Cyclooxygenases (COXs) are crucial rate-limiting enzymes required for the biosynthesis of prostaglandins. COX-2 is an inducible isoform of this enzyme, which is believed to play important roles in the development of atherosclerotic vascular disease. We found that COX-2 expression rapidly increases in response to various signaling events, including activation of the platelet-derived growth factor (PDGF) pathway. Activation of PDGF receptor (PDGFR) in rat aortic vascular smooth muscle cells leads to c-Src-dependent stabilization of COX-2 mRNA requiring an AU-rich region within the 3′-untranslated region of this transcript. This regulation correlates with tyrosine phosphorylation of the RNA-associated protein, CUG-binding protein 2 (CUGBP2), which appears to enhance its interaction with COX-2 mRNA. Site-directed mutagenesis of putative tyrosine phosphorylation sites in CUGBP2 identified tyrosine 39 as a c-Src target, and a CUGBP2 with a mutated tyrosine 39 displayed an attenuated ability to bind COX-2 mRNA. We further show that silencing of CUGBP2 with specific small interference RNAs significantly reduces PDGF-dependent induction of COX-2 at both mRNA and protein levels. Furthermore, forced expression of CUGBP2 or constitutively active c-Src leads to stabilization of co-expressed COX-2 mRNA. Finally, in vitro RNA decay assay demonstrates that CUGBP2 is functionally required for the stabilization of COX-2 mRNA. Therefore, our data suggest that tyrosine phosphorylation of CUGBP2 is an important underlying mechanism for the ability of PDGFR/c-Src signaling to control the stability of COX-2 mRNA.
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In this study, we report that c-Src is functionally required for the PDGF-induced COX-2 mRNA stabilization. Furthermore, activation of PDGFR causes c-Src-dependent tyrosine phosphorylation of CUGBP2, which enhances its ability to bind COX-2 mRNA. Because CUGBP2 is essential for maintaining the stability of the COX-2 mRNA, c-Src-mediated phosphorylation of this RNA-binding protein is shown as a novel trans-acting pathway underlying the post-transcriptional mechanism of PDGF-induced increases in COX-2 expression.

MATERIALS AND METHODS

Growth Factors, UTP, and Kinase Inhibitors Used—PDGF-BB (10 ng/ml, Calbiochem) was used to stimulate the various cell types. 100 mM UTP stock solution was made in 1× phosphate-buffered saline. Kinase inhibitors against PDGFR (STI571, kindly provided by Dr. Jean Y. J. Wang, University of California at San Diego) and c-Src (SU6656, Calbiochem) were made in concentrated Me2SO stock solutions. Working concentrations of each inhibitors used in the experiments are noted in the figure legends. Vehicle (Me2SO only) was used as a negative control where noted.

Cells Used and Culture Conditions—Rat thoracic aorta smooth muscle (RASM) cells were harvested and grown in DMEM containing 10% calf serum, as previously described in detail (27). Cells were typically used between passages 7 and 16. Prior to experiments involving addition of PDGF, the RASM cells were serum-starved (no serum) in DMEM for 24 h. Phoenix (amphotropic retroviral packaging line) and HEK293 cells were also grown in DMEM supplemented with 10% fetal bovine serum.

Introduction of Virus, Plasmids, and siRNA into Cells—For the retroviral infection experiments, Phoenix cells were transfected with each retroviral vector by a CaPO4 precipitation method described previously (28). The RASM cells were successively infected with conditioned supernatants from these retroviral packaging cells three times at ~12-h intervals. For transient transfection of HEK293, expression constructs were introduced into HEK293 cells by standard CaPO4 transfection techniques or using Hyfect transfection reagent (Denville Scientific Inc.). For transient transfection of RASM cells, expression constructs were introduced into the cells using FuGENE HD transfection reagent (Roche Applied Science). 40 h after transfection, the cells were used for the indicated experiments. A custom-designed siRNA oligonucleotide (siRNA1) targeting the amino terminus of rat CUGBP2 (5′-AACGCTGTGGAAAGACCGTAGCAGGAGCTTTGAGATCA-3′) was designed with the assistance of and purchased from Qiagen. A predesigned siRNA (siRNA2) targeting the different region of rat CUGBP2 (Rn_Cugbp2_4_HP siRNA) was also purchased from Qiagen. The Stealth RNA interference medium GC duplex negative control (Invitrogen) was used to control for sequence-independent effects of introducing short RNA duplexes into cells. For siRNA delivery, RASM cells were grown in 6-well plates to ~80% confluence and transfected with the CUGBP2-specific siRNAs or control-scrambled siRNA using FuGENE HD transfection reagent (Roche Applied Science). 40 h after transfection, cells were used in the indicated experiments. Transfection efficiency was confirmed by visualization of enhanced green fluorescent protein (EGFP) expression on an inverted fluorescent microscope.

Plasmids Used—The kinase-inactive c-Src (Src I) and constitutively active c-Src (Src A) inserted in the mammalian expression plasmid pCDNA3 (Invitrogen) were kindly provided by Dr. Yue Feng (Emory University) (26). An XbaI site was introduced by site-directed mutagenesis changing GCG to AGA at bases 139–141 of the 5′-UTR of rat COX-2 cDNA to allow discrimination between recombinant and endogenous COX-2 on RPA analysis. The resulting COX-2 cDNA, referred to as full-length recombinant COX-2 cDNA, was subcloned downstream of the CMV promoter in an MuLV-based retroviral vector to create pKX104 (28). The 356-base most distal portion of the 3′-UTR of COX-2 cDNA was removed by digestion with Hpal and Xhol to create pKX104A. The 2219-base most distal portion of the 3′-UTR of COX-2 cDNA was removed by digestion with EcoRV and Xhol to create pKX104B. To remove the entire 3′-UTR, the Accl site at base 1961 of COX-2 cDNA was converted to SalI site by site-directed mutagenesis, and the entire 3′-UTR, including the proximal region, was removed by digestion with SalI and Xhol to create pKX104C. Rat CUGBP2 cDNA was generated by RT-PCR with the following primers, L18 (forward primer): 5′-ACCGTGATGAAAGGTTCGGTTTGGATCA-3′ with MluI site (in italics) and L21 (reverse primer): 5′-AAATGACGGAGGAGCTTCTGGG-3′, and then inserted by TA cloning into pcCR2.1 to make a new plasmid denoted pKMX26. The integrity of this rat CUGBP2 was verified by full-length sequencing of the cDNA. The CUGBP2 cDNA was excised from KMX26 by BamHI and Xhol and subcloned into the bacterial expression vector pET28a(+) to allow production of a His-tagged CUGBP2 that permits purification of this bacterially produced fusion protein with nickel-nitrilotriacetic acid Superflow columns (Qiagen) according to the manufacturer’s recommendations. The FLAG epitope sequence was also introduced between BamHI and MluI in pKMX26 to create a FLAG-tagged CUGBP2 cDNA that was subsequently subcloned into an MuLV-based retroviral vector downstream of the CMV promoter. The resulting construct was named pKMX31A. The putative tyrosine phosphorylation sites of CUGBP2 at Tyr-453, Tyr-410, Tyr-384, and Tyr-39 were mutated to alanine or phenylalanine by site-directed mutagenesis. The cDNAs of mutant CUGBP2s (Y453A, Y410A, Y384A, and Y39F) were subsequently subcloned into the same retroviral vector used above and referred to as pKMX31A, -A2, -A3, and -A5, respectively, or subcloned into pcDNA3 vector and referred to as pcDNA31-A1, -A2, -A3, and -A5, respectively. To make RNA for the in vitro RNA decay assay, an MluI site was first introduced into pBluescript (Stratagene, La Jolla, CA) by adding Xhol-HindIII-MluI-KpnI oligonucleotide between the Xhol and KpnI sites of pBluescript and then a 25-mer poly(A) sequence with Xhol and MluI sequences on the 5′- and 3′-ends, respectively, was cloned between Xhol and MluI in the modified pBluescript. The 831-base proximal COX-2 3′-UTR was excised with AccI/HindIII and subcloned upstream of the poly(A) sequence at Sall/HindIII in the modified pBluescript. A 104-bp sequence of cyclophilin cDNA was similarly subcloned upstream poly(A) in this vector and used as a control, unregulated mRNA sequence.
Immunoprecipitation and Immunoblotting Assays—In the HEK293 cell experiments, 293 cells were plated on 60-mm dishes at ~70% confluence 1 day before transfection. Two days after transfection, the cells were lysed in 1 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 100 μl of phosphatase inhibitor mixture 11 and proteinase inhibitor mixture (Sigma-Aldrich). Immunoprecipitation of the FLAG-tagged CUGBP2 was performed from these protein extracts using 20 μl of anti-FLAG-M2-agarose affinity gel (Sigma). The bound FLAG-tagged CUGBP2 was eluted in 100 μl of lysis buffer containing 10 μg of FLAG peptide at 4 °C for 2 h. In the RASM cell experiments, RASM cells were plated onto 100-mm dishes and lysed for immunoprecipitation as described above. For immunoblotting experiments, either immunoprecipitations or total lysates (prepared using standard boiling SDS lysis) were used and resolved by SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride membrane and probed with antibodies against CUGBP2 (OriGene, San Diego, CA), phosphorylated-PDGFR-β (Santa Cruz Biotechnology, Santa Cruz, CA), phosphotyrosine (PY20, Upstate Biotechnology, subsidiary of Millipore, Charlotte, VA), Stat3/phosphorylated-Stat3 (Cell Signaling Technology, Beverly, MA), and ERK1/2/phosphorylated ERK1/2 (Cell Signaling Technology). The bound proteins were visualized with ECL Plus (Amersham Biosciences, subsidiary of GE Healthcare). The cyclophilin mRNA signals were used to normalize the signal.

Results

Src Family Kinases Are Required for PDGF-induced COX-2 Expression—Our and other’s previous studies show that PDGF stimulation causes a rapid increase in COX-2 mRNA levels in RSAM cells and NIH3T3 cells (9, 31, 32). Activation of PDGFR signaling leads to activation of many downstream kinases, including the Src family kinases. To determine the contribution of Src family kinases to PDGF-induced COX2 expression, we examined PDGF-induced COX-2 expression at both the mRNA and protein levels in the presence of the PDGFR kinase inhibitor STI571 and the Src family kinase-selective inhibitor SU6656 (33). As expected, pretreatment of RASM cells with STI571 reduced PDGF-stimulated phosphorylation of
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PDGFR-β and, in parallel, expression of COX-2 mRNA in a concentration-dependent manner with 5 μM STI571 being able to completely block both of these processes (Fig. 1A). This result demonstrates that the PDGFR kinase mediates PDGF-induced COX-2 expression. To explore the role of the Src family kinases in PDGFR-induced COX-2 induction, SU6656, a small molecule Src family kinase inhibitor with good selectivity for the c-Src kinase over the PDGFR kinase, was used (33). First, we determined the effective concentration of SU6656 required for inhibition of c-Src kinase activity but not PDGFR kinase activity in our system. Stat3 phosphorylation is a good surrogate for c-Src function because Stat3 has been shown to be a cellular substrate of c-Src kinase in the PDGF-signaling cascade (34, 35) and can be phosphorylated by c-Src in rat vascular smooth muscle cells (36). Through immunoblot analysis, we found that, although 10 μM SU6656 was still insufficient to inhibit PDGF-stimulated phosphorylation of PDGFR-β and its downstream kinase ERK1/2, signaling to Stat3 (as evidenced by its phosphorylation), a process known to be dependent on c-Src activity, was reduced significantly after treatment with 1 μM of SU6656 and completely blocked by 5 μM of drug (Fig. 1B). These results indicate that, in RASM cells, c-Src kinase activity can be partially or completely blocked with 1 or 5 μM SU6656, respectively, whereas these concentrations had no detectable effect on PDGFR phosphorylation status. Thus, the effective concentration of SU6656 required for blocking c-Src activity but not PDGFR kinase activity is between 1 and 5 μM in RASM cells, consistent with previous results obtained using other cell lines (33). RNA analysis by RPA showed that SU6656 blocked PDGF-induced expression of COX-2 mRNA in a dose-dependent fashion with 5 μM drug being sufficient to completely inhibit this induction (Fig. 1B). Consistent with our RPA results, immunoblotting analysis using anti-COX-2 antibody confirmed that COX-2 protein level was significantly increased following PDGF stimulation, and this induction was nearly completely blocked by 5 μM SU6656 (Fig. 1C). These data indicate that PDGF-induced COX-2 expression is dependent on PDGFR and Src family kinases.

Previously, we demonstrated that stimulation with PDGF results in increased transcription of COX-2 mRNA (9). However, given the degree of COX-2 promoter activation, we determined that the increased transcription rate was not sufficient to fully account for the robust induction of COX-2 mRNA that was observed. Therefore, we hypothesized that PDGF-induced stabilization of COX-2 mRNA likely also contributes to the observed rapid accumulation of COX-2 mRNA. To confirm this hypothesis, RASM cells were stimulated with UTP for 1 h to induce COX-2 mRNA, and then transcription was stopped with addition of actinomycin D. RNA was isolated at different time points after treatment with actinomycin D and subjected to RPA. As shown, treatment with PDGF caused a significant increase in the half-life of COX-2 mRNA (Fig. 2A). Actinomycin D has been shown to artificially stabilize some mRNAs, likely due to sequestration of key mRNA export factors (28, 37–39). To overcome this potential difficulty in understanding the role of PDGF in stabilization of COX-2 mRNA, RASM cells were engineered to express recombinant full-length COX-2 mRNA under the control of the constitutively active CMV promoter (Fig. 2B), referred to as RASM104 cells. Upon treatment of these cells with PDGF, a 4.5-fold increase in COX-2 mRNA levels, as assessed by RPA, was seen (Fig. 2, C and D). Pretreatment of these cells with STI571 or SU6656 prior to PDGF stimulation resulted in near complete blockade of exogenous COX-2 induction (Fig. 2D). Similar results were also found for endogenous COX-2 mRNA. By contrast, another RASM cell line generated to express EGFIP mRNA driven by the CMV promoter displays no significant change in expression in response to PDGF stimulation (Fig. 2E). These results indicate that the CMV promoter activity was not affected by PDGF and suggest that the increase in recombinant COX-2 mRNA level is most
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likely due to stabilization of the COX-2 mRNA. Therefore, PDGFR and Src family kinase activities are required for PDGF-dependent stabilization of COX-2 mRNA in a process that, at least partially, contributes to the rapid induction of COX-2 expression in response to this growth factor.

We sought to determine whether c-Src itself could alter the stability of COX-2 mRNA by transient cotransfecting HEK293 cells with expression plasmids containing Src and the rat COX-2 cDNA. Cotransfection of expression plasmids of either c-Src A (a constitutively active form of Src) or c-Src I (a kinase-inactive form of Src) with PKX104 containing rat full-length COX-2 cDNA and pcDNA3/EGFP in 293 cells revealed that COX-2 mRNA levels but not EGFP mRNA levels could be increased by Src A expression (Fig. 2F). These observations presented above indicate that PDGFR/c-Src kinase signaling is involved in stabilization of COX-2 mRNA. CUGBP2 has been shown to stabilize COX-2 mRNA through direct binding interactions (21, 22). We found that CUGBP2 is expressed in RASM cells, but its levels are not affected by PDGF signaling (data not shown). Therefore, we hypothesized that CUGBP2 is a trans-acting factor and the c-Src effector for regulating COX-2 mRNA. To test this hypothesis, we first examined whether PDGFR stimulates the phosphorylation of CUGBP2 by in vivo 32P-labeling of RASM cells. RASM cells either expressing FLAG-tagged CUGBP2 (RASM31A) or containing the empty vector only (WT RASM) were metabolically labeled with [32P]orthophosphate prior to PDGF or mock treatment. The lysates were then generated, immunoprecipitated with anti-

complement the kinase inhibitor results and further argue for a role of c-Src in the stabilization of COX-2 mRNA.

**c-Src-dependent Stabilization of COX-2 mRNA Requires a Cis-acting Regulatory Element within the Proximal AU-rich Region of the 3′-UTR**—Next, mRNA deletion analysis was performed to identify potential cis-acting regulatory elements that mediate the effects of c-Src on COX-2 transcript stabilization. A series of deletion mutants of COX-2 3′-UTR were generated for this analysis (Fig. 3A). We show that deletion of either the distal 356 (KX104a) or 2219 (KX104b) bases of the 3′-UTR did not abolish PDGF-induced and c-Src-dependent stabilization of the recombinant COX-2 mRNA (Fig. 3B). However, when the entire 3′-UTR was deleted, the recombinant COX-2 mRNA (KX104c) lost responsiveness to PDGF stimulation and could not be further decreased with inhibition of c-Src activity (Fig. 3B). Furthermore, PKX104a, PKX104b and PKX104c were, respectively, transfected into HEK293 cells with pcDNA3 Src A or Src I and pcDNA3/EGFP. RNA analysis by RPA showed that the mRNA levels of recombinant COX-2 KX104a and KX104b but not KX104c could be obviously increased by Src A expression (Fig. 3C). Therefore, our results demonstrate that the 231-base proximal 3′-UTR is required for c-Src-dependent stabilization of COX2 mRNA.

**PDGF Stimulates the Phosphorylation of CUGBP2**—The results presented above indicate that PDGFR/c-Src kinase signaling is involved in stabilization of COX-2 mRNA. CUGBP2 has been shown to stabilize COX-2 mRNA through direct binding interactions (21, 22). We found that CUGBP2 is expressed in RASM cells, but its levels are not affected by PDGF signaling (data not shown). Therefore, we hypothesized that CUGBP2 is a trans-acting factor and the c-Src effector for regulating COX-2 mRNA. To test this hypothesis, we first examined whether PDGFR stimulates the phosphorylation of CUGBP2 by in vivo 32P-labeling of RASM cells. RASM cells either expressing FLAG-tagged CUGBP2 (RASM31A) or containing the empty vector only (WT RASM) were metabolically labeled with [32P]orthophosphate prior to PDGF or mock treatment. The lysates were then generated, immunoprecipitated with anti-

**FIGURE 2. PDGF stimulation stabilizes COX-2 mRNA in a process requiring PDGFR and c-Src activity.** A, RPA was used to determine COX-2 mRNA stability in RASM cells after treatment with or without PDGF (10 ng/ml). Basal COX-2 expression was increased by inducing transcription with UTP (100 μM) for 1 h prior to addition of actinomycin D (10 μg/ml) for differing times (0–3 h). The amount of COX-2 mRNA at each time point is expressed as a percent relative to the level at the initial time point. B, the full-length recombinant rat COX-2 and EGFP cDNA expression cassettes with the CMV promoter are depicted. Of note, point mutations generated in the 5′-UTR of the recombinant COX-2 expression cassette allows differentiation of recombinant and endogenous COX-2 expression by RPA. C, RPA was used to determine COX-2 mRNA expression in RASM cells containing the PKX104 COX-2 expression construct (KX104 cells). Cells were untreated or treated with 10 ng/ml PDGF for 1 h, as indicated. A recombinant COX-2 probe was used yielding a full-length protected band representing the recombinant COX-2 mRNA and two faster migrating (long and short) protected bands representing the endogenous COX-2 mRNA. The first lane contains probe only and shows the position of migration of undigested COX-2 and cyclophilin (Cyp, normalization control) probes. For C and D, RPA was used to determine COX-2 and EGFP mRNA expression. Cells were treated with vehicle (DMSO only), STI571 (STI), or SU6656 (SU) for 1 h prior to 1-h stimulation with PDGF, as indicated. In D, KX104 cells were used and the recombinant (Rec) and endogenous (Endo, long form) COX-2 mRNAs are labeled. In E, RASM cells containing the EGFP expression construct are used. F, RPA was used to determine expression of COX-2 and EGFP in HEK293 cells transiently transfected by Hyfect transfection reagent with 1 μg of pcDNA3/Src A or Src I, 1 μg of PKX104 (expressing rat full-length COX-2), and 0.1 μg of pcDNA3/EGFP. Cyclophilin (Cyp) or EGFP normalization control was used for these RPAs in (A, C, D, and F) or in E. Each autoradiograph and graph shown are representative of the average of two to three independent experiments. Error bars, ±S.E.
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A.

B.

C.

FIGURE 3. The proximal ARE in the 3′-UTR of COX-2 is required for PDGFR- and c-Src-dependent stabilization of COX-2 mRNA. A, derivatives of the full-length recombinant rat COX-2 cDNA expression cassettes downstream of the CMV promoter are depicted. These deletions include removal of the distal ARE (ARE2) only (pKX104a), a 2219-base distal portion of 3′-UTR (pKX104b), and the entire 3′-UTR (pKX104c). B, RPA was used to determine expression of COX-2 mRNA in RASM cells expressing pKX104a (KX104a), pKX104b (KX104b), and pKX104c (KX104c). Cells were treated with vehicle (Me2SO only), STI571 (STI), or SU6656 (SU) for 1 h prior to 1-h stimulation with PDGF, as indicated. The recombinant (Rec) and endogenous (Endo, long form) COX-2 mRNAs are labeled. Cyclophilin (Cyp) was used as the normalization control. The displayed autoradiographs are representative of two independent experiments. C, 1 μg of pKX104a, pKX104b, and pKX104c were, respectively, transfected by Hfect transfection reagent into HEK 293 cells with 1 μg of pcDNA3/Src A or SrcI and 0.1 μg of pcDNA3/EGFP. 24 h after transfection, total RNAs were isolated from the transfected cells and analyzed by RPA. EGFP normalization control was used for these RPAs. Each autoradiograph and bar graph shown is representative of the average of two independent experiments. Error bars, ±S.E.

body directed against FLAG, and resolved on SDS-PAGE for both autoradiography as well as immunoblot analysis using anti-FLAG antibody. As shown in Fig. 4A, the phosphorylation of CUGBP2 was increased following PDGF stimulation compared with control cells, whereas the amounts of CUGBP2 proteins were similar in each sample.

We further tested whether PDGFR activity and c-Src signaling are involved in tyrosine phosphorylation of CUGBP2 in RASM cells. RASM31A cells were pretreated for 1 h with Me2SO only, SU6656, or STI571 prior to PDGF treatment. In each case, FLAG-CUGBP2 was immunoprecipitated using anti-FLAG antibody and then analyzed by immunoblotting with anti-phosphotyrosine (PY20) or anti-CUGBP2 antibody. First, our results showed that PDGF stimulated tyrosine phosphorylation of CUGBP2 (Fig. 4B, lanes 1 and 2). Furthermore, each of the two inhibitors (STI571 and SU6656) completely abrogates PDGF-stimulated tyrosine phosphorylation of CUGBP2 (Fig. 4B, lanes 3 and 4). Thus, both PDGFR and c-Src appear necessary for PDGFR-induced tyrosine phosphorylation of CUGBP2.

We next wanted to determine whether c-Src can directly phosphorylate CUGBP2. To test this hypothesis, we performed in vitro kinase assays using bacterially expressed CUGBP2 and purified, active c-Src protein, which is available commercially. This experiment demonstrates that c-Src kinase can directly phosphorylate CUGBP2 in vitro (Fig. 4C). Furthermore, cotransfection of 293 cells with pcDNA3/FLAG-CUGBP2 and either Src I (kinase-dead) or Src A (constitutively active) revealed that introduction of Src A but not Src I with FLAG-CUGBP2 results in detectable tyrosine phosphorylation of CUGBP2 in vivo (Fig. 4D). Therefore, our data identify CUGBP2 as a previously unknown substrate of the c-Src kinase.

Identification of the c-Src Tyrosine Phosphorylation Site in CUGBP2—Based upon sequence analysis of consensus phosphorylation motifs for c-Src, putative sites of phosphorylation at tyrosine 39 and 384 were identified in CUGBP2 (Fig. 5A). Tyrines 453 and 410 were additional sites that could potentially be phosphorylated by other tyrosine kinases downstream of PDGFR. To determine whether these tyrosines could serve as true phosphorylation sites, site-directed mutagenesis of the FLAG-CUGBP2 expression construct was created. This involved changing tyrosine to either alanine or phenylalanine at each position (Y39F, Y384A, Y410A, and Y453A). The Wt and each mutant FLAG-CUGBP2s were introduced by transfection, in concert, with constitutively active c-Src into 293 cells. We found that the Y39F mutation resulted in significantly decreased c-Src-dependent phosphorylation of CUGBP2 (Fig. 6B). Other mutations, including Y384A, did not have obvious effects on the phosphorylation of CUGBP2 by c-Src (Fig. 5B). Therefore, our data indicate that Tyr-39 in CUGBP2 is likely the major phosphorylation site for c-Src.

Involvement of CUGBP2 in COX-2 mRNA Turnover—To directly test whether CUGBP2 mediates PDGF-induced COX-2 expression in RASM cells, CUGBP2 was silenced by two specific siRNAs targeting different regions of CUGBP2. Following two consecutive transfections of these siRNAs within 24 h, the protein expression of CUGBP2 could be significantly reduced (Fig. 6A). These cells were then assessed for COX-2 induction at both the mRNA and protein levels following treatment with PDGF, and the degree of induction was found to be significantly reduced at both the RNA and protein levels (Fig. 6A). This result indicates that CUGBP2 is required for PDGF-induced expression of COX-2.

To further confirm the role of CUGBP2 in COX-2 mRNA stabilization, we performed an in vitro RNA decay assay using 32P-labeled COX-2 3′-UTR and S100 extracts prepared from
PDGF-induced phosphorylation of CUGBP2 in a process requiring PDGFR and c-Src activity. A, RASM31A (cells engineered to stably express FLAG-tagged CUGBP2) were metabolically labeled with \([32P]\)orthophosphate as described under “Materials and Methods.” Cells were incubated without or with PDGF (10 ng/ml) as indicated. Wild-type RASM (WT RASM) cells were used as a negative control. Labeled FLAG-tagged CUGBP2 was immunoprecipitated using anti-FLAG IgG, resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and exposed to a phosphorimaging screen. A representative image of phosphorylated CUGBP2 (P-CUGBP2) from two independent experiments is shown. After exposure to the phosphorimaging screen, immunoblotting with anti-CUGBP2 antibody was performed to show comparable levels of CUGBP2 protein in each lane. B, immunoprecipitation with anti-FLAG antibody was performed on lysates from RASM31A cells treated with vehicle (V, Me2SO only), STI571 (S1, 5 μM), or SU6656 (SU, 5 μM) for 1 h prior to 1-h stimulation with PDGF (10 ng/ml), as indicated. Immunoblots were probed with anti-phosphotyrosine (PY20) and anti-CUGBP2 antibodies. The blot is representative of two independent experiments. C, an in vitro kinase assay was performed by incubating bacterially expressed CUGBP2 with commercially available purified c-Src with \([γ-32P]ATP\). The reaction was resolved on SDS-PAGE and exposed to a phosphorimaging screen. A representative image of three independent experiments is shown. D, FLAG-tagged CUGBP2 expression plasmid was cotransfected into HEK293 cells with kinase-dead (Src I) or constitutively active (Src A) c-Src constructs. Cells were harvested 48 h after transfection, immunoprecipitated with anti-FLAG antibody, and analyzed by immunoblotting using PY20 and anti-CUGBP2 antibodies. EGFP expression construct was used as a negative control, as indicated. The displayed result is representative of three independent experiments.

PDGF-treated RASM cells. Cyclophilin RNA was used as a control. First, we demonstrate that CUGBP2 can be depleted from the S100 extract by successive rounds of immunoprecipitation with anti-CUGBP2 IgG (Fig. 6B). Next, using S100 extract immunoprecipitated with normal rabbit serum or anti-CUGBP2 IgG, we find that COX-2 mRNA was more stable in S100 extracts containing CUGBP2 than with CUGBP2-depleted S100 extracts (Fig. 6C). This result suggests that CUGBP2 is an important component of the machinery responsible for COX-2 mRNA turnover. To examine CUGBP2-mediated effects further, we again cotransfected 293 cells with a constant amount of full-length COX-2 cDNA-expressing plasmid and increasing amounts of FLAG-CUGBP2-expressing plasmid. The results showed that COX-2 mRNA levels increased proportionally to the protein expression of CUGBP2 (Fig. 6D), indicating that overexpression of CUGBP2 can stabilize COX-2 mRNA. Immunoblot analysis using an antibody against COX-2 revealed that COX-2 protein level also increased with increasing CUGBP2 protein level.

**PDGF Treatment Increases the Ability of CUGBP2 to Bind COX-2 mRNA**—CUGBP2 has been shown to bind to the first 60-base AU-rich region of COX-2 mRNA in human HT29 cells following radiation-induced cell stress (21). To determine whether CUGBP2 can bind to the same region of the COX-2 mRNA in response to receptor signaling, we first performed UV-cross-linking experiments using \([32P]\)-labeled full-length 3′-UTR of COX-2 and FLAG-CUGBP2 that was purified by immunoprecipitation using anti-FLAG antibody. Our results showed that FLAG-CUGBP2 purified from untreated RASM31A cells has basal binding activity, and this binding appeared to increase following PDGF treatment despite the input of similar amounts of protein used for the UV-cross-linking assay (Fig. 7A). This result suggests that phosphorylation of CUGBP2 by PDGFR signaling enhances the ability of CUGBP2 to bind the 3′-UTR of COX-2 mRNA. To further confirm that this interaction with COX-2 mRNA occurs in vivo, lysates from RASM31A cells treated with or without PDGF for 1 h were immunoprecipitated with anti-FLAG IgG to pull down CUGBP2/COX-2 mRNA complex, and the RNA isolated within the protein-RNA complex was quantified by RPA. This experiment revealed that significant levels of COX-2 mRNA were only present in lysates of PDGF-treated RASM31A cells. By contrast, negligible amounts of COX-2 mRNA coimmunoprecipitated with CUGBP2 using anti-FLAG antibody in both the WT RASM controls that lacked FLAG-CUGBP2 expression and the untreated RASM31A controls (Fig. 7B).

To obtain further evidence that phosphorylation of CUGBP2 affects its binding ability, we performed immunoprecipitation analysis with lysates derived from PDGF-treated RASM31A cells (expressing FLAG-CUGBP2) or RASM31A5 cells (expressing FLAG-CUGBP2/Y39F). Our results show that significantly less COX-2 mRNA associated with the immunoprecipitated products (using anti-FLAG antibody) from RASM31A5 compared with RASM31A cells, although the
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A.

**FIGURE 6. CUGBP2 is involved in the stability of COX-2 mRNA.** A, RASM cells were twice transfected within 24 h with control (siCtrl) or CUGBP2-targeting (siRNA1 and siRNA2) siRNAs. 40 h after transfection, cells were treated with PDGF for 1 h for RPA of COX-2 mRNA or 2 h for immunoblot analyses. Immunoblots were separately probed using antibodies against CUGBP2, COX-2, and EIF5α. The displayed blots and autoradiographs are representative of two independent experiments. The amount of COX-2 mRNA normalized to expression of cyclophilin (Cyp) is expressed as a relative percentage with the PDGF-treated controls arbitrarily set at 100%. Error bars, ±S.E. B, S100 extract is prepared and subjected to CUGBP2 depletion by successive immunoprecipitation (Fig. 7D) as described under “Materials and Methods.” Extracts are resolved by SDS-PAGE and analyzed by immunoblotting using antibodies directed against CUGBP2 and actin. Blots demonstrate successful depletion of CUGBP2 from the S100 extract. C, COX-2 RNA decay assay is performed by incubating at 30 °C for the indicated times in vitro transcribed, 32P-labeled, capped, and polyadenylated COX-2 proximal 3′-UTR (871 bp) and cyclophilin (Cyp) RNAs with control and CUGBP2-depleted S100 extracts prepared from PDGF-stimulated RASM cells. After incubation, RNAs were isolated, resolved on a denaturing gel, and visualized by autoradiography. The displayed autoradiograph is representative of three independent experiments. The graph shows the average quantity of COX-2 RNA levels at each time point normalized by the amount of Cyp RNA present (n = 3). Error bars, ±S.E. D, HEK293 cells are cotransfected by standard CaPO4 transfection techniques with empty vector (g), as indicated. Empty vector is used to bring total DNA in transfections up to 18 μg, as necessary. COX-2 mRNA and protein expression and CUGBP2 expression were assessed by RPA and immunoblot analysis 48 h after transfection. The results shown are representative of two (immunoblots) and four (RPAs) independent experiments with mRNA quantitation being the average of the RPA experiments. Error bars, ±S.E.

amount of CUGBP2 protein was found to be similar in each immunoprecipitation (Fig. 7C). Thus phosphorylation of Tyr-39 appears critical for increasing the ability of CUGBP2 to in vivo bind COX2 mRNA in PDGF-treated RASM cells.

Next, we sought to determine whether PDGF-dependent induction of endogenous COX-2 mRNA is altered in RASM cells transiently transfected with the wild-type (pcDNA 31A) or Tyr-39 phosphorylation mutant (pcDNA 31A5) FLAG-CUGBP2. Following two consecutive transfections within 24 h, wild-type and mutant FLAG-CUGBP2 could be expressed at high level as assessed by immunoblot analysis using anti-FLAG antibody (Fig. 7D). These cells were then assessed for COX-2 mRNA induction following treatment with PDGF. We found that the degree of COX-2 mRNA induction was enhanced in RASM cells transiently expressing wild-type CUGBP2 compared with those transiently expressing mutant CUGBP2 (Fig. 7D).

**DISCUSSION**

In RASM cells, activation of PDGFR leads to c-Src-dependent stabilization of COX-2 mRNA via the 231 nucleotide AU-rich region in the proximal 3′-UTR. This regulation involves tyrosine phosphorylation of CUGBP2 by c-Src, and this phosphorylation appears to enhance the interaction of CUGBP2 with the COX-2 mRNA. Furthermore, CUGBP2 is involved in PDGF-dependent COX-2 mRNA turnover in RASM cells as assessed by siRNA as well as by in vitro RNA decay assay. Therefore, our data suggest that CUGBP2 is a trans-acting effector of the PDGFR/c-Src signaling cascade that post-transcriptionally regulates COX-2 expression in RASM cells.

Our previous study showed that PDGF stimulation can cause profound increases in COX-2 mRNA levels despite only moderate increases in the rate of transcription (9). We reasoned that post-transcriptional regulatory mechanisms, in addition to transcriptional activation, likely contribute to the observed increase in COX-2 expression. Receptor tyrosine kinases initiate many downstream signaling cascades resulting in activation of various effectors, including the Src family kinases. Src family kinases have been shown to be involved in PDGF-induced mRNA stabilization of specific early response genes, including c-myc, monocyte chemotactic protein 1 and 3 and endothelial nitric-oxide synthase (40–42). In the present report, we have demonstrated that the induction of COX-2 by PDGF in RASM cells is, in large part, regulated by the PDGFR/c-Src signaling pathway. Using actinomycin D to analyze RNA decay, PDGF treatment was found to significantly stabilize COX-2 mRNA. Pretreatment with the Src family kinase inhibitor SU6656 nearly abolishes PDGF-stimulated expression of both endogenous and CMV promoter-driven recombinant COX-2. In contrast, CMV promoter-driven EGFP mRNA expression is not influenced by PDGF, further supporting the role of PDGF/Src signaling in the regulation of COX-2 mRNA stability. In addition, forced expression of constitutively active...
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c-Src increases the level of recombinant COX-2 mRNA containing an AU-rich proximal 3′-UTR element. These data suggest that c-Src kinase can regulate COX-2 mRNA stability, strongly contributing to the rapid induction of COX-2 mRNA by PDGF.

The p38 MAPK signaling pathway has also been implicated in the regulation of stability of several transcripts, including COX-2 mRNA (25, 43–50). However, the p38 MAPK signaling pathway does not appear to be involved in PDGF-induced stabilization of COX-2 mRNA in RASM cells, because inhibition of p38 MAPK by SB203580 does not alter PDGF-induced accumulation of COX2 mRNA. However, the same concentration of SB203580 could block angiotensin II and UTP-induced COX-2 expression, indicating that, although the p38 MAPK pathway is active in RASM, it does not play a role in PDGF-regulated COX-2 expression.

The 3′-UTR of COX-2 mRNA has been demonstrated to be a critical regulator of COX-2 mRNA stability. Our data indicate that the proximal 231 nucleotides of the COX-2 mRNA 3′-UTR contain a cis-acting element for c-Src-dependent COX-2 mRNA stabilization in RASM cells. Several constructs lacking this proximal 3′-UTR region could not be regulated by c-Src. This region is highly conserved in different species, and contains six tandem repeats of AUUUA. The corresponding region of human COX-2 mRNA has been identified as binding sites for decay-promoting RNA-binding proteins such as AUF1 and stabilizing RNA-binding proteins, including HuR and CUGBP2 (12, 20, 21). This region has also been shown to be associated with p38 signaling-dependent stabilization of COX-2 mRNA in response to lipopolysaccharide in human HeLaTO cells (12). Our findings and those of others suggest that distinct mechanisms for stabilization of COX-2 mRNA in different cell types are initiated in response to specific stimuli.

Multiple RNA-binding proteins, such as HuR, AUF1, TTP, hnrNPs, and CUGBP2 (51), are capable of binding to the ARE of COX-2 mRNA. The binding of TTP leads to a decrease in the stability of COX-2 mRNA (18), whereas the binding of HuR and, as shown here and by others, CUGBP2 stabilizes COX-2 mRNA (14, 17, 19, 21). There is mounting evidence that these RNA-binding proteins are effectors for discrete signaling pathways. Recently, Briata et al. (23) reported that p38 MAPK directly phosphorylates the destabilizing RNA-binding protein KSRP, leading to attenuation of its decay-promoting function. Similar to KSRP, TTP is also phosphorylated in response to p38 MAPK activation, resulting in decreased binding to tumor necrosis factor-α mRNA and increased tumor necrosis factor-α mRNA stability (24).

In the current study, we have demonstrated that CUGBP2 phosphorylation occurs as a result of PDGFR activation, and that CUGBP2 may be a direct substrate for c-Src kinase. Phosphorylation of CUGBP2 leads to an increase in its ability to bind COX-2 mRNA, whereas mutation of the phosphorylation site (Y39F) attenuates its COX-2 mRNA-binding ability. In addition, inhibition of CUGBP2 phosphorylation by SU6656 is accompanied by abrogation of COX-2 mRNA stabilization. Furthermore, silencing of CUGBP2 significantly reduces the PDGF-dependent induction of COX-2 mRNA. In vitro RNA decay assay further confirms the importance of CUGBP2 in the stabilization of COX-2 mRNA in RASM cells. Although transient transfection of wild-type CUGBP2 into RASM cells enhanced PDGF-stimulated COX-2 mRNA expression to a lesser degree than that seen in 293 cells (Figs. 6D and 7D), the enhancement in RASM cells was consistently obtained on independent experiments. We believe factors such as transfection efficiency (significantly higher with 293 versus RASM cells) and differences in the baseline expression of CUGBP2 may account for some of the differences seen in these cells. Overall, these findings suggest that the phosphorylation of CUGBP2 by PDGFR/c-Src signaling is linked to PDGF-induced stabilization of COX-2 mRNA. To our knowledge, this is the first evidence that demonstrates the phosphorylation of CUGBP2 by c-Src. This phosphorylation has profound effects on CUGBP2 RNA-binding activity and plays an indispensable role in PDGF/c-Src signaling-dependent stabilization of COX-2 mRNA. Precedent for an Src kinase signal regulating the function of other RNA-binding proteins has been reported. For instance, the selective RNA-binding protein QKI can be phosphorylated by Src family kinases, which negatively modulates its activity for binding and stabilizing the myelin basic protein mRNA (26). In contrast, activation of c-Src in our system results in the phosphorylation

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K. Xu, C. M. Kitchen, H.-K. G. Shu, and T. J. Murphy, unpublished data.

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**FIGURE 7. CUGBP2 binds to COX-2 mRNA both in vitro and in vivo.**

A, lysates from Wt RASM and RASM31A (with or without PDGF stimulation for 1 h) were harvested and immunoprecipitated with anti-FLAG antibody (to isolate FLAG-tagged CUGBP2). The immunoprecipitates were incubated with in vitro transcribed, [32P]-labeled COX-2 3′-UTR RNA for the UV cross-linking assay as described under “Materials and Methods.” Additional aliquot of the immunoprecipitates was determined by immunoblot analysis using anti-CUGBP2 antibody. B, lysates from RASM 31A cells treated with or without PDGF for 1 h were immunoprecipitated for FLAG-tagged CUGBP2:RNA complexes using anti-FLAG antibody. The RNA was isolated from the immunoprecipitates, and the amount of COX-2 mRNA was determined by RPA using a COX-2-specific Riboprobe. C, lysates from Wt RASM, RASM31A, and RASM31A5 cells treated with PDGF for 1 h, as indicated, were immunoprecipitated for FLAG-CUGBP2:RNA complexes using anti-FLAG antibody. Again, quantification of COX-2 mRNA within the immunoprecipitates was performed by RPA analysis using a COX-2-specific Riboprobe. Immunoblotting with anti-CUGBP2 antibody was performed as well to show the presence of CUGBP2 in the anti-FLAG immunoprecipitates. All results in this figure are representative of three independent experiments in each case. D, transient transfection of wild-type CUGBP2 into RASM cells enhanced PDGF-stimulated COX-2 mRNA expression. The RASM cells were twice transfected within 24 h with pcDNA (vector only control, Ctrl), pcDNA31A (wild-type CUGBP2), and pcDNA 31AS (mutant CUGBP2). 40 h after initial transfection, the cells were harvested to assess the FLAG-tagged CUGBP2 expression by immunoblot analysis or were treated with PDGF for 1 h for RPA analysis of COX2 mRNA. The displayed blots and autoradiographs are representative of two (immunoblots) and four (RPAs) experiments. Error bars, ± S.E.
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of CUGBP2 and rapid accumulation/stabilization of COX-2 mRNA, thus demonstrating the pleiotropic effect of Src-dependent regulation on distinct RNA-binding proteins.

CUGBP2 has three RNA recognition motifs (RRMs), two near the N terminus and a third near the C terminus (52). Tyrosine 39 resides within the first RRM of CUGBP2. It has been proposed that RNA-binding activity of CUGBP2 is mediated via the first two RRMs (53, 54). Therefore, one reasonable hypothesis is that the phosphorylation of tyrosine 39 on CUGBP2 alters its binding activity, and perhaps, access of template mRNAs to the cellular decay machinery.

Mukhopadhyay et al. (21) found that the binding of CUGBP2 to COX-2 mRNA can result in inhibition of its translation in the epithelial cells undergoing radiation-induced apoptosis, although such binding stabilizes COX-2 mRNA. In our RASM cells, however, despite PDGF-stimulated binding of CUGBP2 to COX-2 mRNA (Fig. 7, A and B), translation is not obviously inhibited, because activation of PDGFR/c-Src signaling still leads to increases in COX-2 expression at both the mRNA and protein levels (Fig. 1). Furthermore, silencing of CUGBP2 by siRNAs leads to a significant decrease in PDGF-dependent induction of COX-2 expression at both mRNA and protein levels (Fig. 6A). Recent studies have shown that the function of some RNA-binding proteins is modulated by their partner protein. Sureban et al. (22) reported that HuR-mediated induction of COX-2 translation can be antagonized by CUGBP2 through its competitive binding to COX-2 mRNA. In addition, Katsanou et al. (55) found that HuR synergizes with the translational silencer TIA-1 to reduce the translation of cytokine mRNAs. Therefore, it is possible that modulation of translation by CUGBP2 depends on its protein partners, which could differ based on cellular context and/or the stimuli that cells are exposed to. CUGBP1 is a related protein that has also been shown to be phosphorylated by EGFR signaling, which increases its binding to C/EBPβ mRNA and the expression of C/EBPβ-LIP (56). Thus, a related RNA-binding protein can also be regulated by specific growth factor receptor signaling.

In summary, we find that PDGFR-dependent signalling is capable of inducing COX-2 expression in RASM in a process that involves mRNA stabilization. We have defined the mechanism for this increase in message stability with data that support a model whereby probable direct phosphorylation of the RNA-binding protein CUGBP2 by c-Src leads to increased binding to the proximal ARE in the 3′-UTR of the COX-2 transcript. This work defines a new mechanism by which COX-2 expression can be controlled and, in particular, defines a novel link between growth factor signaling and this inductive mediator of inflammation. Because COX-2 expression is elevated in plaque lesions and may play a role in the premature development of atherosclerotic disease, our hope is that further definition of the regulatory mechanisms in play for COX-2 expression may allow novel approaches for the treatment and/or prevention of such diseases.

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