a canonical CRE can control transcriptional activity independently of both cAMP and CREB.

One explanation for the apparent dispensability of CREB with respect to CRE-dependent activation of the EphA2 promoter could

be redundancy. CREB belongs to the basic leucine zipper (bZIP) family of transcription factors [Vinson et al., 2002]. Within this family, ATF-1 and CREM are closely related to CREB and bind canonical CREs either as homodimers or as heterodimers. Other, more distant bZIP tanscription factors, such as the five members of the Oasis family also have a preference for binding canonical CREs [Vinson et al., 2002]. Therefore, a combined knockdown of several bZIP transcription factors may be necessary to inhibit the transcriptional activation of some CRE-containing genes, as was suggested recently for C/EBP β [Fox et al., 2006].

In addition to bZIP transcription factors, E4F, a zinc finger protein of the Gli-Krüppel family, can bind CRE-like sequences [Fajas et al., 2001; Tessari et al., 2003] and was identified as a likely candidate for binding to the EphA2 CRE by our analysis with MatInspector (data not shown). Preliminary studies indicate that E4F mRNA is expressed in 293 cells and that levels remain unchanged after induction of Fyn overexpression (data not shown). Whether E4F actually binds the EphA2 promoter or contributes to its regulation by Src family kinases remains to be determined.

A recent oligonucleotide-based in vitro binding study has suggested the intriguing possibility that p53 might be recruited to a novel, 10-nucleotide long palindrome found in at least 27 genes, including EphA2 [Jin et al., 2006]. In fact, this putative p53-binding palindrome contains the canonical CRE (GTGACGTCAC; CRE underlined) described in the current paper. However, direct evidence of p53 binding to promoters containing this sequence is currently lacking. Also, as noted earlier, only the first nine nucleotides of the palindrome are phylogenetically conserved in the EphA2 gene. Moreover, the failure of siRNA-mediated knockdown of p53 to alter basal or Src kinase-induced activation of the EphA2 promoter in the current study argues against a critical role of p53 in regulating EphA2 in our model system, although possible redundancy by the p53-related transcription factors p63 and/or p73 could potentially account for our results.

Finally, the possible role of the transcription factors Myc, HIF-1α, and TCF/LEF was not directly addressed in the current study. However, sequence analysis did not identify any binding sites for these factors in the minimal Src kinase-responsive EphA2 promoter construct ($-145/+137$). This suggests to us that the apparent role of Myc and HIF-1α in EphA2 regulation [Vihanto et al., 2005; Yekkala and Baudino, 2007] may be indirect. However, this will require experimental verification.

Recent evidence suggests that EphA2 can regulate epithelial and endothelial permeability both in culture and in vivo [Tanaka et al., 2005; Larson et al., 2008]. We believe that EphA2 functions as an injury-induced sensor of epithelial cell–cell contact and that its upregulation in renal IRI may constitute an important adaptive response whereby loosening of cell–cell junctions is necessary to limit cell loss and permit sufficient plasticity for rapid tubular remodeling and functional recovery. Understanding the mechanisms of EphA2 regulation may therefore have important implications for the development of interventions aimed at modulating various epithelial repair processes. The current study demonstrates that the endogenous EphA2 promoter is highly upregulated in renal IRI in vivo and shows that a highly conserved CRE regulates basal and Src kinase induced activation of this promoter, but that it does

so independently of both cAMP and CREB. In the future, it will be important to determine the identity of the transcription factors involved and to define the elements that impose this atypical mode of regulation of a CRE. It will also be interesting to determine whether our observation reflects the existence of a larger array of similarly regulated, but as yet undefined CRE-containing genes.

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