Cross-talk between Different Enhancer Elements during Mitogenic Induction of the Human Stromelysin-1 Gene*

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Platelet-derived growth factor (PDGF) induces the expression of human stromelysin-1, a matrix metalloproteinase involved in tumor invasion and metastasis. Here it is shown that stromelysin-1 gene induction by PDGF depends on Ras and involves three previously identified promoter elements (the stromelysin-1 PDGF-responsive element (SPRE) site, the two head-to-head polyomavirus enhancer A-binding protein-3 (PEA3) sites, and the activator protein-1 (AP-1) binding site). During mitogenic induction, these responsive elements appear to be organized in two independent transcriptional units, SPRE-AP-1 and PEA3-AP-1, which result from specific element cross-talking. Interestingly, expression of a dominant negative mutant of Raf-1 significantly interfered with the induction through PEA3-AP-1 but not with that operating through SPRE-AP-1. Conversely, only the induction operating through SPRE-AP-1 was affected significantly by the expression of a dominant negative mutant of the atypical λι protein kinase C (λι PKC). These data strongly suggest that the signal triggered by PDGF flows through Ras and bifurcates toward two distinct pathways, one operating through Raf and involving PEA3-AP-1 and the other one Raf-independent, operating through λι PKC and SPRE-AP-1. Furthermore, we present evidence suggesting that the novel SPRE-binding transcription factor SPBP cross-couples with c-Jun to transactivate the SPRE site.

A number of mitogenic molecules including growth factors, proto-oncogenes, oncogenes, and tumor promoters induce the expression of stromelysin-1, an extracellular matrix-degrading metalloproteinase (Matrisian et al., 1985; Kerr et al., 1988; McDonnel et al., 1990; Diaz-Meco et al., 1991; Wasylyk et al., 1991). These observations together with studies showing that this gene is also induced in transformed cells (Matrisian et al., 1985; Breathnach et al., 1987; Nicholson et al., 1989) suggest that stromelysin-1 induction has important consequences in tumor invasion and metastasis (Matrisian et al., 1986; Sreenath et al., 1992).

Three specific DNA elements in the promoter of the human stromelysin-1 gene have been implicated in mitogenic induction: the SPRE (stromelysin-1 PDGF-responsive element) (Sanz et al., 1994), the PEA3 site (polyomavirus enhancer A-binding protein-3 site) (Wasylyk et al., 1991; Buttice and Kurkinen, 1993), and the AP-1 site (activator protein-1 binding site) (Kerr et al., 1988). The AP-1 site (centered at −67) is the most proximal element and is the target of the Fos and Jun transcription factors (Curran and Franzi, 1988). In the stromelysin-1 gene, two PEA3 elements are arranged head to head (Quinones et al., 1989; Buttice and Kurkinen, 1993), resulting in a palindromic element (centered at −208) with two binding sites for Ets transcription factors (Wasylyk et al., 1991). Finally, SPRE, the most distal of the three elements (centered at −1573), is the binding site of a new transcription factor, SPBP (SPRE-binding protein) (Sanz et al., 1994, 1995).

Studies on rat and human stromelysin-1 gene expression have independently identified two of the above elements, SPRE and AP-1, as playing major roles in PDGF induction (Kerr et al., 1988; Sanz et al., 1994). Similar studies have implicated each of the three elements in the Ras activation of the stromelysin-1 gene (Imler et al., 1988; Wasylyk et al., 1989, 1991; Diaz-Meco et al., 1991). Because it has been established that Ras is an intermediate in the mitogenic cascade triggered by PDGF, it was of interest to investigate whether, in fact, these three elements all contribute to the induction of the human stromelysin-1 gene by PDGF. In this report, by transient gene expression experiments using promoter constructs extending upstream to −2311 and mutants thereof, we show that both PDGF and c-Ha-ras induce transcription of the human gene through two different functional units, one comprising the SPRE and AP-1 sites and the other involving the two head-to-head PEA3 elements and the AP-1 site. Moreover, we present evidence supporting the idea that SPBP and c-Jun, a critical component of the AP-1 transcription factor, cross-couple to yield heteromers that efficiently transactivate the SPRE site.

The abbreviations used are: SPRE, stromelysin-1 platelet-derived growth factor-responsive element; PDGF, platelet-derived growth factor; PEA3, polyomavirus enhancer A-binding protein-3; AP-1, activator protein-1; SPBP, SPRE-binding protein; PKC, protein kinase C; GST, glutathione S-transferase.
and therefore have potential relevance in the transactivation of the SPRE-AP-1 unit. It has been established that phospholipase C-mediated hydrolysis of phosphatidylcholine plays a crucial role in mitogenic signaling of mammalian cells (Larrrodera et al., 1990 and references therein) and suggested that phosphatidylcholine-hydrolyzing phospholipase C acts downstream of Ras in the PDGF-triggered mitogenic cascade (Lopez-Barahona et al., 1990; Diaz-Meco et al., 1991). Furthermore, recent evidence supports the existence of a bifurcation of the PDGF-mitogenic signaling pathway downstream of phosphatidylcholine-hydrolyzing phospholipase C with 1/2PKC and Raf-1 located in separated branches (Sanz et al., 1994; Bjorkoy et al., 1995; Diaz-Meco et al., 1996). In this study we show that each of the PDGF-mitogenic branched pathways is specifically involved in the activation of a single transcriptional unit. Thus, the PEA3-AP-1 unit activation is essentially Raf-dependent, whereas the alternative 1/2PKC-dependent pathway operates through the SPRE-AP-1 unit.

MATERIALS AND METHODS

Cell Culture—NIH 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C.

To obtain XXB1, the 1,833-base pair construct (XXB1) used in the experiments detailed below (data not shown). The corrected sequence of the promoter fragment extending upstream to −2311 has been submitted to the GenBank/EMBL Data Bank with accession number U43511.

Expression and Purification of Recombinant Fusion Proteins—pGEX constructs were transformed into Escherichia coli J M101 and the corresponding GST-fusion proteins expressed and purified following the manufacture's instructions (Pharmacia).

RESULTS

Two Different and Independent Transcriptional Units Promote the Expression of Human Stromelysin-1 in Response to Mitogens—We have reported previously a sequence obtained from a subclone of the human stromelysin-1 gene promoter (Quinones et al., 1989). Based on the recent observation of a partial inversion and an additional 1 kilobase of promoter sequence interrupting the 2311 to −479 fragment of the human stromelysin-1 promoter was subcloned upstream of the palCAT, pcDNA3HASPB plasmids, and RSV-c-Jun expression vector (Quinones et al., 1990 and references therein) and suggested that phosphatidylcholine-hydrolysis phospholipase C acts downstream of Ras in the PDGF-triggered mitogenic cascade (Lopez-Barahona et al., 1990; Diaz-Meco et al., 1991). Furthermore, recent evidence supports the existence of a bifurcation of the PDGF-mitogenic signaling pathway downstream of phosphatidylcholine-hydrolyzing phospholipase C with 1/2PKC and Raf-1 located in separated branches (Sanz et al., 1994; Bjorkoy et al., 1995; Diaz-Meco et al., 1996). In this study we show that each of the PDGF-mitogenic branched pathways is specifically involved in the activation of a single transcriptional unit. Thus, the PEA3-AP-1 unit activation is essentially Raf-dependent, whereas the alternative 1/2PKC-dependent pathway operates through the SPRE-AP-1 unit.

2 By using a cDNA probe encoding the regulatory domain of the atypical rat 1/2PKC, we cloned a PKC isotype from Xenopus laevis which was initially named pPKC because it displayed an overall 72% identity with the rat counterpart. The more recent description of other atypical PKC isoforms has allowed the comparison of the X. laevis enzyme with all known subspecies. On the basis of their sequence alignment, the X. laevis atypical PKC shows the highest identity with 1/2PKC (90%).

nucleotides used were ON SPRE, 5'-AGCTTATAGAATACTAGTTTAG-3'; ON SPREMut, 5'-AGCTTATAGAATACTACTTTTAG-3'; ON PEA3, 5'-AGCTTACAGAACGACCTTCGAGG-3'; ON PEA3mut, 5'-AGCTTACAGAACGACCTTGCAGG-3' (Wasylk et al., 1991); ON AP-1, 5'-AGCTTATAGAATACTTTGAG-3'; ON AP-1mut, 5'-AGCTTATAGAATACTTTGAG-3' (Angel et al., 1987). The small letters in the sequence indicate the mutated positions. All of the constructs were characterized by nuclease sequencing and restriction mapping. Dominant negative expression constructs were as described previously (Cai et al., 1990; Diaz-Meco et al., 1993; Sanz et al., 1994). The production of palCAT, pcDNA3HASPB plasmids, and RSV-c-Jun expression vector have been described elsewhere (Sanz et al., 1994; Angel et al., 1996). The GST-constructs were generously provided by Bernd Stein, and the c-Ha-Ras expressing construct was a gift from Scott Powers.

Gel Shift Mobility Assays—Mobility assays were performed as described previously (Sanz et al., 1995) using nuclear extracts from each PDGF or serum-stimulated NIH 3T3 cells. Where indicated the precipitation mixture contained anti-jun antibodies, which recognize c-jun, J unB, and J unD, or anti-Fos antibodies, which recognize c-Fos, Fra1, and Fra2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

In Vitro Transcription and Translation—The SPB2 mRNA was obtained from linearized pcDNA3HASPB plasmid (10 μg) using T7 RNA polymerase in the presence of the cap analog GpppG and further translated (1 μg) in a nucleotide-treated rabbit reticulocyte lysate system which contained methionine-free amino acid mixture supplemented with [35S]methionine according to the manufacturer's instructions (Promega). Control mixtures contained no mRNA.

In Vitro Protein Interaction Assays—Equal amounts of GST-c-Fos or GST-c-jun immobilized on glutathione-Sepharose beads in LSBT buffer (20 mM Hepes, pH 7.9, 10 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Nonidet P-40, 1.5% Triton X-100) were incubated with 5 μl of 35S-labeled translation mixture in a final volume of 200 μl in the presence of 1 mM dihydrothiocol and 1 mM phenylmethylsulfonyl fluoride. After 10 min at 37°C and 40 min at room temperature in an effective end-to-end mixing device, the beads were successively washed, washed with 0.5 mM NaCl in LSBT, and equilibrated in 50 mM Tris, pH 6.8. The bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

Expression and Purification of Recombinant Fusion Proteins—pGEX constructs were transformed into Escherichia coli J M101 and the corresponding GST-fusion proteins expressed and purified following the manufacturer's instructions (Pharmacia).
important intermediary step in the PDGF signaling cascade. Similar to PDGF, cotransfection with c-Ha-ras resulted in increased stromelysin-1 gene promoter activity, surpassing the fold induction observed with saturating amounts of PDGF at concentrations of Ras of 0.15–0.3 μg/well (Fig. 2).

Using several different human stromelysin-1 promoter constructs, we and others have identified three positive DNA elements (the SPRE site, the two head-to-head PEA3 sites, and the AP-1 site) which activate transcription in response to various stimuli, including PDGF and Ras (Wasylyk et al., 1991; Buttice et al., 1991, 1993; Quiñones et al., 1994; Sanz et al., 1994). To assess the contribution of each element in transcriptional activation by PDGF, we first assayed constructs containing the individual elements or nonfunctional mutant counterparts, attached to the heterologous promoter of thymidine kinase. The results demonstrate that each element, but not the corresponding mutant (not shown), was able to confer inducibility to the thymidine kinase promoter in response to PDGF (Fig. 3) and c-Ha-ras (not shown) and that expression of a dominant negative Ras mutant abolished the observed induction (Fig. 3). In the next series of experiments we assayed the same elements in the context of a large native promoter. Thus, the activity of mutant constructs in which one or more of the above elements was altered was compared with that of the wild type promoter (Fig. 4). Mutations disrupting the SPRE or PEA3 sites resulted in approximately a 40% and 60% decrease, respectively, in induction by either Ras (Fig. 4) or PDGF (not shown). Interestingly, in the mutant where the AP-1 site was removed (ΔAP1), no significant induction was observed. This indicates that in the context of the human stromelysin-1 promoter, PDGF and Ras require a functional AP-1 site to induce the human stromelysin-1 gene and suggests that SPRE and PEA3 sites contribute to different extents to this induction. Furthermore, it appears that the major induction is directed through the SPRE and PEA3 elements since transfection of double-stranded oligonucleotides representing either of these elements, but not the corresponding nonfunctional mutants (not shown), can efficiently inhibit transcription when the promoter harbors a mutation in the other element. Under these conditions the levels of promoter activity were similar to those of the mutant in which the AP-1 site was deleted (Fig. 4) or to those in which the inducer was missing (not shown). Consistently, a construct containing inactivating mutations of both SPRE and PEA3 elements and harboring a functional AP-1 was noninducible (Fig. 4). These data strongly suggest that the human stromelysin-1 gene promoter contains at least two different functional transcriptional units responsive to PDGF and Ras, one involving the SPRE and the AP-1 sites and the other involving the two head-to-head PEA3 and the AP-1 sites.

Each Transcriptional Unit Is Controlled by Different Kinases Located Downstream of Ras—Recently it has been reported that there is a bifurcation of the mitogenic signals downstream of Ras toward Raf-1 and ηPKC (Sanz et al., 1994; Bjorkoy et al., 1995). To investigate the involvement of these kinases in the induction of each transcriptional unit, different stromely-
Similar results were obtained when using PDGF-activated SPBP dramatically inhibited the SPRE nuclear binding activity. Data are expressed as percent induction with respect to the induction of the wild type promoter, which was set at 100%, and are the mean ± S.D. The expression values of noninduced control cells were at 10% ± 4 (not shown).

Transcriptional Regulation of Human Stromelysin-1

c-Fos is a Critical Component of the SPRE-binding Nuclear Complex—The previously recognized synergism between PEA3 and AP-1 sites in the transcriptional activation of specific genes (Wasylyk et al., 1989, 1990) is due to physical interaction between Ets and AP-1 transcription factors (Bassuk and Leiden, 1995). To investigate whether a physical association between the corresponding transcription factors could also occur during transcriptional activation through SPRE, we used specific antibodies to determine the presence of SPBP and AP-1 proteins in the nuclear complexes associated with SPRE (Fig. 6A). Incubation of serum-activated nuclear extracts with anti-j un caused a characteristic supershift of the retarded band, whereas the presence of anti-Fos produced little or no effect, suggesting that j un proteins but not Fos are critical components of the SPRE-binding nuclear complex. In parallel control studies using oligonucleotides representing the AP-1 site, both anti-Fos and anti-j un antibodies caused a characteristic supershift consistent with the known composition of the AP-1 complex (not shown). Consistent with previous data (Sanz et al., 1995) incubation of nuclear extracts with anti-SPBP dramatically inhibited the SPRE nuclear binding activity. Similar results were obtained when using PDGF-activated nuclear extracts (data not shown). Further analysis using antibodies specific for different j un proteins indicates that c-Jun is most likely the factor present in the SPRE-binding nuclear complex (data not shown). These results strongly suggest that a physical interaction between SPBP and c-J un occurs. To
Fig. 7. SPBP and c-Jun cooperate to transactivate a promoter under SPRE control. Subconfluent NIH 3T3 fibroblasts were transfected with different concentrations of SPBP (panel A) or c-Jun (panel B) expression vectors along with 5 μg of palCAT reporter plasmid. In panel B, transfections were performed either in the absence (circles) or in the presence of 1 μg of SPBP expression plasmid (squares). Results are means ± S.D. of three independent experiments with transfections done in duplicate.

To evaluate the functional significance of SPBP-c-Jun interaction, we performed specific transactivation studies of a minimal promoter construct under SPRE control (Fig. 7). The transient expression of increasing amounts of SPBP in the transfected fibroblasts resulted in a strong transactivation of this construct with a sigmoidal profile, suggesting that SPBP transactivates this element through a cooperative process (panel A). Similarly, transient expression of c-Jun also transactivated the SPRE-controlled promoter, although the induction was much more limited than with SPBP (panel B, circles). To investigate whether these transcription factors can in fact cooperate in this specific transactivation, we expressed suboptimal levels of SPBP (1 μg) along with variable amounts of c-Jun (panel B, squares). In these conditions, expression of c-Jun strongly induced gene reporter expression, indicating that SPBP and c-Jun can in fact cooperate and elicit a synergistic action in the transactivation of the SPRE site.

DISCUSSION

Transient gene expression assays are valuable tools to understand gene transcription despite their limited scope when extrapolating these results to the native environment, mainly due to the inherent difficulty in defining the precise DNA regions involved in the transcriptional regulation of a specific eukaryotic gene. Nevertheless, because promoters often contain regulatory elements within a few hundred nucleotides upstream of the transcriptional start site, functional studies usually are performed with relatively small promoters. However, because recent studies demonstrate the existence of DNA elements specifically responding to mitogenic signals far upstream in the human stromelysin-1 promoter (Diaz-Meco et al., 1991; Sanz et al., 1994), we used a large (~2311) promoter construct derived from the human gene (XXB1) to study stromelysin-1 gene expression in response to PDGF and Ras. PDGF induces transient expression of the ~2311 promoter similarly to the extent that it induces the endogenous gene in cultured human fibroblasts (data not shown), suggesting that this promoter fragment contains the bulk of the regulatory elements involved in PDGF induction. By using different stromelysin-1 promoter fragments from various species, four major regulatory elements and the corresponding transcription factors have been characterized, and the existence of additional elements has been suggested (Kerr et al., 1988, 1990; Wasylyk et al., 1991; Sanz et al., 1994; Quinones et al., 1989, 1994). Specifically, these elements are in proximal to distal order: AP-1 site, PEA3 site, TIE (for transforming growth factor-β1 inhibitory element), and SPRE. With the exception of TIE, a negative element that responds to transforming growth factor-β1 characterized in the rat gene promoter, the other three elements exist in the ~2311 promoter fragment of the human stromelysin-1 gene and have been implicated independently in the transcriptional induction of the stromelysin-1 gene in response to mitogenic stimuli (Kerr et al., 1988; Wasylyk et al., 1991; Sanz et al., 1994). Consistently, minimal sequences representing these individual elements can drive the expression of a minimal heterologous promoter in response to PDGF or Ras. These individual responses were notably different in the context of a more “native” promoter construct (XXB1), although the differences were not evident when assaying specific 5′ deletion clones thereof (data not shown) or the inversion mutant HB1 (Diaz-Meco et al., 1991). This indicates that the stromelysin-1 gene promoter structure is more critical than element-flanking regions to integrate the control of individual responsive elements in the native environment and that large native-like promoter constructs are required to authenticate gene regulation mechanisms experimentally. This may explain why Wasylyk et al. did not observe synergy between the PEA3-cassette and AP-1 site in the rat stromelysin-1 gene promoter in contrast to their pioneer findings showing such element cooperation in other promoters (Wasylyk et al., 1990; Gutman and Wasylyk, 1990). Accordingly, we have found that the PEA3-AP-1 unit is indeed disrupted in a ~255 to ~11 promoter construct in which both elements are present (data not shown).

Our functional studies using minimal XXB1 mutants in which one of the two functional transcriptional units are preserved, strongly suggest the existence of two independent transcriptional units, one positively controlled by λPKC and the other by Raf-1, two downstream intermediates of the mitogenic PDGF/Ras cascade thought to be located in different branches. It was also determined that the PEA3-AP-1 unit under Raf-1 control contributes to approximately 60% of the total activity, whereas the SPRE-AP-1 unit accounts for the remaining 40%. This is supported by competition studies using the XXB1 construct and double-stranded oligonucleotides representing the active DNA elements. However, even though the data indicate that λPKC is involved in the induction of stromelysin-1 gene through the SPRE-AP-1 unit, the expression of a dominant negative mutant of λPKC resulted in an unexpectedly strong inhibition of Ras-induced activity when both transcription units were functional, suggesting that the kinase is involved in additional positive control(s) other than the regulation of the SPRE-AP-1 unit and that such control only operates when the transcriptional complex is fully assembled and thus able to influence PEA3-AP-1 activity. Studies using 5′ deletion clones
of XXB1 have revealed the presence of a negative regulatory sequence immediately 5’ of the PEA3 site which plays a central role in the human stromelysin-1 gene transcription in response to PDGF. The presence of this negative regulatory element has been reported previously by us and others in similar studies using human or rat promoter constructs in response to other mitogens and cytokines (Wasylk et al., 1991; Quinones et al., 1994). PKC could conceivably be exerting a second positive control by causing a release of the negative transcription machinery, thereby positively influencing transcription. The existence of crucial negative controls governing human stromelysin-1 gene expression was suggested previously by the demonstration that protein synthesis inhibitors strongly induced its transcription (Otani et al., 1990).

Very recently, a direct physical association has been reported between Ets and AP-1 transcription factors to form a trimeric protein complex that transactivates enhancer elements containing PEA3 and AP-1 sites (Bassuk and Leiden, 1995). In the same report, the authors show that the interaction occurs between the DNA binding domains of Ets and J un. It is known that a single PEA3 site is not sufficient for Ets induction and that target genes for the trimeric Ets-AP-1 complex contain a single PEA3 site either nearby or adjacent to an AP-1 site (Wasylk et al., 1990; Gutman and Wasylk, 1990; Bassuk and Leiden, 1995). Thus it appears that this structural arrangement, allowing simultaneous DNA binding and transcription factor interaction, is required for Ets induction. The stromelysin-1 gene contains two PEA3 sites forming a palindromeic cassette that is sufficient for Ets induction and is distanced more than 100 nucleotides from the AP-1 site. Therefore it is not evident how similar interactions might occur to activate the PEA3-AP-1 unit. Additional factors might be necessary to permit these elements to associate in a ternary complex. Consistent with this, specific XXB1 deletion doses demonstrate the lack of cooperation between these elements. SPBP is sufficient to transactivate a minimal promoter driven by SPRE. These transactivations exhibit cooperative profiles suggesting that SPBP oligomers are needed for induction. In contrast, c-j un caused limited transactivation, and cooperation with other endogenous factors appears to be required for induction. Consistently, c-j un efficiently induced minimal promoters when coexpressed with suboptimal SPBP levels, demonstrating a synergistic effect of SPBP and c-j un. This and the hyperbolic profiles of these transactivations with respect to the levels of c-j un suggest that SPBP and c-j un aggregate to generate a transcription factor that binds the SPRE site with more affinity than SPBP oligomers. Accordingly, SPBP and c-j un are both present in the transcriptional complex bound to the SPRE site and produce aggregates efficiently in vitro. It is premature to postulate that such interactions mediate the cooperation observed between AP-1 and SPRE sites in the context of the full promoter. However, because SPBP is unusually large and the putative DNA binding and leucine zipper domains are far apart in the polypeptide chain (Sanz et al., 1995), a single SPBP-J un aggregate might be able to contact both the SPRE and AP-1 sites and to induce transcription. Alternatively, if SPBP does not interact with the c-j un leucine zipper, c-j un could be the center of a trimeric transcription factor, SPBP-J un-Fos, which links the SPRE and AP-1 sites and results in the observed transcriptional induction.

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