CUX1 Transcription Factor Is a Downstream Effector of the Proteinase-activated Receptor 2 (PAR$_2$)*

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Proteinase-activated receptors (PARs) are G-protein-coupled receptors that have been linked to an array of cellular processes, including inflammation, migration, and proliferation. Although signal transduction downstream of PARs has been actively investigated, little is known about the mechanisms that lead to changes in transcriptional programs. Here we show that the CUX1 homeodomain protein is a downstream effector of PAR$_2$. Treatment of epithelial and fibroblastic cells with trypsin or the PAR$_2$-activating peptide (PAR$_2$-AP) caused a rapid increase in CUX1 DNA binding activity. The stimulation of CUX1 was specific to PAR$_2$ because no effect was observed with thrombin or the PAR$_1$-AP. Using a panel of recombinant CUX1 proteins, the regulation was found to involve the cut repeat 3 (CR3) and the homeodomain, two DNA binding domains that are present in all CUX1 isoforms. Expression analysis in murine CUX1$^{−/−}$ mouse embryo fibroblasts led to the identification of three genes that are regulated downstream of PAR$_2$, and CUX1 as follows: interleukin-1$\alpha$, matrix metalloprotease-10, and cyclooxygenase-2. p110 CUX1 was able to activate each of these genes, both in reporter assays and following the infection of cells. Moreover, the treatment of Hs578T breast tumor cells with trypsin led to a rapid recruitment of p110 CUX1 to the promoter of these genes and to a concomitant increase in their mRNA steady-state levels. Altogether, these results suggest a model whereby activation of PAR$_2$ triggers a signaling cascade that culminates with the stimulation of p110 CUX1 DNA binding and the transcriptional activation of target genes.

The proteinase-activated receptors (PARs)$^3$ are a subfamily of G-protein-coupled receptors that are activated by serine-proteinases (reviewed in Ref. 1). PARs include four family members, with PAR$_1$, PAR$_2$, and PAR$_3$ being activated predominantly by thrombin, and PAR$_2$ by trypsin or other serine proteinases (2–4). These receptors are activated by cleavage of an extracellular portion of the receptor, exposing a new N-terminal sequence, which then acts as a ligand for the receptor, thereby mediating autoactivation. Synthetic peptides corresponding to the newly cleaved PAR N-terminal sequences are able to activate these receptors selectively, without proteolysis (5, 6). PARs have been linked to a variety of physiological processes, including inflammation (7), wound healing (8), cellular proliferation (9, 10), and cell migration (11–13). Inactivation of specific PAR genes in knock-out mouse models has confirmed the role of PAR$_2$ in neovascularization and of PAR$_2$ in inflammation (14–18). There has been much study of PAR activation at the macroscopic animal/tissue level, and at the cellular level some signal transduction pathways have been elucidated as having putative end point targets, including monocyte chemoattractant protein 1 (19, 20), cyclooxygenase-2 (21–23), interleukins 6 and 8 (20, 24), matrix metalloproteinases (25–27), and vascular endothelial growth factor (28, 29).

Although a large number of studies have examined the signaling pathways activated in response to PAR$_2$ activation, in comparison only a few studies have investigated PAR$_2$-mediated signaling. Yet treatment of cells with PAR$_2$-specific proteases or a PAR$_2$-activating peptide was shown to lead to the activation of phospholipase C, PKC, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (MAPK) (30–32). Very little is known regarding the transcription factors that could mediate changes in gene expression patterns in response to PAR activation. Nuclear factor $\kappa$B (NF-$\kappa$B) has been shown to be activated downstream of PAR$_2$ in endothelial cells (24), pulmonary epithelial cells (33), and NCTC2544 keratinocytes (30), and of both PAR$_2$ and PAR$_3$ in bovine smooth muscle cells (34). NF-$\kappa$B was also found to be activated in HeLa and NIH3T3 cells after PAR$_2$ stimulation with factor Xa or thrombin (35, 36). In vivo, however, NF-$\kappa$B was shown only to play a minor role in a mouse model of PAR$_2$ cutaneous inflammation (37). Another transcription factor, the peroxisome proliferator-activated receptor-$\gamma$, has been implicated in the increase in cyclooxygenase-2 expression downstream of PAR$_2$ stimulation (38). A study has also linked PAR activation with activation of STAT-1 (signal transducer and activator of transcription-1) in...
DU145 and other cell lines (39). Collectively these studies have tentatively linked activation of PARs with the stimulation of a small number of transcription factors and expression of target genes.

CUX1 (cut homeobox 1) proteins are a family of transcription factors present in all metazoans and involved in the control of many cellular processes, including determination of cell identity, cell cycle progression, cell-cell communication, and cell motility (reviewed in Refs. 40, 41). Cux1−/−/ mice as well as cut mutants in Drosophila displayed phenotypes in many organs and tissues (42–45). Globally, the data indicated that Cux1 is involved in tissue homeostasis in several organs through both cell-autonomous and non-cell-autonomous mechanisms. The DNA binding and regulatory activities of CUX1 have been shown to be regulated by a vast array of post-translational modifications, including phosphorylation by PKC (46), CKII (47), cAMP-dependent protein kinase (48), and cyclin A/Cdk1 (49), dephosphorylation by Cdc25A (50), acetylation by PCAF (51), and proteolytic processing by a nuclear isoform of caspase 3 (52–54) and a caspase (55). In most cell types, two CUX1 isoforms are expressed as follows: the full-length protein, called p200, and an N-terminally truncated isoform generated by proteolytic processing, p110. Four DNA binding domains are present within p200 CUX1 as follows: cut repeats 1–3 and the cut homeodomain. Both p200 and p110 CUX1 represent heterogeneous sets of proteins that display various post-translational modifications and, in the case of p110, different N termini because caspase 3 cleaves at several positions between cut repeats 1 and 2 (52). Although p200 CUX1 functions exclusively as a transcriptional repressor, p110 CUX1 appears to function both as a repressor or activator depending on promoter context (55–57).

This study was triggered by an observation we made by serendipity. As CUX1 is regulated in a cell cycle-dependent manner, we established various methods to obtain populations of cells enriched for different phases of the cell cycle. One such approach, centrifugal elutriation, consists in the fractionation of asynchronously growing cells based on their size and density. In the standard procedure with adherent cells, a treatment with trypsin is performed to detach cells from the plate and separate them from each other. In contrast, when preparing protein extracts for transcriptional and DNA binding assays, adherent cells are generally harvested by scraping. We therefore performed an experiment to ensure that the treatment with trypsin would not affect CUX1 expression or activity. Surprisingly, trypsin did increase CUX1 activity, very rapidly. We thus hypothesized that trypsin might cause its effects by activating PAR2, a well recognized trypsin target. Our results suggested that trypsin triggers a signaling cascade involving activation of PAR2, which in turn stimulates CUX1 DNA binding activity and transcriptional regulation of a number of downstream targets.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—NIH3T3, NIH3T3/CUX1-HA, NIH3T3/CUX1-(831–1336)-HA, NIH3T3/CUX1-(1029–1505)-HA, Cux1−/−, and Cux1−/− 3T3 cells were grown in DMEM supplemented with 10% FBS. Caco-2 cells were grown in DMEM supplemented with 20% FBS, whereas KNRK and Hs578T cells were supplemented with 5% FBS. All cells were supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were routinely passed without the use of trypsin, employing Pucks EDTA cell dissociation solution (140 mM NaCl, 5 mM KCl, 5.5 mM dextrose, 4 mM NaHCO3, 13 μM phenol red, 9 mM HEPES, pH 7.3).

**Transfections and Nuclear Extracts**—Cells were seeded into a 15-cm plate (NUNC) the night before transfection with GeneJuiceTM (Novagen), with the pXJ42/CUX1-HA-expression vector. Cells were collected 24 h later with standard 5-min trypsinization (1× trypsin/EDTA, Invitrogen) or scraping in PBS. Cells were washed once in PBS, and nuclear extracts were prepared according to the procedure of Lee et al. (58), except that nuclei were obtained by submitting cells to three freeze/thaw cycles in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol, 5 mM NaVO4, and 5 mM NaF). Nuclei were then resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol 1.5 mM MgCl2, 420 mM NaCl2, 0.2 mM EDTA, 5 mM NaVO4, and 5 mM NaF) and incubated at 4 °C for 30 min. Buffers A and C were supplemented with protease inhibitor mix tablet purchased from Roche Applied Science. After 15 min of centrifugation, the supernatant was collected.

**PAR-activating Peptides**—SLIGRL-NH2- and TFLLR-NH2-activating peptides and LRGILS-NH2 reverse peptide were obtained from the University of Calgary Peptide Synthesis Core Facility, Canada.

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSA were performed as described previously (56), with these modifications. 2 μg of nuclear extract was used for stable cell lines expressing recombinant CUX1 isoforms. For transient transfections, 1–4 μg of nuclear extract was used, depending on the cell line. For supershift, antibodies were incubated for 10 min after DNA and proteins had been in contact. Oligonucleotides used in EMSA contained the CUX1 consensus binding site (underlined), TCGAGCATATCGATAAGCTTCTTTTC.

**Western Blot Analysis**—Immunoblotting was performed as detailed previously (49). CUX1 polyclonal antibodies have been described: 403 and 1300 (56) and 861 (59). HA antibody was obtained from Covance.

**Reverse Transcripase (RT)-PCR**—Cell pellets were harvested in TRIzol (Invitrogen), extracted in chloroform, precipitated in isopropyl alcohol, and resuspended in DEPC water for 1 h of DNase treatment (Promega RQ1 DNase) in DNase buffer. Ethanol precipitation then followed, and RNA was resuspended in DEPC water. For the reverse transcription reaction, 4 μg of RNA was annealed with 0.1 μg of oligo(dT) before incubation with Superscript II (Invitrogen) and RNaseOUT™ (Invitrogen) according to the manufacturer’s instructions. Incubation was for 1 h at 42 °C followed by 1 h at 37 °C and boiling for 10 min to inactivate the enzyme. DEPC water was added to a final volume 50 μ. 1 μl of cDNA or 1 μl of a 1:30 dilution was used for PCR (final volume 30 μl). PCRs were performed with Taq polymerase (Promega) in 1× buffer containing 1.5 mM MgCl2 and 0.6 mM dNTPs with 10 pmol of each primer. Initial denaturation was 94 °C for 4 min, and the cycles were as follows: 94 °C for 45 s; annealing temperature varied from 55 to 58 °C for 45 s; extension temperature was 72 °C for 50 s; 22 cycles for GAPDH;
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Figure 1. Treatment of cells with trypsin or the PAR2-activating peptide (SLIGRL-NH2) leads to a rapid stimulation of DNA binding by CUX1. A and B, cells from the Caco-2 epithelial cell line were transfected with a vector expressing recombinant full-length CUX1 protein with a C-terminal hemagglutinin tag (HA). 24 h later, cells were incubated for 5 min in the presence of either 1× trypsin/EDTA (A) or the PAR2, activating peptide SLIGRL (B). Cells that were not treated with trypsin were harvested by scraping. Nuclear extracts were prepared and analyzed by EMSA with double-stranded oligonucleotides containing a consensus binding site for CUX1 (left panel) and in Western blot with the HA antibody. GST, glutathione S-transferase. C, NIH3T3 cells endogenously expressing CUX1 were incubated for 5 min in the presence of either 1× trypsin EDTA, the PAR2-activating peptide, SLIGRL, or the reverse peptide, LRGLS, as a control. Cells that were not treated with trypsin were harvested by scraping. Nuclear extracts were prepared and analyzed in EMSA as above and in Western blot with the 861 CUX1 antibody. D, NIH3T3 cells stably expressing a recombinant p110 CUX1 protein were incubated for 1 h in the presence of PAR2, GST antibody (Ab) (2.5 μl per ml of medium), PAR2 FSSLRY-NH2 antagonist (Antag) peptide (200 μM), or medium alone (control). As indicated cells were then stimulated with trypsin for 5 min (lanes 3, 5, and 7) or were scraped (lanes 2, 4, and 6), and nuclear extracts were prepared for EMSA and CUX1 Western blot. Supershift (lane 1) was performed with an antibody specific to CUX1 isoforms. Quantiﬁcation of CUX1 DNA binding was performed with the phosphorimaging analysis software. E, schematic representation of p200 and p110 CUX1, indicating antibody epitopes including the C-terminal HA tag for the recombinant CUX1.

30–35 cycles for other primers, and the final extension was 72°C for 7 min. Mouse oligonucleotides were as follows: Cox2 (582 bp), forward ACTCACTCAGTTTGTGAGTCTTC and reverse TTGCAATGACTTGTTAGGTTAATG; IL-6 (598 bp), forward CTGGTGACAACCACCCGGCTTTCC and reverse ATGCATTAGGCTATAACGCTTTG; MMP9 (456 bp), forward AGCACAACAGCTGACTACGATAAG and reverse GGCTTGGCCACGCTGGAATGATCTAA; PAR2 (625 bp), forward TCCCCGTTGGACACCAGCTTGG and reverse CGATGTTCCTTCTTCTTCTTG; VEGF (spliced isoforms of 431, 563, and 635 bp), forward ACATCTCAAGCCACCACCCAGGC and reverse AAATCGGGAATCGTCCCCACGAG; FGF-2 (344 bp), forward AAGCGGCTCTACTGCAAGA-ACG and reverse TTTCTGAGTGTCGGCTTTTGG; GAPDH, forward ACCACTCTGATGCCACTCA-TCAC and reverse TCCACACCTCCTTGTGCTA. The human genes were as follows: PAR1 (572 bp), forward GGTGCTGGGACTCTTTGG; PAR2 (520 bp), forward GACTTTCTTCGTTGGCT and reverse CCCCATAAATCCAGTTTGTG.

Luciferase Assays and Reporters—25,000 Hs578T cells were seeded the night before into 12-well plates, transfected as above with 100 ng of reporter construct, 500 ng of pXJ expression vector alone or expressing CUX1-(1–1505) or -(878–1505), corresponding to p200 and p110. β-Galactosidase protein (Sigma) was co-transfected as a transfection efficiency control. Cells were harvested 24 h post-transfection. Results are an average of three internal repeats, and the experiments were performed in triplicate. Errors are mean ± S.E. The Cox2 reporter has been described (60). For the MMP10 and IL1α reporters, promoter fragments were obtained by PCR amplification and inserted into the Nhel–Xhol or Xhol-HindIII sites, respectively, of the pGL3-Basic vector (Promega). The primers used are as follows: for MMP10, forward CACCATGCCCCAGCAAGCCTATATTCCT and reverse TCTCACGCTTTACTTCTTGTCTAC; for IL1α, forward TGCCTTTCTTTCCAACTCT.
binant p110-Tag<sup>2</sup> protein was purified by the Taptag purification method with some modifications (62). The IgG matrix-bound p110 Tag<sup>2</sup>/DNA were washed with the above-mentioned wash buffers I, II, and III then TEV buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% Triton X-100, 0.5 mM EDTA, 10% glycerol, 1 mM DTT). After TEV protease digestion, the released protein-DNA complexes were purified by affinity chromatography on calmodulin beads in the presence of calcium and then eluted with EGTA. After de-cross-linking, samples were treated with RNase A and proteinase K. Each purification step was monitored by immunoblotting with polyclonal antibodies against the calmodulin binding domain epitope tag or against CUX1. PCR amplification was performed with the following primers: COX2, forward TCTGAGCGGCCCTGAGGT and reverse ACAGCCTATTAAGGTCGTC (this amplifies from −584 to −410 relative to the transcription start site); MMP10, forward GGAAATGGAGAATGAGTGAAC and reverse AGAATTAAGTAGGTCACCGGAATA (−610 to −396 relative to the transcription start site); ILL1a, forward GCATGCCATCACACCTAGTT and reverse TTCCTTCATGAGAATGAGTGAAC and reverse AGAATTAAGTAGGTCACCGGAATA (−610 to −396 relative to the transcription start site); GAPDH, forward GGACCTTGTGCTGCGATTT and reverse CTTTCTGGGATTGCTT.

**RESULTS**

**Trypsinization of Epithelial and Fibroblastic Cells Stimulates DNA Binding by CUX1**—We compared the DNA binding activity of CUX1 after harvesting cells by two distinct methods, only one of which involved trypsin exposure. Cells of the Caco-2 colonic epithelial cell line expressing a recombinant CUX1 protein with an HA tag at its C terminus were collected either by scraping in a PBS solution or by incubation for 5 min in a trypsin/EDTA solution. In both cases, cells were then washed in PBS, resuspended in the same lysis buffer, and processed in identical fashion to purify nuclear extracts (see details under “Experimental Procedures”). An electrophoretic mobility shift assay was performed using equal amounts of nuclear extracts and double-stranded oligonucleotides containing a consensus binding site for CUX1 (Fig. 1A). As reported previously, two main retarded complexes were observed (52, 54, 59, 63). A sharp band at the top of the gel was previously ascribed to the full-length p200 CUX1 isoform (59). A more diffuse band lower in the gel was found to involve smaller CUX1 isoforms, globally called p110, that are generated by proteolytic processing at three different positions and are post-translationally modified in various ways (see details in the Introduction) (52, 54, 59). Both retarded complexes were more intense in the sample prepared using trypsin treatment (Fig. 1A, compare lanes 1 and 2). Western blot analysis indicated that the amount of CUX1 protein was not greater in the trypsin-treated sample (Fig. 1A, lanes 3 and 4). Similar results were obtained using NIH3T3 fibroblastic cells (Fig. 1C, compare lanes 2 and 3; and data not shown). Altogether, these results revealed that the DNA binding activity of CUX1 was stimulated following treatment of cells with trypsin.

**Treatment of Epithelial and Fibroblastic Cells with PAR<sub>2</sub>-activating Peptides Stimulates DNA Binding by CUX1**—As both trypsin/EDTA and Pucks/EDTA solutions cause cells to detach and round up, we concluded that cell detachment-rounding up per se was not responsible for the increase in CUX1 DNA binding. However, because trypsin is well recognized as an activator of PAR<sub>2</sub>, we hypothesized that CUX1 binding might be stimulated by a PAR<sub>2</sub> signaling mechanism. To test this hypothesis, Caco-2 cells were incubated for 5 min in the presence of the receptor-selective PAR<sub>2</sub>-activating peptide (PAR<sub>2</sub>-AP), SLIGRL-NH<sub>2</sub>, and then harvested by scraping. DNA binding by CUX1 was increased by the PAR<sub>2</sub>-AP, whereas the steady-state level of the proteins remained unchanged (Fig. 1B, compare lane 4 with 5 and lane 6 with 7). The supershifts obtained upon addition of both the HA and the CUX1 861 antibodies confirmed that the retarded complexes involve CUX1 proteins (Fig. 1B, lanes 1 and 2). In contrast, an unrelated antibody, anti-glutathione S-transferase, did not induce a supershift but increased the intensity of the retarded complexes (Fig. 1B, lane 3). This effect is because of the increase in protein concentration and the stabilization of protein-DNA complexes.

Similarly, treatment of NIH3T3 cells with either trypsin or the PAR<sub>2</sub>-activating peptide, but not with the receptor-inactive PAR<sub>2</sub>-reverse peptide LRGILS-NH<sub>2</sub>, stimulated DNA binding by the endogenous CUX1 protein without affecting its level of expression (Fig. 1C compare lanes 2–4 with lanes 5–7). The slowly migrating retarded complex was supershifted by the CUX1 861 antibody (Fig. 1C, lane 1). To verify the implication of the PAR<sub>2</sub> receptor, NIH3T3 cells stably expressing a recombinant CUX1 protein were incubated for 1 h in the presence of the PAR<sub>2</sub> A5 antibody, a PAR<sub>2</sub> antagonist peptide or medium
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FIGURE 3. DNA binding by CUX1 can be stimulated by trypsin or the PAR2-activating peptide, but not by thrombin or the PAR1-activating peptide. A–C, cells were treated for 5 min with either 1 unit/ml thrombin or the 1× trypsin/EDTA solution. Cells that were not treated with trypsin were harvested by scraping. Nuclear extracts were prepared and analyzed in Western blots with the indicated antibodies and in EMSA with a consensus binding site for CUX1. A, immunoblotting was also performed with a phosphoserine PKC substrate antibody (Cell Signaling Technology) and α-tubulin as a loading control. Where indicated in the EMSA, CUX1 antibodies were added to the reactions to generate supershifts. The regions recognized by each antibody are shown in the diagram of Fig. 1E, D, NIH3T3 cells stably expressing recombinant HA-tagged CUX1 were treated for 5 min with the PAR2-activating peptide, SLIGRLNH2, the PAR1-activating peptide, TFLLRNH2, or both. Cells were collected by scraping and analyzed as in A.

alone prior to the trypsin treatment. Both the antibody and the antagonist peptide hampered the stimulation of CUX1 DNA binding activity (Fig. 1D, compare lanes 3, 5, and 7 and see quantitation). These results suggest that the PAR2 receptor is involved in the stimulation of CUX1 DNA binding.

Increase in CUX1 DNA Binding Correlates with the Concentration of PAR2-AP—To verify whether the PAR2-AP would stimulate CUX1 DNA binding activity in a concentration-dependent manner, NIH3T3 cells were treated with increasing concentrations of the receptor-activating peptide, and CUX1 DNA binding was monitored using the EMSA approach. As a control, cells were treated with the receptor-inactive reverse-sequence peptide at the highest concentration used for the active PAR2-AP. Western blot analysis demonstrated that the steady-state level of CUX1 protein did not vary significantly (Fig. 2B), whereas the DNA binding by CUX1 (densitometry analysis) was progressively stimulated with increasing concentrations of the PAR2-AP (Fig. 2A). In contrast, at the highest concentration used for the active PAR2-AP, the reverse-sequence peptide did not stimulate DNA binding (Fig. 2A, lane 5).

PAR2 Is the Only Protease-activated Receptor That Can Stimulate CUX1 DNA Binding—We asked whether activation of all protease-activated receptors could lead to the stimulation of CUX1 DNA binding. The thrombin proteinase is able to signal via PAR1 (coordinately with PAR3) and PAR4 but cannot signal via PAR2. To determine whether any of the thrombin-triggered PARs might affect CUX1 DNA binding, we compared the effect of thrombin and trypsin on three different cell lines as follows: NIH3T3 fibroblastic cells, Hs578T breast tumor cells, and KNRK-rat PAR2, a fibroblastic cell line expressing a recombinant rat PAR2 receptor. As controls, cells were maintained in the medium and then harvested by scraping. In each of the three cell lines, thrombin had no significant effect, whereas trypsin-stimulated DNA binding by CUX1 (Fig. 3, A–C). To ensure that thrombin was able to trigger a signaling response, protein extracts from NIH3T3 cells were submitted to immunoblotting with a phosphoserine PKC substrate antibody (Cell Signaling Technology). As a loading control immunoblotting was performed with an α-tubulin antibody. The results show that both thrombin and trypsin stimulated the activity of PKC, a downstream effector of phospholipase C (Fig. 3A, right panel) (64, 65). In accordance with these results, incubation of NIH3T3 cells with a receptor-selective PAR2-activating peptide, TFLLR-

Mapping of CUX1 Sequences Required for the Stimulation of DNA Binding Downstream of PAR2—To map the region of CUX1 that is the target of regulation downstream of PAR2, we utilized a series of vectors expressing smaller recombinant CUX1 proteins (Fig. 4B). NIH3T3 cells expressing recombinant CUX1 proteins were treated or not with trypsin for 5 min, and nuclear extracts were prepared and analyzed using EMSA and Western blotting. We observed stimulation of DNA binding with recombinant proteins that lacked the N-terminal region, the C-terminal region, or an internal deletion from amino acids 659–878 (Fig. 4A, see constructs 659–1505, 1029–1505, and 831–1336). Stimulation of DNA binding was seen with proteins containing all possible combinations of DNA binding domains as follows: CR1CR2CR3HD (1–1505, 1–1505, and 1029–1505) and CR2CR3HD (659–1505, 831–1336), or CR3HD (1029–1505) (Fig. 4A). Taken together, the results indicated that the region between amino acids 1029 and 1336 contains all the elements required for the stimulation downstream of PAR2. This region contains two DNA binding domains as follows: the cut repeat 3 (CR3) and the cut HD.
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Some PAR2 Target Genes Are Differentially Regulated in cux1+/+ and cux1−/− Immortalized MEF Cells—The results presented above raised the possibility that CUX1 might mediate, at least in part, some of the changes in transcriptional program that take place in response to PAR2 activation. As a first step to test this hypothesis, we compared the steady-state mRNA levels of a number of putative PAR2 targets in immortalized mouse embryo fibroblasts derived from a cux1−/− knock-out mouse and a cux1+/+ littermate (42). As expected, expression of a housekeeping gene, GAPDH, was identical in the cells derived from the two cell backgrounds (Fig. 5A). We observed minor differences, if any, in the expression of the genes for matrix metalloproteinase 9 (MMP9), PAR2, and fibroblast growth factor 2 (FGF2) (Fig. 5A). In contrast, in the cux null cells, relative to the wild-type cells, we observed a reduction in the expression of genes coding for cyclooxygenase-2 (COX2), interleukin 6 (IL6), vascular endothelial growth factor (VEGF), interleukin 1α (IL1α), and MMP10 (Fig. 5A). We verified that the expression of these genes was up-regulated in cux1+/+ MEF cells in response to a 5-min treatment with trypsin or a 2-h treatment with the PAR2-activating peptide, SLIGRL-NH2, but not with the reverse sequence PAR2-inactive peptide, LRGILS-NH2 (27). Cells were returned to the incubator. At the indicated time, cells were harvested by scraping and pipetting, and total RNA was purified. The mock-treated cells were maintained in their medium for 5 min and then returned to the incubator for another 15 min. RT-PCR analysis was performed to measure expression of Cox2, IL-6, VEGF, IL1α, MMP10, and GAPDH. The products of the reactions were separated by electrophoresis on agarose gel and stained with ethidium bromide.

Observed minor differences, if any, in the expression of the genes for matrix metalloproteinase 9 (MMP9), PAR2, and fibroblast growth factor 2 (FGF2) (Fig. 5A). In contrast, in the cux null cells, relative to the wild-type cells, we observed a reduction in the expression of genes coding for cyclooxygenase-2 (COX2), interleukin 6 (IL6), vascular endothelial growth factor (VEGF), interleukin 1α (IL1α), and MMP10 (Fig. 5A). We verified that the expression of these genes was up-regulated in cux1+/+ MEF cells in response to a 5-min treatment with trypsin or a 2-h treatment with the PAR2-activating peptide, SLIGRL-NH2, but not with the reverse sequence PAR2-inactive peptide, LRGILS-NH2. RNA was prepared after various periods of time, and RT-PCR was performed. Although expression of the housekeeping gene, GAPDH, did not vary at any time point (Fig. 5B and C), expression of COX2, IL6, VEGF, IL1α, and MMP10 were induced following treatment with trypsin (Fig. 5B). Similarly, following treatment with the PAR2-AP, we observed a reproducible increase in the expression of COX2, IL1α, and MMP10 (Fig. 5C). Thus both trypsin, presumably acting via PAR2, and the PAR2-activating peptides that selectively activate PAR2, were able to trigger the induction of the same genes. Interestingly, expression of these three genes was increased following the infection of cux1−/− MEF cells with a retroviral vector expressing p110 CUX1 but not with a vector.
Treatment of Cells with Trypsin Induces the Recruitment of CUX1 to the Promoters of the COX2, IL1α, and MMP10 Genes—We investigated whether CUX1 could directly activate the COX2, IL1α, and MMP10 genes following stimulation of PAR2. For reasons of economy, cells were stimulated with the PAR2-activating enzyme, trypsin, rather than with the PAR2-AP. First, scanning ChIP analysis was performed to verify whether CUX1 would bind to their promoters (data not shown). In each case, CUX1 was found to bind to a region situated between 100 and 600 bp upstream of the transcription start site. Examination of the genomic DNA sequences bound by p110 CUX1 revealed the presence of a CUX1 consensus binding site (ATCAAT) within the MMP10 gene promoter, whereas the COX2 and IL1α gene promoters contained sequences (ATCCAT, ATACAT, and ATTCAT) that had previously been identified as high affinity binding sites for CUX1 in PCR-mediated site selection performed with various combinations of CUX1 DNA binding domains (66). We then investigated the recruitment of p110 CUX1 to the promoter of these genes in response to the activation of cells with trypsin, reflecting PAR2 stimulation. Because there are no antibodies that selectively recognize p110 CUX1 but not the full-length protein, we opted to purify chromatin by tandem affinity purification, using a method that we recently developed and validated to identify p110-specific targets (57). Following a 5-min treatment with trypsin, RNA and chromatin were isolated at various time points from Hs578T breast tumor cells stably expressing physiological levels of a p110 CUX1 protein with two epitope tags at its C terminus. Expression of the three genes was increased in response to trypsin treatment (Fig. 6A). In parallel, p110 CUX1 was recruited to the promoters of these genes after 15 min and was still present on these promoters after 60 min (Fig. 6B). Thus, trypsin-stimulated binding of p110 CUX1 to these promoters correlated with gene induction. We then verified whether the promoter regions that included the CUX1-binding site were sufficient for transcriptional activation by CUX1. The promoter sequences were introduced into a luciferase reporter plasmid, and luciferase activity was measured in the presence of effector plasmids expressing either p200 or p110 CUX1. Expression was not significantly affected by p200 CUX1 but was stimulated ~5-fold in the presence of p110 CUX1. Altogether, these results suggest a model whereby proteinase-mediated activation of PAR2 triggers a signaling cascade that culminates with the stimulation of p110 CUX1 DNA binding and the transcriptional activation of target genes.

affinity purification: cells were treated or not with trypsin for 5 min, returned to the incubator for 15 or 60 min, and then treated with a chemical cross-linking agent (see “Experimental Procedures”). Chromatin was purified by affinity chromatography. The total and the affinity-purified DNAs were used as templates in PCR amplification using primers derived from the promoter regions of the COX2, IL1α, MMP10, and GAPDH genes. C, reporter assays. The promoter regions of the COX2, IL1α, MMP10, and GAPDH genes were inserted into the pGL3-luciferase reporter plasmid. Each reporter plasmid was introduced into Hs578T cells together with an effector plasmid expressing p200 or p110 CUX1 or with an empty vector. Cytoplasmic extracts were prepared the following day and processed to measure luciferase activity. The values are the mean of three independent experiments, and error bars represent standard deviation.

FIGURE 6. p110 CUX1 activates expression of COX2, IL1α, and MMP10 after trypsin treatment. A, Hs578T breast tumor cells stably expressing physiological levels of a p110 CUX1 protein with two epitope tags at its C terminus were treated for 5 min with trypsin/EDTA. 10 volumes of DMEM +10% FBS was added to the plate, and cells were returned to the incubator. At the indicated time, cells were harvested by scraping and pipetting, and total RNA was purified. The mock-treated cells were maintained in their medium for 5 min and then returned to the incubator for another 15 min. RT-PCR analysis was performed to measure expression of COX2, IL1α, MMP10, and GAPDH. The products of the reactions were separated by electrophoresis on agarose gel and stained with ethidium bromide. B, chromatin expressing p200 CUX1 (Fig. 5D). Altogether, these results singled out genes that are regulated downstream of both PAR2 and the CUX1 transcription factor.
DISCUSSION

The data we have obtained support the hypothesis that trypsin-mediated activation of PAR₂ leads to a rapid increase in the ability of multiple CUX1 isoforms to bind to DNA (Figs. 1, 3, and 4), thereby stimulating the transcription of a number of PAR₂-stimulated genes (Figs. 2 and 5). Presumably, other serine proteinases capable of activating PAR₂ in the setting of tumors, like kallikrein-related peptidase-14 (KLK14), will also trigger CUX1-mediated transcription (3). The speed with which DNA binding was stimulated as well as the lack of change in the steady-state level of CUX1 protein isoforms both concur to suggest that post-translational modifications were probably responsible for the modulation of CUX1 DNA binding activity (Figs. 1–4). This conclusion is in keeping with a number of studies describing the regulation of CUX1 by various types of post-translational modifications (46–51). Although the precise modification(s) of CUX1 that are induced following PAR₂ activation remain to be investigated, our mapping analysis implicates a targeting of the region of the cut repeat 3 and the cut homeodomain. We note that these two DNA binding domains are the subject of many modifications, including phosphorylation and acetylation, which have already been shown to affect DNA binding (46–51). The importance of this region is illustrated by the complete conservation of its amino acid sequence among mammalian organisms not only within the DNA binding domains themselves but also within the linker region between them (67–69). Future work will aim to define each step of the signaling pathway leading from PAR₂ activation to CUX1-mediated gene transcription. Results from our study point to a specific branch of the PAR₂ signaling pathway that does not involve mediators (e.g., elevated intracellular calcium) that are in common with both the PAR₂ and PAR₁ signaling pathways, because neither thrombin nor a PAR₂ activating-peptide had any effect on CUX1 DNA binding activity (Fig. 3).

A number of genes have been reported to be regulated in response to PAR₂ activation. In this study, we identified three genes whose transcriptional activation was mediated by the p110 isoform of the CUX1 transcription factor. The evidence for this activation by p110 was obtained from multiple experimental approaches. First, the mRNA steady-state level of COX2, MMP10, and IL1α was reduced in cux1⁻/⁻ as compared with cux1⁺/⁺ MEFS (Fig. 5A). Second, expression of these genes was increased following the treatment of cux1⁺/⁺ MEFS with either trypsin (Fig. 5B) or the PAR₂-activating peptide (Fig. 5C), or following the infection of cux1⁻/⁻ MEFS with a p110 CUX1 but not a p200 CUX1, retroviral vector (Fig. 5D). Third, reporter plasmids for each of these genes were activated by p110 CUX1 but not p200 CUX1 (Fig. 6C). Finally, to investigate whether the COX2, MMP10, and IL1α genes were direct transcriptional targets of CUX1, we performed scanning ChIP analysis with multiple primer pairs to map the regions where CUX1 would bind within each transcription unit (data not shown). Armed with this information, we were then able to test whether CUX1, and in particular its p110 isoform, would be induced by trypsin to bind to the regulatory regions of the three genes. Using Hs578T cells stably expressing a recombinant p110 CUX1 protein with two tags, a correlation was established between the recruitment of p110 CUX1 to the promoters of these genes and their transcriptional activation following a trypsin treatment (Fig. 6, A and B).

Importantly, the increase in CUX1 DNA binding following trypsin-mediated PAR₂ activation was demonstrated not only in vitro in the electrophoretic mobility shift assay, but also in the chromatin affinity purification assay that monitors directly in cells the recruitment of transcription factors to specific regulatory sequences (Fig. 6). Although other studies have described the involvement of transcription factors downstream of PAR₂ using various in vitro assays, to our knowledge this study for the first time provides evidence for the recruitment of a specific transcription factor in vivo. Genome-wide location arrays (ChIP-chip) should be used in the future to identify all the targets to which CUX1 is recruited as part of physiological processes that are induced following PAR₂ activation. In particular, it is striking that both PAR₂ and CUX1 have been implicated in proliferation as well as in motility (9–13). Constitutive expression of p110 was shown to stimulate cell proliferation by accelerating entry into S phase (70), and functional annotation of targets identified by genome-wide location arrays revealed an over-representation of genes with various functions required for cell cycle progression, notably DNA replication and mitosis (57). Moreover, CUX1 was identified as a pro-migratory factor in a high-throughput RNA interference screen aimed at identifying genes involved in cell motility (71). Presumably in the setting of proteinase-producing cancer cells, PAR₂ activation by serine proteinases in addition to trypsin will have a comparable impact on CUX1-mediated effects. In a panel of human cancer cell lines, short interfering RNA-mediated knockdown of CUX1 expression caused a decrease in cell motility, as measured in a wound-healing assay and video time-lapse microscopy, in invasion through Matrigel, and in pulmonary colonization after caudal vein injection (71). Therefore, we would predict that many effects of proteinase-triggered PAR₂ activation on cell proliferation and cell motility are mediated via the stimulation of CUX1 DNA binding and its subsequent recruitment to specific target genes. More broadly, the comparison of results obtained from expression profiling and ChIP-chip performed with several transcription factors should allow us to dissect the changes in transcriptional program that are imparted following activation of the PAR₂ signaling pathway.

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