Identification of a Thrombin Response Element in the Human Platelet-derived Growth Factor B-chain (c-sis) Promoter*

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Thrombin is a coagulation system protease that also serves as a potent stimulator of gene expression in several cell types, including endothelial cells (EC). We and others have previously demonstrated that the transcription of platelet-derived growth factor (PDGF) B-chain (c-sis) by EC is stimulated severalfold by thrombin. Here we examine the molecular mechanism of this regulatory process using bovine aortic EC transiently transfected with a vector containing the chloramphenicol acetyltransferase (CAT) gene under the control of a 400-base pair fragment of the human PDGF B-chain promoter. Thrombin treatment of these cells caused a severalfold increase in CAT expression. Deletion analysis and site-directed mutagenesis revealed that the region spanning nucleotides −61 to −53 from the transcription initiation site (referred to as the thrombin response, or ThR, region) was critical for the transcriptional response to thrombin. Electrophoretic mobility shift assays with an oligonucleotide corresponding to the region −64 to −44, which contained the ThR region, led to the identification of a thrombin-inducible nuclear factor (TINF) in extracts from thrombin-treated, but not control, EC. TINF was formed as early as 40 min post-thrombin treatment, persisted for at least 7 h, but was no longer present after 24 h. TINF appeared in the absence of de novo protein synthesis. The ThR region consists of a repeat of a CCACCC element in an ABBA configuration, which, based on mutation analysis and transfection assays, appears to be critical in mediating thrombin stimulation of the PDGF B-chain gene. The conservation of the ThR region in the promoter of the PDGF B-chain among three species (human, feline, and murine) further supports the importance of this region as a cis-acting regulatory element.

Endothelial cell (EC) injury or activation and the subsequent proliferation of vascular smooth muscle cells (SMC) are central to the development of atherosclerosis (1). While the triggering molecule(s) responsible for stimulating the multiplication of SMC is not known, a viable candidate is platelet-derived growth factor (PDGF), the major mitogen in human serum (2-5). PDGF, although originally purified from human platelets, is produced in a regulated manner by numerous transformed cells and by several types of normal diploid cells, including EC, SMC, mesangial cells, activated macrophages, and cytotoxophoblasts (6–8). PDGF consists of a disulfide-linked heterodimer or homodimer of two distinct but homologous sub-units designated the A- and B-chains (6). The B-chain is the protein product of the c-sis proto-oncogene (9, 10).

Regulated EC expression of PDGF production in vivo may be important in stimulating proliferation of the underlying perivascular cell types and in the recruitment of both leukocytes and SMC in response to vascular injury (11, 12). A probable physiological modulator of PDGF release from the endothelium is α-thrombin, a multifunctional serine protease generated at sites of vascular injury, which has been demonstrated in vitro to induce the expression and release of PDGF A- and B-chains from EC (13–17). Thrombin, at physiologically relevant concentrations, stimulates a number of EC functions including the generation of prostacyclin (18), platelet-activating factor (19), von Willebrand factor (20), plasminogen activator (21), and its inhibitor (22). The molecular mechanism underlying thrombin stimulation of transcription of the PDGF B-chain gene or, in fact, thrombin induction of any gene in EC, as well as in any other cell type, remains unknown.

Regulation of PDGF B-chain gene transcription has been explored in multiple cell types. Specific sequences have been defined within the 5′ untranslated region of the B-chain gene, which may be important in the regulation of its transcription (23). Transfection experiments in K-562 cells (a hematopoietic cell type) have defined a minimal promoter region which includes the sequence extending 400 bp 5′ of the transcription initiation site (24). In addition, several DNase I-hypersensitive sites have been located in the human PDGF B-chain gene, although localization of these sites has focused on the first intron and the region downstream of the coding sequence with little known about the 5′ flanking region (25, 26). Recently, Kachigian et al. (27) have defined a minimal promoter for basal expression of the PDGF B-chain gene in EC. This promoter region contains several consensus sequences for binding such transcriptional factors as Ets family members and AP-1 complexes. These investigators, however, have not examined the role of any of these elements in agonist-stimulated transcription of this gene in EC. Others have used linker scanning of the PDGF B-chain core promoter to identify an element essential for TPA-induced activation of this gene in K562 cells (28).

In this report, we present evidence supporting the identification of a 9-bp region in the PDGF B-chain gene as being responsible for thrombin-induced transcription of this gene. We further demonstrate that a thrombin-inducible nuclear factor (TINF) and the transcription factor Sp1 are two distinct factors that bind to this region of DNA.
EXPERIMENTAL PROCEDURES

Materials—Bovine thrombin was purchased from Miles, Inc. (Kankakee, IL). Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F-12) culture medium was from Irvine Scientific (Santa Anna, CA) and fetal bovine serum (FBS) was obtained from Whittaker Bioproducts (Walkersville, MD). Rabbit polyclonal IgG to Sp1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and human recombinant Sp1 was from Promega (Madison, WI). Tissue culture plastic was from Costar (Cambridge, MA). All other chemicals, unless otherwise noted, were purchased from Sigma.

Cell Culture—Bovine aortic EC were isolated as described previously (29) and grown in DMEM/F-12 containing 5% FBS. At confluence the EC were routinely subcultured at 1:3 or 1:5 ratios by trypsin-EDTA treatment. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were included in the media of cells for assay. Bovine aortic EC were used at passages 3–12. Human umbilical vein EC were isolated from undamaged segments of 1–3-day-old umbilical cords, as described previously (29), and grown in DMEM/F-12 supplemented with 20% FBS. Human umbilical vein EC were used in primary culture.

PCR Amplification of Fragments of the PDGF B-chain Promoter, Cloning, and Plasmid Preparation—Based on the published sequence of the 5' flanking region of the PDGF B-chain gene (24), we synthesized several primers, which were used for amplification of regions of the promoter. A 400-bp region upstream of the transcription initiation site, as well as several shorter fragments, were amplified using human EC DNA (200 ng) (30) and primers (130 pmol each) shown in Table I in a total volume of 100 μl of 1 X Taq DNA polymerase buffer (Promega). The amplification was carried out using a Perkin-Elmer Cetus thermal cycler. Cycling times were 3 min at 95°C (denaturation), 3 min at 40°C (annealing), and 3 min at 72°C (extension). Twenty-five cycles were performed, concluding with a 3-min extension at 72°C. The resulting PCR products were resolved by low melting point agarose gel electrophoresis (31). The purified DNA was digested with the restriction enzyme PstI in order to cleave the synthetic sites flanking the amplified DNA and then cloned into pBluescript (KS- vector (Stratagene, Inc., La Jolla, CA) and into a promoterless plasmid vector from Promega. Plasmid DNAs were purified using Qiagen columns (Qiagen, Chatsworth, CA) according to the manufacturer's directions. All the amplified DNA fragments were confirmed by sequencing (Sequenase version 2.0, U. S. Biochemical Corp.).

Transfection Protocol and Chloramphenicol Acetyltransferase (CAT) Assay—Bovine aortic EC were grown in six-well plates to about 80% confluence and washed with Opti-MEM I reduced-serum media (Life Technologies, Inc.) prior to transfection. The optimal transfection efficiency was obtained using 5 μg of DNA and 15 μg of Lipofectin (Life Technologies, Inc.) in 1 ml of Opti-MEM I as described by the manufacturer. Following incubation for 16 h at 37°C, 5% CO2, the medium was replaced with fresh Opti-MEM I with or without bovine thrombin (10 units/ml or as specified). At the time indicated in the figure legends, cells were harvested by scraping from the plates and lysed by repeated freeze-thaw cycles. The CAT assay was performed using [14C]chloramphenicol and Tris buffer according to the method of Rosenthal (32), and the reaction products were extracted by simple phase-extraction (33). All determinations were obtained within the linear range of the assay, typically between 0.7 and 30% conversion of [14C]chloramphenicol. Presented data represent the mean of at least duplicate transfection wells and are normalized to protein content per well, as determined by the BCA protein assay (microtiter plate protocol) (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared as described by Dignam et al. (34). Double-stranded synthetic probes were labeled by random priming with [γ-32P]ATP (Du Pont NEN). The binding buffer solution contained Tris-HCl (10 mM, pH 7.5), NaCl (50 mM), MgCl2 (1 mM), EDTA (0.5 mM), dithiothreitol (0.5 mM), glycerol (4%, v/v), poly(dI-dC) (0.1 mg/ml) (Pharmacia Biotech Inc.), and protease inhibitors (100 μM PMSF, 0.1 mM leupeptin, 5 μg/ml aprotinin, 5 μg/ml antipain, and 5 μg/ml) (Boehringer Mannheim) added immediately before use, and 5 μg of nuclear extract protein in a final volume of 10 μl. Labeled oligonucleotide (~50,000 cpm/35 fmol) was added, and the incubation was continued for 20 min at room temperature. DNA-protein complexes were separated by electrophoresis on a 6% polyacrylamide gel in 22.5 mM Tris base, 22.5 mM boric acid, and 0.5 mM EDTA at room temperature in a cooled circulating electrophoresis apparatus and visualized by autoradiography. Where indicated, competing oligonucleotides at a 100-fold molar excess (3.5 pmol) were used.

RESULTS

Thrombin Induces CAT Expression Driven by the p400 PDGF B-chain Promoter Region in Bovine Aortic EC—To investigate the effects of thrombin on the promoter region of the PDGF B-chain gene, we first generated a 400-bp promoter fragment directly 5' to the transcription initiation site of this gene using PCR and primer oligonucleotides, the sequences of which were derived from the published sequence of Pech et al. (24). For functional studies, the amplified fragment was subcloned into the pCAT-basic vector upstream of the reporter gene chloramphenicol acetyltransferase (CAT). Thrombin responsiveness of the promoter region of the PDGF B-chain gene was evaluated by transiently transfecting bovine aortic EC with the synthesized 400-bp CAT construct, subsequently treating these cells with increasing concentrations of bovine α-thrombin (0–8 units/ml) for 24 h, and ultimately quantitating CAT activity in the cell lysates. A 24 h-period following thrombin addition was sufficient to allow for readily detectable CAT in the lysates of transfected EC. Increased levels of CAT were observed in the bovine aortic EC containing the 400-bp CAT construct in response to as little as 0.1 units/ml thrombin, and maximal CAT expression was achieved at 8 units/ml thrombin (Fig. 1). EC transfected with the pCAT-basic vector alone showed no increase in response to thrombin (data not shown). In a study with several independently isolated strains of bovine aortic EC transfected with the p400-CAT construct, we reproducibly observed a 3–7-fold increase in CAT expression induced by thrombin. These thrombin-stimulated levels of CAT correlated quantitatively with thrombin induced levels of PDGF in the conditioned media of these cells, as measured by radioreceptor assay (data not shown).

Identification of a Thrombin Response (Thr) Element in the PDGF B-chain Promoter—To define further the region corresponding to the thrombin response element, we generated deletion mutants of the 400-bp promoter region using PCR technology and the primers described in Table I. A schematic representation of these constructs appears in Fig. 2A. Promoter fragments were ligated into our reporter vector, transfected into bovine aortic EC, and the cultures tested for the ability to respond to thrombin. As shown in Fig. 2B, the smallest construct that retained thrombin responsiveness was p86. Construct p43, which contained the minimal promoter activity...
Thrombin Response Element in the PDGF B-chain Promoter

Table I

Oligonucleotides employed in this study

In the case of oligonucleotides denoted with a mix of uppercase and lowercase letters, uppercase refers to nucleotides identical to those in wild type PDGF B-chain promoter and lowercase indicates the introduction of restriction sites. Bold characters indicate substitution nucleotides to produce mutations. Nucleotide positions with respect to the transcription initiation site within the PDGF-B promoter are in parentheses.

A. Oligonucleotides used in EMSA experiments (coding strand)

|     | Sequence                        |
|-----|---------------------------------|
| A   | 5′-aactgcagGAAGAGAAAGCTTgtcgactt-3′ |
| B   | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| C   | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| Thr | 5′-catatgcatacCCACCCACACTgtagcttgac-3′ |
| B mut | 5′-aactgcagCTTAACTTTGAGACCTGACGACTgtcgactt-3′ |
| S64 | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| S55 | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| S610587 | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| S60 | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| S-ABBA | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| AP1 (c-j un) | 5′-GCCTGGAGAGAGCAAGCCGGAAG-3′ |
| AP2 | 5′-GATCGAACTGACGCGGCGGCGGCGCCTG-3′ |
| NFκB | 5′-AGTTGAGAGGACTTCCAGGCGG-3′ |
| CREB | 5′-AGAGAATTGCCTGAACTGAGACGACT-3′ |
| TF1ID | 5′-GCAGACATATAAGGGAGGAGGAAG-3′ |
| Spi | 5′-AAGGCATCTGACGCGGCGGCGGCGGCGGCGGCGGCGGCGGCG-3′ |

B. Oligonucleotides used to generate promoter fragments by PCR

| Primer set | Sequence                        |
|------------|---------------------------------|
| p248       | 5′-aactgcagCTTGGTGAGGAGGAGGAC-3′ |
| p141       | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| p86        | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| p43        | 5′-aactgcagCTTCCCCACCTTCTGCGACTgtcgactt-3′ |
| p6       | 5′-ctgcactgtcgacGATTTTGAAGGAC-3′ |
| p64mutant  | 5′-ctgcactgtcgacGATTTTGAAGGAC-3′ |
| p64S610587 | 5′-ctgcactgtcgacGATTTTGAAGGAC-3′ |

Primer used in all the above reactions for the reverse direction

5′-gtcgacGACGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCG-3′

required for expression in these cells, demonstrated no increased CAT expression in response to thrombin. This observation suggested that the region spanning oligonucleotides –86 to –44 (see Fig. 2A) was involved in the transcriptional response to thrombin.

To further identify the specific sequence within the p86 construct of the PDGF B promoter responsible for thrombin induction, we employed an alternative approach. Transcription factors present in the nuclear extracts of thrombin-treated, but not in untreated EC, should bind to their specific DNA sequence, allowing visualization by EMSA. Oligonucleotides corresponding to two regions of the segment containing the thrombin response element, one corresponding to the 5′-79 to –66 and the second, the 3′-31 sequence, outside of the thrombin response region, designated C was prepared as a control (Fig. 3). Each oligomer was radiolabeled for use in EMSA. Incubation of oligonucleotide A or C with nuclear extracts prepared from bovine aortic EC that had been treated with thrombin did not reveal any novel shifted bands compared with nuclear extracts from untreated EC (data not shown). The B oligomer, however, yielded a thrombin-dependent DNA-protein complex that migrated more rapidly than several other complexes that were constitutively present in nuclear extracts (Fig. 4). The specificity of these DNA-protein complexes was demonstrated by competition experiments with 100-fold molar excess of unlabeled oligonucleotides, representing several consensus binding site sequences for known transcription factors as well as other sequences that are proximal to the B region (Fig. 4). These oligonucleotides are fully defined in Table I. The competition study showed that among the many oligomers tested, only an excess of the unlabeled oligonucleotide B competed efficiently with labeled B oligomer in binding to TINF.

To demonstrate that the B region was a functional thrombin

Fig. 2. Identification of the THR in the PDGF B-chain promoter using deletion analysis. A, schematic diagram indicating the location of the deletion constructs in the PDGF B-chain promoter. Each construct label indicates the remnant size (bp) of the promoter region cloned into the expression vector (based on the transcription initiation site described by Pech et al. in Ref. 24). TIS indicates the transcription initiation site. B, bovine aortic EC were transfected with the deletion mutant constructs depicted in A, treated with or without thrombin (10 units/ml) for 16 h, and CAT activity was determined. Results represent mean of triplicate cultures ± S.E. Figure is representative of more than four independent experiments.
response element, we generated a construct that contained the B region attached to the thrombin-unresponsive minimal promoter region p43 (Fig. 2B) and assayed for thrombin-stimulated CAT activity in EC. This new PCR-generated construct, designated p64, was linked to the CAT reporter gene and used for transfection into bovine aortic EC. As shown in Fig. 5, EC containing the p64 construct responded to thrombin with greater than a 3-fold increase in CAT expression over similarly transfected controls that were not treated with thrombin, and this level was comparable to cells containing the fully thrombin-responsive p86 construct. In addition, thrombin treatment of the p64 construct-transfected EC yielded 3 times the fold induction in CAT activity than was seen with the p43 construct-transfected EC, indicating that the B region was sufficient to restore thrombin responsiveness to the p43 construct. Taken together, these data strongly suggested that the B region contained the element(s) necessary to increase the transcription of the PDGF B-chain gene as a result of thrombin stimulation.

Interaction of SP1 and TINF with the B Region of the PDGF B-chain Promoter—With the observation that an oligomer corresponding to the B region formed a specific protein-DNA complex when incubated with nuclear extracts prepared from thrombin-treated EC, we were led to investigate further the properties of this nuclear factor. First, we examined the kinetics of TINF appearance in the nucleus following thrombin treatment. As visualized by EMSA, the TINF complex was apparent in EC nuclear extracts as early as 40 min after thrombin addition, remained at a peak level for up to 7 h, and became undetectable by 24 h (Fig. 6). When EC were pretreated with the protein synthesis inhibitor cycloheximide (10 μg/ml) for 30 min, followed by 1 h with the inhibitor plus thrombin, TINF still appeared in the nuclear extracts, indicating that de novo protein synthesis was not required (data not shown). The rapid appearance of this binding protein in the nucleus of EC following thrombin treatment is consistent with a lack of requirement of new protein synthesis.

In an attempt to identify other nuclear proteins in thrombin-stimulated EC that bind to the B region and to determine the novelty of TINF, competition assays were performed using oligonucleotides containing consensus binding site sequences for several known nuclear factors. As shown in Fig. 4, many consensus sequence oligonucleotides failed to cause any reduction in the TINF-DNA complex. Effective competition was observed, however, with a 100-fold molar excess of unlabeled

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**Fig. 3.** Region of the PDGF B-chain promoter containing the thrombin response element. Diagram showing the sequence of the region involved in thrombin responsiveness. Oligonucleotide sequences used as probes for EMSA are shown in boxes. The arrows indicate the beginning position of the PCR constructs. The ThR element is underlined.

**Fig. 4.** Characterization of the thrombin response region by EMSA. Nuclear extracts prepared from bovine aortic EC treated without (control) or with thrombin (10 units/ml) for times indicated were used in EMSA. The 5′ end-labeled B sequence oligomer (see Fig. 3) was incubated with nuclear extracts (5 μg) in the absence or presence of a 100-fold molar excess of unlabeled oligonucleotides corresponding to the B region, C region, A region, AP1 binding sequence, NF-kB binding sequence, AP2 binding sequence, TFIID binding sequence, and the CREB binding sequence. The TINF complex is indicated.

**Fig. 5.** Functional analysis of the thrombin response region. Bovine aortic EC were transfected with the p43 and p64 CAT constructs as well as p86-CAT as positive control, treated with or without thrombin (10 units/ml) for 16 h, and CAT activity was determined as described under "Experimental Procedures." Results represent mean of triplicate cultures ± S.E. Figure is representative of four independent experiments. Fold induction of CAT activity is expressed as a ratio of cpm of thrombin-treated cultures/cpm of media-treated cultures.

**Promoter Constructs**

- p86
- p43
- AP1
- NF-kB
- AP2
- TFIID
- CREB

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Thrombin Response Element in the PDGF B-chain Promoter
Fig. 6. Time course of TINF appearance in EC nuclear extracts. Nuclear extracts prepared from bovine aortic EC treated without (control) or with thrombin (10 units/ml) for the times indicated were heat treated (10 min, 47°C) to eliminate Sp1 binding and then incubated with labeled double-stranded oligomer B and used in EMSA. Arrow indicates the position of the TINF complex.

oligomer representing the consensus binding site for Sp1 (Fig. 8A). This result suggested that in addition to the TINF complex, Sp1-like proteins may bind to the B region. To determine whether TINF and Sp1 are distinct proteins, a “supershift” experiment was performed in which Sp1 antibody, Egr-1 antibody, or purified IgG was incubated with the nuclear extract-labeled oligomer mixture prior to the mobility shift assay. One of the major constitutively present bands (band 1) was shifted to a lower mobility in the gel (band ss-Sp1) with the Sp1 antibody, whereas TINF migration was unaltered (Fig. 7). The Egr-1 antibody as well as the IgG control had no effect on any of the bands from the EC extracts. Evidence that TINF is distinct from Sp1 includes its gel mobility, which is greater than the Sp1 proteins, its insensitivity to heat treatment (#47°C for 10 min; data not shown) (35), and the inability of an Sp1 polyclonal antibody to either deplete nuclear extracts of TINF or to supershift TINF in the EMSA.

Detailed Characterization of the B Region—Oligonucleotides containing several substitutions in the B region were tested as competitors in an EMSA in order to identify more specifically the DNA sequence requirements for TINF-DNA complex formation. As shown in Fig. 8A, oligonucleotides corresponding to the B region, ThR, and the Sp1 consensus binding site efficiently competed with TINF in binding to the B region oligomer. Common to all of these binding site oligomers was a CCACCC element, which has been reported to have high affinity for Sp1-like proteins (36–38). Included in the B region is also an inverted repeat of the sequence CCACCC in an ABBA configuration (Fig. 8B). To test whether this element may be involved in mediating thrombin responsiveness of the PDGF B-chain promoter, a series of mutant oligonucleotides were synthesized and tested in an EMSA. Fig. 8A shows that the B region substitution mutant oligomers, S64–59 and S55–50, each of which had only one end of the ABBA motif mutated but still contained a single intact CCACCC sequence, competed with TINF, as well as the Sp1-like proteins for binding to the B oligomer. However, binding of these nuclear proteins to the labeled B oligonucleotide was not inhibited by an excess of the oligomer S-ABBA, in which both the forward and inverted repeat formed by the CCACCC elements had been disrupted. Additionally, oligomer S610587, which contained four point mutations within the CCACCC sequence, and oligomer S60–56, in which the CCACCC (position −60 to −56) of the B region had been replaced with AGTTGT, did not compete for TINF binding. Direct nuclear factor binding studies, visualized in EMSA, showed that the labeled B mutant and S610587 failed to bind TINF, as well as the lower mobility, constitutively expressed, nuclear proteins. These results suggest that the ABBA configuration of bases CCACCC, which constitutes the CCACCCACC sequence, referred to as the ThR element within the PDGF B-chain promoter B region, is responsible for the binding of both TINF and Sp1-like proteins and that the minimum sequence required for a thrombin-induced response is CCACCC (Fig. 9).

To confirm that the CCACCC element is mediating thrombin-induced transcription of the PDGF B-chain gene in bovine aortic EC, we generated several mutants of the PDGF B-chain promoter by PCR and ligated them into the CAT expression vector in order to perform functional analyses. These mutants included a p64 mutant plasmid, in which the CCACCCACC (ThR region) sequence was fully substituted by AAGTTTGAA, and p64S610587, in which four base substitutions were made within the CCACCC element. These mutant plasmid constructs were used to transfect bovine aortic EC, and CAT activity was measured in replicate cultures following treatment with thrombin or no stimulator. Cells transfected with the mutant constructs did not demonstrate thrombin responsiveness, although EC transfected with either of two wild-type constructs, p86 and p64, exhibited a severalfold increase in CAT activity in response to thrombin (Fig. 10). These results further support a role for the CCACCC element in thrombin stimulation of PDGF B-chain gene expression.

DISCUSSION

We have employed a combination of reporter gene constructs-transfection experiments and gel shift assays to map the region of the PDGF B-chain gene that controls the transcriptional response to thrombin in EC. The ThR region is localized to nucleotides −61 to −53 and contains sequences capable of binding to a thrombin-induced nuclear factor, denoted as TINF, as well as constitutively expressed protein(s). The TINF complex appears rapidly in nuclear extracts of EC treated with thrombin, as early as 20–40 min post-stimulation, is relatively long-lived, being maintained up to 7 h, and is absent by 24 h after treatment. The appearance of the TINF complex in the
nucleus does not require de novo protein synthesis, which may indicate that it pre-exists in an inactive form in the cell and that thrombin either directly or indirectly causes its activation and/or translocation.

We show by competition in EMSA that the interaction between the thrombin-induced nuclear proteins and the B region of the PDGF B-chain promoter is specific. Among a panel of nuclear factor oligomers representing the consensus sequence binding sites for AP1, AP2, NFκB, TFIID, CREB, Sp1, or oligonucleotides flanking the B region and the B region itself, only an excess of unlabeled oligomer B and consensus sequence Sp1 oligonucleotide were effective competitors. Moreover, recombinant Sp1 protein bound to labeled B oligomer and yielded a band that corresponded in electrophoretic mobility to band 1 from the profile obtained using nuclear extract from thrombin-stimulated EC. When HeLa cell nuclear extracts (used as a known source of Sp1) (39) were incubated with B region oligomer, two distinct bands were observed that had similar gel mobilities to two bands seen with thrombin-stimu-

FIG. 8. Characterization of the specificity of TINF complex formation by competition in EMSA. Nuclear extracts were prepared from bovine aortic EC treated without (control) or with thrombin (10 units/ml) for 5 h. The 5'-end-labeled B oligonucleotide was incubated with nuclear extracts (5 μg) in the absence or presence of 100-fold molar excess of various mutated B oligonucleotides as indicated. EMSA was performed as described under “Experimental Procedures.” Arrow indicates the position of the TINF complex.

FIG. 9. Characterization of the specificity of TINF complex formation by competition in EMSA by use of radiolabeled mutated oligomers. Nuclear extracts were prepared from bovine aortic EC treated without (control) or with thrombin (10 units/ml) for 5 h. Labeled double-stranded wild type oligomer (B) or mutated oligomers (B mutant and S-610587), were incubated with EC nuclear extracts (5 μg). EMSA was performed as described under “Experimental Procedures.” Arrow indicates the position of the TINF complex.

FIG. 10. Transfection assay using PDGF B-chain promoter-CAT constructs with mutations in the thrombin response region. Bovine aortic EC were transfected with the p86, p64, p64 mutant, and p64S610587 PDGF B-chain promoter constructs, treated with or without thrombin (10 units/ml) for 16 h, and CAT activity was determined as described under “Experimental Procedures.” Results represent mean of triplicate cultures ± S.E. Figure is representative of at least six independent experiments. -Fold induction of CAT activity is expressed as a ratio of cpm of thrombin-treated cultures/cpm of medium-treated cultures.

Promoter Mutant Constructs

The nucleus does not require de novo protein synthesis, which may indicate that it pre-exists in an inactive form in the cell and that thrombin either directly or indirectly causes its activation and/or translocation.

We show by competition in EMSA that the interaction between the thrombin-induced nuclear proteins and the B region of the PDGF B-chain promoter is specific. Among a panel of nuclear factor oligomers representing the consensus sequence binding sites for AP1, AP2, NFκB, TFIID, CREB, Sp1, or oligonucleotides flanking the B region and the B region itself, only an excess of unlabeled oligonucleotide B and consensus sequence Sp1 oligonucleotide were effective competitors. Moreover, recombinant Sp1 protein bound to labeled B oligomer and yielded a band that corresponded in electrophoretic mobility to band 1 from the profile obtained using nuclear extract from thrombin-stimulated EC. When HeLa cell nuclear extracts (used as a known source of Sp1) (39) were incubated with B region oligomer, two distinct bands were observed that had similar gel mobilities to two bands seen with thrombin-stimu-
an EMSA. In a supershift assay, in which the same nuclear extracts were allowed to bind to the B region oligomer first and then allowed to react with Sp1 antiserum, we observed retarded mobility of the Sp-1 bands without a change in TINF mobility.

The GC box is the classic high affinity Sp1-binding site (40). However, this is not the nuclear factor’s target sequence in the ThR region. In the ThR region the sequence CCACCC is present as an overlapping, inverted repeats. Extensive reports exist in the literature that show Sp1 is capable of binding to a CCACCC sequence (36–38, 41), which is present in each of the inverted repeats of the ThR region of the PDGF B-chain gene. Although Sp1 is widely distributed through many cell types and species, binding sites for this strong transcriptional activator have been reported in the promoters of many cell-specific genes. Direct interactions between Sp1 and a variety of other binding proteins have been implicated in several regulated processes, including hormone activation (42), regulation by steroids (43), heat shock response (44), and tissue-specific gene expression (45).

Sp1 has been shown to contain several independent transcriptional activation domains in addition to the zinc finger region located within the C terminus of the protein (46). Regions outside of the DNA binding domain of Sp1 may be responsible for modulating transcriptional activity by allowing for its interaction with other factors in the transcription initiation complex, such as TINF. Sp1 sites have been identified near binding sites for other transcriptional activators, such as CTF/NF-1 (47), AP2 (48), OTF-1 (49), and, in fact, a functional interaction between Sp1 and AP-1 has been demonstrated (50). Recently, Sanchez et al. (51) have shown cooperation of a sterol regulatory element-binding protein and Sp1 in regulating the transcription of the low density lipoprotein receptor gene.

We have demonstrated that the ThR segment within the B region of the PDGF B-chain gene is involved in the transcriptional response to thrombin. The conservation of the ThR region of the PDGF B-chain gene across different species (human (52), feline (52), and murine (53)) suggests that the region may serve as a binding site for important cis-acting elements. If this region is in fact a site responsive to a thrombin-induced transcriptional activator, then one might anticipate finding the CCACCC motif within the promoter region of other thrombin-responsive genes. In fact, the CCACCC-motif is also present in the promoters of other EC genes that are regulated by thrombin. The PDGF A-chain gene, which is induced by thrombin, contains this motif both in the sense and “antisense” DNA strands (positions 269 and 233 bp from the TATA box, respectively). The CCACCC motif is also present in the promoters of other thrombin-regulated genes, such as tissue factor (GGGTGG at 85 bp and CCCACC at 291 bp from the TATA box), thrombomodulin (CCACCC at 56 bp and GGGTGG at 246 bp from the TATA box), VCAM-1 (at 117 bp from the TATA box), and von Willebrand factor (at 403 bp from the TATA box). The PAI-1 gene, another EC gene that is induced by thrombin, contains a GGGTGG at positions 46 and 401 from the TATA box, and it has been shown that the sequence GGGTGG in the sense strand at position 46 is responsible for TPA induction of this gene (50).

Our studies with mutant sequence oligomers of the ThR region suggest that in thrombin-stimulated EC the CCACCC motif of the PDGF B-chain promoter is the recognition site for both TINF and Sp1, and the orientation is of no consequence to nuclear factor binding. The mechanism by which these two nuclear factors mediate induction of transcription by thrombin is not known. In gel shift assays, we observed a reduction of Sp1 binding to the B region oligomer in thrombin-treated nuclear extracts, possibly because TINF and Sp1 bind in a mutually exclusive fashion and therefore compete for the oligomer. J in et al. (28) have recently identified a site in the PDGF B-chain promoter that was essential for phorbol ester-induced PDGF B-chain transcription in K-562 cells. Subsequent in vivo footprinting analyses by Dirks et al. (54) have confirmed that this region binds a transcriptional activator in phorbol ester-treated K562 cells. This reported TPA response element coincides with the region that we report as the ThR region. Our evidence indicates that TINF is a different nuclear factor than the TPA-induced protein identified by J in et al. and referred to as band “m” (28). TINF has substantially greater gel mobility than band m and, unlike band m, appearance of TINF in the nucleus is independent of de novo protein synthesis. It should be noted that there is a difference in the numbering of the position of the ThR region in our report and the PMA-responsive region in the report of J in and co-workers, which is based on a discrepancy in the literature of the position of the transcription initiation site of the PDGF B-chain gene. We have followed the mRNA initiation site described by Pech et al. (24) rather than that of Rao et al. (23).

In conclusion, we have identified a sequence in the PDGF B-chain promoter which is involved in thrombin-induced transcription of this gene in bovine aortic EC. Mutational analysis of the ThR region has revealed that the CCACCC-motif is required for a thrombin response. We also provide evidence of a specific thrombin-induced nuclear factor that binds to this region. Sp1 protein is also capable of binding to the CCACCC sequence, but is distinct from TINF. The mechanism by which these transcription factors interact to regulate transcription of the PDGF B-chain gene is the subject of further investigation.

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REFERENCES

1. Ross, R. (1993) Nature 362, 801–809
2. Antoniades, H. N., and Williams, L. T. (1983) Fed. Proc. 42, 2630–2634
3. Deuel, T. F., and Huang, J. S. (1983) in Progress in Hematology (Brown, E. B. et al., eds) Vol. 13, Grune & Stratton, Inc.
4. Heldin, C.-H., Wasteson, A., and Westermark, B. (1985) Mol. Cell. Endocrinol. 39, 169–187
5. Ross, R. (1986) N. Engl. J. Med. 314, 488–500
6. Ross, R. (1989) Lancet i, 1179–1182
7. DiCorleto, P. E., and Fox, F. P. (1988) in Endothelial Cells (Ryan, U. E., ed) Vol. II, pp. 51–61, CRC Press, Boca Raton, FL
8. Shultz, P. J., DiCorleto, P. E., Silver, B. J., and Abboud, H. E. (1988) Am. J. Physiol. 255, F674–F680
9. Doolittle, R. F., Hunkapiller, M. W., Hood, L. E., Devare, S. G., Robbins, K. C., Aaronson, S. A., and Antoniades, H. N. (1983) Science 221, 275–277
10. Deuel, T. F., Huang, J. S., Huang, S. S., Stroobant, P., and Waterfield, M. D. (1983) Science 221, 1348–1350
11. Ross, R., Raines, E. W., and Bowen-Pope, D. F. (1986) Cell 46, 155–169
12. Heldin, C.-H., and Westermark, B. (1990) Cell Regul. 1, 555–566
13. Waterfield, M. D., Srapec, G. T., W. N., Stroobant, P., Johnson, A., Waterson, A., Westermark, B., Heldin, C.-H., Huang, J. S., and Deuel, T. F. (1983) Nature 304, 35–39
14. Heldin, C.-H., Gibbs, V. C., Milly, D. F., Garovoy, M. R., and Williams, L. T. (1986) J. Biol. Chem. 261, 9579–9582
15. Daniel, T. O., Gibbs, V. C., Milly, D. F., Garovoy, M. R., and Williams, L. T. (1987) J. Biol. Chem. 262, 11893–11899
16. Starksen, N. F., Harsh, G. R., IV, Gibbs, V. C., and Williams, L. T. (1987) J. Biol. Chem. 262, 14381–14384
17. Shankar, R., de la Motte, C. A., and DiCorleto, P. E. (1992) Am. J. Physiol. 262, C190–C206
18. Wessler, B. B., Lye, C. W., and Jaffe, A. E. (1978) J. Clin. Invest. 62, 923–930
19. Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3703–3707
20. Levine, J. D., Harlan, J. M., Harker, L. A., Joseph, M. L., and Counts, R. B. (1982) Blood 60, 531–534
21. Levine, E. G., Marquez, U., Anderson, J., and Harker, L. A. (1984) J. Clin. Invest. 74, 1988–1995
22. Gelehrter, T. D., and Sznycer-Laszuk, R. (1986) Biochemistry 25, 5087–5094
23. Rao, C. D., Igarashi, H., Robbins, K. C., and Aaronson, S. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2392–2396
24. Pech, M., Rao, C. D., Robbins, K. C., and Aaronson, S. A. (1989) Mol. Cell. Biol. 9, 396–405
25. Franklin, G. C., Donovan, M., Adam, G. J. R., Holmgren, L., Pfeifer-Olsson, S., and Olsson, R. (1991) EMBO J. 10, 1363–1373
3032

Thrombin Response Element in the PDGF B-chain Promoter

26. Dirks, R. P. H., Jansen, H. J., Gerritsma, J., Onnekink, C., and Bloemers, H. P. J. (1993) Eur. J. Biochem. 211, 509–519
27. Khachigian, L. M., Fries, J. W. U., Benz, M. W., Bonthron, D. T., and Collins, T. (1994) J. Biol. Chem. 269, 22647–22656
28. Jin, H.-M., Brady, M. L., and Fahl, W. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7563–7567
29. DiCorleto, P. E., and de la Motte, C. A. (1989) Biochem. J. 264, 71–77
30. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989) Current Protocols in Molecular Biology, Greene Publishing Associates/Wiley-Interscience, New York
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Rosenthal, N. (1987) Methods Enzymol. 152, 704–755
33. Seed, B., and Sheen, J.-Y. (1988) Gene (Amst.) 6, 271–277
34. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
35. Farnham, P. J., and Cornwell, M. M. (1991) Gene Exp. 1, 137–148
36. Hartzog, G. A., and Myers, R. M. (1993) Mol. Cell. Biol. 13, 44–56
37. Gumucio, D. L., Rood, K. L., Blanchard-McQuate, K. L., Gray, T. A., Saulino, A., and Collins, F. S. (1991) Blood 78, 1853–1863
38. Yu, C.-Y., Motamed, K., Chen, J., Bailey, A. D., and Shen, C.-K. J., (1991) J. Biol. Chem. 266, 8907–8915
39. Kadonaga, J. T., Carner, K. R., Masiarz, F. R., and Tjian, R. (1987) Cell 51, 1079–1090
40. Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) Trends Biochem. Sci. 11, 20–23
41. Chang, D. J., Paik, Y.-K., Leren, T. P., Walker, D. W., Howlett, G. J., and Taylor, J. M. (1990) J. Biol. Chem. 265, 9486–9504
42. Schule, R., Muller, M., Otsuka-Murakami, H., and Renkawitz, R. (1988) Nature 332, 87–90
43. Dawson, P. A., Hofmann, S. L., van der Westhuyzen, D. R., Sudhof, T. C., Brown, M. S., and Goldstein, J. L. (1988) J. Biol. Chem. 263, 3372–3379
44. Morgan, W. D. (1989) Mol. Cell. Biol. 9, 4099–4104
45. Morgan, J. G., Courtois, G., Fouriel, G., Chodosh, L. A., Campbell, L., Evans, E., and Crabtree, G. R. (1988) Mol. Cell. Biol. 8, 2628–2637
46. Courey, A. J., and Tjian, R. (1988) Cell 55, 881–888
47. Jones, K. A., Yamamoto, K. R., and Tjian, R. (1985) Cell 42, 559–572
48. Mitchell, P. J., Wang, C., and Tjian, R. (1987) Cell 50, 847–861
49. Janson, L., and Pettersson, U. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4732–4736
50. Descheemaeker, K. A., Wyns, S., Nefles, L., Auwerx, J., Ny, T., and Colen, D. (1992) J. Biol. Chem. 267, 15086–15091
51. Sanchez, H. B., Yi, L., and Osborne, T. F. (1995) J. Biol. Chem. 270, 11611–11616
52. Van den Ouweland, A. M. W., Roebroek, A. J. M., Schalken, J. A., Claesen, C. A. A., Bloemers, H. P. J., and Van de Ven, W. J. M. (1986) Nucleic Acids Res. 14, 765–778
53. Bonthron, D. T., Sultan, P., and Collins, T. (1991) Genomics 10, 287–292
54. Dirks, R. P. H., Jansen, H. J., van Gerven, B., Onnekink, C., and Bloemers, H. P. J. (1995) Nucleic Acids Res. 23, 1119–1126