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Cloning of Human Preprotachykinin-I Promoter and the Role of Cyclic Adenosine 5′-Monophosphate Response Elements in Its Expression by IL-1 and Stem Cell Factor†,‡

Jing Qian,*‡ Ghassan Yehia,‡ Carlos A. Molina,§ Annemarie Fernandes,* Robert J. Donnelly,§ Devashish J. Anjaria,¶ Pedro Gascon,* and Pranela Rameshwar3*

Preprotachykinin-I (PPT-I) gene encodes several peptides with organ-specific functions that link the neuroendocrine-immune-hemopoietic axis. We cloned upstream of the initiation site of human PPT-I promoter and identified consensus sequences for two cAMP response elements (CRE). PPT-I is induced by cytokines including those that signal through the cAMP pathway. Therefore, we studied the role of the two CRE in IL-1α and stem cell factor (SCF) stimulation of bone marrow stroma because both cytokines induce endogenous PPT-I in these cells and activate the cAMP pathway. Furthermore, bone marrow stroma expresses the transcription factors regulated by the cAMP pathways such as the repressor (ICERIIγ) and activator (CREMγ). Mutagenesis of the two CRE and/or cotransfection with vectors that express ICERIγ or CREMγ indicated that the two CRE have major roles in PPT-I expression. The two CRE are also required for optimal promoter activity by SCF and IL-1α. A particular cytokine could concomitantly induce PPT-I and the high affinity G protein-coupled receptor for PPT-I peptides, NK-1R. We showed that SCF, a representative cytokine, induced PPT-I and NK-1R leading to autocrine and/or paracrine cell activation. Because NK-1R activates cAMP through the G protein, the results suggest that the presence of CRE sequences within PPT-I promoter could be important in the regulation of PPT-I expression by cytokines, irrespective of their ability to signal through cAMP. As PPT-I is implicated in hemopoietic regulation, immune responses, breast cancer, and other neural functions, these studies add to the basic biology of these processes and could provide targets for drug development. The Journal of Immunology, 2001, 166: 2553–2561.

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Preprotachykinin-I (PPT-I)† gene encodes a family of peptides that interact with a network of soluble factors in neural and non-neural tissues to exert biological pleiotropism, such as neurotransmission, immune modulation, and hemopoiesis (1–3). The role of PPT-I in hemopoiesis and angiogenesis, and its overexpression in breast and other cancers that metastasize to the bone marrow (BM), suggest that PPT-I could have a central role in BM metastasis (4–6). PPT-I is also associated with pain, asthma, arthritis, aggressive behavior, and depression (1, 7–9). The evolutionary conserved sequence of PPT-I peptides underscores the importance of the pleiotropism that they demonstrate in interactive biological functions (10). Understanding the regulation of PPT-I may help to unravel the molecular mechanisms mediated by this gene that underlie organ-specific functions and provide new insights into human pathology such as tumorigenesis, hemopoietic diseases, nerve damage, and other brain-associated functions and/or behavior (1–3, 11, 12).

In the BM, the two major PPT-I peptides, through their natural receptors, neurokinin-1 (NK-1R) and NK-2R, exert opposing influences, inhibitory and stimulatory, on hemopoiesis at the level of the mature and immature progenitors (13). Therefore, hemopoietic stimulation by one of the major PPT-I peptides may be clinically important in hemologic deficiencies such as in the development of neutropenia and also other inflammatory responses (14). The inhibitory effect could be important in protection of the lymphopoietic stem cells in the BM, where maintaining cell quiescence is often important. Furthermore, PPT-I is involved in the cellular and molecular connection among the immune, neuroendocrine, and hemopoietic systems (1). Thus, understanding the regulation of PPT-I has relevance to BM-associated biology, including the rapidly evolving fields of transplantation and gene therapy, and also inflammatory processes.

In this study, we cloned the genomic sequences upstream of the coding region of human PPT-I and identified the area with promoter activity. Because several modulators of PPT-I regulation activate the cAMP pathways, we hypothesize that the consensus sequences for two cAMP response elements (CRE) have major roles in the regulation of PPT-I expression. Several transcription factors can bind as dimers to CRE: CRE modulator (CREM), CRE binding proteins (CREB), and activator transcription factor-1 (ATF-1) (15, 16). CRE-interacting proteins are mostly constitutive, and their activation requires cAMP-dependent protein kinase

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Abbreviations used in this paper: PPT-I, preprotachykinin-I gene; CRE, cAMP response element(s); SCF, stem cell factor; BM, bone marrow; CREM, CRE modulator; CREB, CRE binding protein(s); PKA, protein kinase A; ICER, inducible cAMP early repressor; SP, substance P; SP-IR, immunoreactive SP; UMDNJ, University of Medicine and Dentistry of New Jersey; NK-1R, neurokinin-1 receptor; ATF-1, activator transcription factor-1; FK, forskolin.

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A (PKA) (15). A second internal promoter in the CREM gene can be induced by cAMP to produce a repressor, inducible cAMP early repressor (ICER), which is a negative regulator of cAMP-induced transcription (15, 16).

In organs where PPT-I is important to maintain steady-state functions, cytokines are important for regulating its expression (1). Therefore, we used representative cytokines to determine the role of CRE and CRE-like sequences in cytokine-mediated PPT-I regulation. Cytokines could activate the cAMP pathway through direct and/or indirect mechanisms (17). Indirect stimulation could occur through the induction of other soluble factors that can stimulate the cells through autocrine and/or paracrine mechanisms. In this study, we showed that IL-1α and stem cell factor (SCF) required the two CRE for optimal promoter activity. The use of specific NK-1R antagonist suggested that SCF could induce PPT-I through direct and/or indirect mechanisms. The involvement of NK-1R in indirect induction of PPT-I by SCF was explained in a two-step mechanism: concomitant induction of PPT-I and NK-1R followed by autostimulation of the expressed, membrane-bound NK-1R with the released peptides derived from PPT-I. Because PPT-I is implicated in several functions, we determined tissue-specific expression. Indeed, two relevant cells, fibroblasts and epithelial, showed cell-specific differences in reporter activity.

Materials and Methods

Cell lines

Undifferentiated neuroblastoma cells (SH-SY5Y) were provided by Dr. Richard Howell, Department of Biochemistry, University of Medicine and Dentistry of New Jersey (UMDNJ)-New Jersey Medical School (Newark, NJ). Cells were cultured in DMEM with high glucose (Life Technologies, Grand Island, NY) containing 10% FCS (HyClone, Logan, UT), Skin fibroblasts (CRL-1510) and normal mammary epithelial cells (MCF-10 and MCF-12A) were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured based on their instructions.

Preparation of BM stroma

Stromal cells and BM fibroblasts were prepared as described (18) from BM aspirates obtained from healthy volunteers at UMDNJ or from the National Disease Research Interchange (Philadelphia, PA). The study was conducted as outlined by the guidelines of the Institutional Review Board, UMDNJ-New Jersey Medical School.

Stimulation of BM stroma

Transfected BM stroma was stimulated with the optimal concentrations of IL-1α (2.5 ng/ml), SCF (8 ng/ml) or 1 mM substance P (SP, Sigma, St. Louis, MO), and/or 10 nM NK-1R-specific antagonist (CP-99,994) in α-MEM with 2% FCS. Because the duration of stromal stimulation depended on the type of experiments (endogenous levels of β-PPT-I vs reporter activity or levels of SP), the stimulation time is stated in the respective figure legend. In other experiments, BM stroma was stimulated with 5 μg/ml forskolin (FK; Sigma) for 2 and 5 h. SCF was purchased from R&D Systems (Minneapolis, MN), and IL-1α was obtained from Hoffman-La Roche (Nutley, NJ). Pfizer (Groton, CT) provided CP-99,994. SP and CP-99,994 were dissolved and stored as described (18). Optimal parameters were determined with dose-response and time-course studies.

Quantitative RT-PCR

BM stroma was stimulated in serum-free α-MEM supplemented with insulin-transferrin-selenium-A (Life Technologies). Quantitative RT-PCR with total RNA extracted from BM stroma and construction of standard DNA were previously described (4). The end sequences of the standard DNA contained gene-specific sequences that are complementary for the reaction primers. Furthermore, the primers in the standard DNA flank neutral DNA. Total RNA (2 μg) was reverse transcribed, and 200 ng cDNA was used in PCR with specific oligonucleotide primers for PPT-I, NK-1R, or NK-2R. Standard DNA, log10-fold dilutions, ranged between 10−2 and 10−6 attomole/L. Each unknown sample was assayed with a particular concentration of standard DNA in the same reaction tube. PCR products (10 μl) were separated by electrophoresis on 1.5% agarose containing ethidium bromide, and the densities of the DNA bands were quantitated with a Fluorimag (Molecular Dynamics, Sunnyvale, CA) and then analyzed with ImageQuant software. A standard curve was established for each unknown sample. Band densities of unknown/standard DNA vs log10 standard DNA concentration was used to determine the concentration of RNA molecules in the unknown samples. The concentration of the unknown sample was selected at the concentration in which the ratio of the unknown and standard were equivalent.

In situ hybridization and immunofluorescence

In situ hybridization for the luciferase reporter vector, pGL3 (Promega, Madison, WI) was performed with a 300-bp ampicillin DNA probe, which was labeled with a random biotin-labeling kit (NEN, Boston, MA). Probe was prepared by PCR with primers specific for ampicillin gene and incubated (Clontech, Palo Alto, CA) as template. The second labeling was performed with Abs for the three major stromal subsets as described (18). Primary Abs for fibroblast, endothelial cells, and macrophages were specific for prolyl 4-hydroxylase (Dako, Carpinteria, CA), von Willebrand factor (Dako), and CD14. After this, cells were incubated for 30 min with rat PE-conjugated anti-α (Becton Dickinson Immunocytochemistry Systems, San Jose, CA) and FITC-avidin (Vector Laboratories, Burlingame, CA). Cells were examined for fluorescence intensity with excitation at 495 nm/ emission at 515 nm for FITC and excitation at 595 nm/emission at 606 nm for PE.

Cloning of PPT-I promoter

Because the cDNA for human β-PPT-I was already cloned (19), we used this sequence as a guide in nested PCR to clone upstream relative to exon 2. PCR was performed with templates from five human genomic libraries (PromoterFinder DNA Walking Kit, Clontech). Outer (AP1) and inner (AP2) adapter sequences linked to the 5′ ends and gene-specific primers, +124/+151 and +88/+116 (19) were used in nested PCR. The procedure followed manufacturer’s instructions. Single bands from three libraries were cloned into pNoTA/T7 (21 Prime, San Jose, CA) and FITC-avidin (Vector Laboratories, Burlingame, CA). Cells were examined for fluorescence intensity with excitation at 495 nm/emission at 515 nm for FITC and excitation at 595 nm/emission at 606 nm for PE.

Transfection and reporter gene assay

pGL3-basic with inserts of different fragments from PPT-I-pl2.2 was co-transfected with pβ-gal-Control (0.5 μg each) in 80% confluent BM stroma using SuperFect (Qiagen, Valencia, CA). After 48 h, cells were scraped in 30 μl 250 mM Tris (pH 8.0) and then lysed by freezing and thawing in a 1:12 ratio of ethanol/2% SDS. Cell-free lysates (24 μl) were obtained by centrifugation at 15,000 × g for 5 min at 4°C and then diluted with 5× cell culture lysis buffer (Promega). Luciferase and β-gal activities were quantitated with 10 μl of lysates using a Luciferase assay system (Promega) and a Luminescent β-galactosidase detection kit II (Clontech), respectively. In the experimental model, the ratios of Lucifierase/β-gal in cells transfected with vector alone ranged from 0.18 to 0.19 and were normalized to 1. Because cytokines induce the promoter upstream of β-gal in cytokine-stimulated cells, luciferase activity was presented per microgram of total protein in the levels normalized with stroma transfected with vector alone. Total protein was determined with a kit purchased from Bio-Rad.

Western blot

DNA-binding proteins were extracted from transfected stromal cells using a rapid micro preparation technique as described (20). Protein extraction for endogenous CRE-binding proteins was performed by boiling for 5–10 min in 100 mM Tris and 4% SDS. Protein concentrations were determined using Bio-Rad DC protein assay. Proteins were separated on 15% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) transfer membrane (NEN). Membranes were incubated overnight with rabbit anti-CREM (21), anti-phospho-CREB (Cell Signaling, Beverly, MA), or anti-CREB (Cell Signaling). The working dilutions of Abs were 1:1000. Anti-CREM cross-reacts with the different isoforms of CREM proteins and ICER. At the end of the incubation period with the primary Abs, membranes were washed and then incubated with HRP-conjugated goat anti-rabbit IgG (1:5000) for 45 min. HRP was developed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).
CRE and CRE-like mutation

Mutations were performed with a mutagenesis kit (Stratagene, La Jolla, CA). The desired mutant sequences (Table I) were synthesized within 40 nt in the forward and reverse directions and then used in PCR with pGL3 containing wild-type Upstream/N0. After PCR amplification, mutation was verified by identification of ApaI I within CRE-like mutant and the loss of XhoI in CRE mutant. Primer synthesis was performed at the Molecular Core Facility, UMDNJ.

EMSA

Mutant or wild-type CRE and CRE-like sequences (20 ng; Table I) were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. Labeled probe was incubated with 2 μg of CREm or ICERIIy (17) in the presence or absence of excess cold competitor for 1 h. Reactions were separated on 4% PAGE, which were dried and then developed by autoradiography after 24 h.

Statistical analysis

Data were analyzed using ANOVA and Tukey-Kramer multiple comparison tests. A p value of <0.05 was considered significant.

Results

Identification of PPT-I promoter

By genomic walk using nested PCR with primers specific for exon 2 of β-PPT-I cDNA (19), we isolated three overlapping sequences from different DNA libraries, upstream of exon 2 of PPT-I. Fig. 1 shows the sequence of the longest fragment (1.225 kb, PPT-I-p1.2), which overlaps with exon 1 of the cloned sequence of PPT-I (22), thus providing insights into the role of this gene in the regulation of PPT-I expression. This indicates that the 5′ end of Upstream/N0 consists of sequences that are important for promoter activity. The relative lack of promoter activity by exon 1 and intron 1 further supported the presence of a promoter in Upstream/N0 (Figs. 2, 3).

We next narrowed the region containing promoter activity by subcloning different fragments of PPT-I-p1.2 into pGL3-basic, resulting in a 480 ± 30 nt (n = 6) increase in normalized luciferase activity (Fig. 2B) and <0.8-fold in the anti-sense orientation (data not shown). These results indicate that PPT-I-p1.2 contains promoter activity. In addition to BM stromal cells, we also observed promoter activity in two other types of cells, CCL64 and skin fibroblasts.

We next narrowed the region containing promoter activity by subcloning different fragments of PPT-I-p1.2 in the sense and anti-sense orientations in pGL3-basic. Fig. 2B shows the ratio of luciferase/β-gal in stroma transfected with Upstream/N0, Upstream/N1 (−722 ∆ −589), Upstream/N2 (−722 ∆ −392), Upstream/N3 (−722 ∆ −230), exon 1, exon 1/intron 1, or intron 1. There was no significant difference in luciferase activities in cells that were transfected with Upstream/N0 (36 ± 2) and Upstream/N1 (30 ± 2), p > 0.5. Further deletion in the 5′ region (Upstream/N2 and Upstream/N3) resulted in <2 normalized luciferase activity. This indicates that the 5′ end of Upstream/N0 consists of sequences that are important for promoter activity. The relative lack of promoter activity by exon 1 and intron 1 further supported the presence of a promoter in Upstream/N0 (Fig. 2B).

In contrast to the individual sequences, exon 1, placed in tandem with intron 1, showed a significant increase in luciferase activity, suggesting that these sequences contain a second, but weak promoter and/or regions that might be stabilizing the DNA. These latter findings are significant, and are the subject of ongoing studies, because further elucidation would provide an explanation for the
enhanced activity by PPT-I-p1.2 compared with Upstream/N0. Together, the results shown in Fig. 2B indicate that PPT-I-p1.2 has a strong (Upstream/N0) and possibly a weak (exon 1/intron 1) promoter. Regulatory regions in exon 1/intron 1 are interesting because the protein-coding region for each of the four PPT-I transcripts is within exon 2 (19).

Characterization of CRE and CRE-like in Upstream/N0

Computer analyses of Upstream/N0 indicated consensus sequences for two CRE that we termed CRE and CRE-like (Fig. 1). We first established whether these two sequences could bind CRE-binding proteins using ICERIIγ in gel shift assay (Ref. 21 and Fig. 3A). The results indicated that ICERIIγ binds to wild-type CRE and CRE-like, indicating that the latter could be a CRE site (Table I). However, ICERIIγ did not bind to the mutants (Fig. 3A), indicating that the particular mutation could adequately prevent interaction with the specific proteins. These results justify the use of mutants in studies to determine the specificity of CRE and CRE-like in the analyses of PPT-I promoter.

Because the experimental model included transfection of BM stroma to study CRE and CRE-like, we next determined whether these cells express CRE-binding proteins and whether they could be phosphorylated by FK, a cAMP-inducing agent. Consistent with other cell types, the levels of CREM did not show any significant change regardless of cell stimulation (Fig. 3B: lanes 1–3, top arrow). However, stromal cell stimulation with FK resulted in the presence of ICER after 2 h and an increase by 5 h (Fig. 3B, lanes 2 and 3, lower arrow). ICER was not detected in unstimulated cells (Fig. 3B, lane 1, lower arrow). Fig. 3C showed constitutive expression of CREB proteins. FK stimulation resulted in phosphorylation of CRE-binding proteins (CREB, CREM, and ATF); CREB and CREM comigrated together (Fig. 3D, top bands, lanes 2 and 3) and ATF-1 (lower bands, Fig. 3D, lanes 2 and 3). The manufacturer provided information on anti-phospho CRE-binding proteins. The results shown in Fig. 3, B–D, showed that similar cells to several sources (23, 24), BM stroma expresses CRE-binding proteins, thus justifying the next set of studies with stromal cells.
Before addressing the importance of CRE and/or CRE-like in the induction of PPT-I by cytokines, we determined whether these sequences are important for PPT-I promoter activity using two different approaches. In the first approach, we cotransfected BM stroma with pGL3-Upstream/N0 and/or the transcription factors that interact with CRE: CREM<sup>t</sup> (activator) or ICERII<sup>g</sup> (repressor). Because activation of CREM requires PKA phosphorylation (15), we included PKA expression vectors. Cells were transfected with PKA<sup>1</sup>, CREM<sup>t</sup>, PKA, or PKA<sup>1</sup>, CREM<sup>t</sup>, ICERII<sup>g</sup>, and the levels of luciferase activities were quantitated. In the second approach, we mutated CRE and/or CRE-like (Table I) in Upstream/N0 and then performed similar cotransfection. Western analysis confirmed the expression of CREM<sup>t</sup> and ICERII<sup>g</sup> in the transfected stromal cells (Fig. 4A). The results of both approaches are shown in Fig. 4B. Cotransfection of pGL3-Upstream/N0 with wild-type or mutant CRE showed no change in luciferase activity (open columns). This indicated that sequences other than CRE and CRE-like are involved in baseline promoter activity. Cotransfection with PKA showed no change in luciferase activity in the wild-type or single mutant. Because PKA phosphorylates proteins other than those that bind to CRE sites, the data shown for cotransfection with PKA and single mutants indicated that either one CRE site could mediate optimal luciferase activity or that non-CRE sites are involved in activation of the PPT-I promoter. There was significant reduction of luciferase activity (<i>p</i> < 0.05) when PKA was cotransfected with CRE double mutant. This suggests that although other sites might be involved in the activation of PPT-I promoter, an available CRE site is required for optimum activity given the appropriate activation signal. The specificity of CRE-mediated responses is shown by the significantly reduced activity (<i>p</i> < 0.01) when ICERII<sup>g</sup> was cotransfected with PKA and CREM<sup>t</sup>. Comparing luciferase activity in the cotransfectants of the double mutants with wild-type CRE and CRE-like indicated that, in the presence of the appropriate transcription factors, both

**FIGURE 3.** A. Representative gel shift of four different experiments showed that CRE and CRE-like can bind ICERII<sup>g</sup> using wild-type or mutant oligonucleotide probes. Lane 1, Wild-type; lane 2, mutant; lanes 3 and 4, 200 and 50 ng mutant cold competitor, respectively; lanes 5, 6, and 7, 200, 100, and 50 ng wild-type cold competitor, respectively. =, Protein-DNA interactions. B–D, Representative Western blots of three different experiments were determined for CRE-binding proteins in BM stroma with an Ab that reacts with CREM (B, top arrow) and ICERII<sup>g</sup> (B, bottom arrow), anti CREB (C), or anti-phospho CRE-activators (D, top lanes, CREM and CREB; bottom lanes, ATF-1). For left lanes, m.w. marker. Lane 1, Unstimulated stroma; lane 2, FK-2 h; lane 4, FK-5 h.

**FIGURE 4.** Representative data of six different experiments performed with BM stroma, transiently transfected with pGL3 basic-Upstream/N0 that contained wild-type or mutant CRE and/or mutant CRE-like (Table I). Cells were cotransfected with 200 ng/μl of pSV that expressed 1) PKA and CREM<sup>t</sup>; 2) PKA; or 3) PKA, CREM<sup>t</sup>, and ICERII<sup>g</sup>. A. Western blots with rabbit Abs verified that the transfected CREM<sup>t</sup> and ICERII<sup>g</sup> were expressed in the cotransfected cells; representative figure shows lane 1, no transfection; lanes 2 and 5, CREM<sup>t</sup>; lanes 3 and 6, ICERII<sup>g</sup>; and lane 4, salmon sperm DNA. B. Reporter activity was determined 48 h after transfection in cell lysates. *, <i>p</i> < 0.05 vs Upstream/N0 or PKA, CREM<sup>t</sup>, ICERII<sup>g</sup>.
CRE sites could contribute in either a synergistic or additive manner with other transcription factors to induce Upstream/N0. However, CRE and CRE-like binding factors demonstrated synergistic rather than additive effects.

Role of CRE and CRE-like in PPT-I induction: model by IL-1α and SCF

Cytokines are important inducers of PPT-I (1). We used two representative cytokines (IL-1 and SCF) to determine the physiologic significance for CRE and CRE-like in PPT-I regulation. These two cytokines were used because they induce PPT-I and the high affinity receptor for PPT-I peptides, neurokinin-1 (NK-1R) in BM stroma, and they also activate the cAMP pathway (1, 25, 26). Table II shows the validity of using IL-1α and SCF in this model. Compared with unstimulated cells, IL-1α and SCF stimulation resulted in a significant increase of endogenous PPT-I mRNA (p < 0.005) and also modulated the mRNA for the receptors NK-1R and NK-2R (Table II). These observations are consistent with the modulation of NK-1R and NK-2R in BM cells to regulate hemopoiesis (1, 13, 27).

To address whether SCF and IL-1α required CRE and CRE-like for PPT-I induction, we transfected BM stroma with pGL3-Upstream/N0 with wild-type or mutant CRE and/or CRE-like and then stimulated the transfectants with SCF or IL-1α. Compared with unstimulated cells, SCF and IL-1α stimulation showed a 4- and 3-fold increase in luciferase activities, respectively (Fig. 5A). SCF and IL-1α showed no significant induction of luciferase in cells transfected with the double mutants (Fig. 5A). However, there was 1.5-fold less luciferase activity in the double mutants compared with cells transfected with wild-type or single mutant (Fig. 5A). These observations indicate that both CRE and CRE-like have roles in the activation of Upstream/N0. Furthermore, the data suggest that with respect to PPT-I induction, the CRE sites could be the dominant regulatory regions for inducers that are associated with stimulation of the cAMP pathway and, in the absence of CRE-binding proteins, cytokine could be repressors of PPT-I induction. Additional experiments are required to dissect the mechanism. It is possible that cytokine-mediated activation and/or induction of CRE-binding proteins could be due to stimulation by one or more cytokines. Additional cytokines could be derived from paracrine and/or autocrine sources. Autostimulation could occur by an initial stimulus inducing one or more cytokines. Because cytokines are central to the biology of PPT-I in several organs, and in inflammatory diseases (1), these findings are intriguing and could contribute to unraveling the ‘black boxes’ associated with these processes.

In general, biological functions do not occur in a microenvironment with only one stimulus. Therefore, we designed the next set of experiments with the aim of obtaining insights into the role of the two CRE in a microenvironment that could have multiple soluble factors that are associated with the activation of cAMP pathway, such as cytokines and neurotrophic factors. Because CREM is activated by many cytokines, we overexpressed this protein as a model to mimic the presence of other stimuli that could activate CRE-binding proteins. Stroma was cotransfected with pGL3-Upstream/N0 and CREM and then stimulated with IL-1α or SCF. For both cytokines, cotransfection with CREM resulted in significant induction of luciferase compared with cells transfected with pGL3-Upstream/N0 alone (Fig. 5B). Luciferase induction by both cytokines was significantly reduced by cotransfection with ICERII (Fig. 5B). These results supported an important role for CRE in PPT-I induction by at least two PPT-I-inducible cytokines that are associated with stimulation of cAMP pathway. Further understanding of PPT-I regulation requires detailed studies with other regulators such as cytokines, neurotrophic factors, and neurotides, and the role of other transcription-sensitive regions within PPT-I promoter. These studies are in progress and are important to pursue because cytokines and PPT-I peptides interact to modulate a vast array of biological responses such as nerve damage, immune modulation, and hemopoiesis. The findings described in this section as well as ongoing studies may be relevant to unraveling the mechanism of multiple cytokines and other modulators of PPT-I.

Autocrine activation of PPT-I through SCF-mediated expression of NK-1R

In the next set of experiments, we investigated the possibility for an indirect mechanism in the activation of PPT-I promoter. We hypothesize that this pathway could occur independently of, or in addition to, other pathways through concomitant induction of endogenous PPT-I and the high affinity receptor (NK-1R) for its peptides by the same stimulus, e.g., cytokine (Table II). The production of PPT-I peptides could interact with the G protein-coupled NK-1R to activate cAMP pathway (28), consequently regulating PPT-I expression through CRE and CRE-like. We first induced NK-1R with optimal SCF for 36 h (5052 ± 50 molecules/µg total RNA). Because SCF induces endogenous PPT-I (Table II), we used ELISA (4) to quantitate the level of its major translation product, immunoreactive SP (SP-IR), in stroma cell extracts. The results are presented as the total levels of SP-IR in 1 ml of cell extract, obtained from one confluent stromal layer, grown in a 25-cm² tissue culture flask. The results showed 115 ± 8 pg/ml (n = 5, ±SD) of SP-IR after 36 h in SCF-stimulated stromal extracts, whereas extracts from unstimulated stroma consisted of <1 pg/ml. Therefore, we asked whether SCF-mediated production of SP could stimulate the cells through an autocrine mechanism. To address this, we incubated the SCF-stimulated cells with 10 nM of an NK-1R-specific antagonist (CP-99,994) and then quantitated luciferase activity. To ensure that the manipulation by the transfection did not blunt the production of endogenous SP, we determined its levels in five different experiments in which stroma was stimulated with SCF. SP-IR at time 0 and 4 h post-transfection were 110 ± 18 and 122 ± 14 pg/ml, ±SD, respectively. Despite the high levels of SP-IR, luciferase activity was significantly reduced in the presence of the antagonist (Fig. 6A). Antagonist alone did not affect luciferase activity compared with transfectants with vector alone. The results of these experiments show that NK-1R is at least partly required for the activation of PPT-I promoter by SCF.

We next determined whether SCF could be initiating a response so that PPT-I is able to autoregulate its own expression. To address this, we stimulated BM stroma with 1 nM of the major PPT-I peptide, SP (Sigma), and then determined the levels of PPT-I mRNA by quantitative RT-PCR. Compared with undetectable PPT-I mRNA in unstimulated cells, cultures with SP showed a

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<tr>
<th>Stimuli</th>
<th>β-PPT-I</th>
<th>NK-1</th>
<th>NK-2</th>
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<tbody>
<tr>
<td>Unstimulated</td>
<td>16 ± 5</td>
<td>&lt;10</td>
<td>2609 ± 70</td>
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<tr>
<td>SCF</td>
<td>1336 ± 134*</td>
<td>5253 ± 170*</td>
<td>273 ± 19*</td>
</tr>
<tr>
<td>IL-1α</td>
<td>4849 ± 121*</td>
<td>8106 ± 91*</td>
<td>968 ± 26*</td>
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</table>

* Confluent BM stroma was stimulated with 8 ng/ml SCF or 2.5 ng/ml IL-1α for 16 h in serum-free α-MEM. Quantitative RT-PCR determined steady state mRNA. *, p < 0.05 vs unstimulated, n = 10.
significant increase in β-PPT-I, \( p < 0.01 \) (Table III). NK-1R-specific antagonist (CP-99,994) did not completely blunt the effects of SP because at 10 nM, there was only a 7-fold reduction (Table III). The data showed that SP, at least partly through NK-1R, could mediate its own expression. Similarly, induction of endogenous PPT-I by SCF is blunted by CP-99,994 (Table III). In summary, the results in this section used two different approaches, transient transfection and induction of endogenous PPT-I, to show that SCF could induce PPT-I directly and/or indirectly through activation of the G protein-coupled NK-1R.

Cell-specific activity of PPT-I promoter

PPT-I is expressed with different efficiency and by different stimuli in particular tissues (1, 5, 11). In BM stromal cells, although macrophage and fibroblasts express endogenous PPT-I when stimulated by IL-1α, the levels in macrophage are relatively higher than fibroblasts (data not shown). Similar differences were shown in IL-1α-stimulated BM and skin fibroblasts. The data described in Fig. 6 indicate that NK-1R expression could regulate PPT-I expression through signaling of cAMP. Because the expression of NK-1R is different in BM stroma, inducible, (1) and neural cells, constitutive (13), we determined whether there is tissue and/or cell specificity in its regulation. We chose relevant cells based on the role of PPT-I in major clinical interests such as breast cancer, hemological disorders, and brain-associated injuries and/or dysfunctions. Thus, we used fibroblasts from BM and skin, undifferentiated neuroblastoma (SY5Y), and mammary epithelial cells. Because intron 1 and exon 1 could have regulatory regions (Fig. 2B), we used PPT-I-p1.2 and Upstream/N0 for cell transfection. Because transfection efficiency could vary depending on the cell source, for comparison purposes, cells were cotransfected with β-gal, and each transfection was normalized with β-gal activity. The results, summarized in Fig. 7, indicate that mammary epithelial cells transfected with pGL3-PPT-I-p1.2 showed a significant increase in luciferase compared with fibroblasts and SY5Y. There was no difference in reporter activity in the two sources of fibroblasts. We also observed comparable luciferase activities in transfected fibroblasts and SY5Y.

FIGURE 5. Effects of CRE and CRE-like in the induction of Upstream/N0. BM stroma was transfected with pGL3-Upstream/N0 containing wild-type CRE, mutant CRE, mutant CRE-like, or double mutant (CRE and CRE-like) and then stimulated with optimal SCF or IL-1α (A) (Table II). In parallel studies, stroma was cotransfected with Upstream/N0 containing wild-type CRE, CRE-like, and CREM or ICERIIγ. Stroma was stimulated 24 h after transfection with optimal SCF or IL-1α (A) (B). After 10 h, luciferase activity was determined in cell lysates. Stimulation time was deduced from time-course studies ranging from 2 to 48 h. A, **, \( p < 0.05 \) vs unstimulated stroma; *, \( p < 0.05 \) vs wild-type CRE. B, **, \( p < 0.05 \) vs Upstream/N0 or Upstream/N0, CREM. Each experimental point is the mean (±SD) of duplicate transfections with seven BM donors.
Table III. Induction of PPT-I by SP

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>β-PPT-I (molecules/μg total RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated or CP-99,994</td>
<td>&lt;1</td>
</tr>
<tr>
<td>SP</td>
<td>284 ± 12*</td>
</tr>
<tr>
<td>SP + CP-99,994</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>SCF</td>
<td>1324 ± 76**</td>
</tr>
<tr>
<td>SCF + CP-99,994</td>
<td>119 ± 8</td>
</tr>
</tbody>
</table>

* BM stroma were stimulated with 1 nM SP, 8 ng/ml SCF, and/or 10 nM CP-99,994 for 16 h. After this, β-PPT-I levels were quantified using total RNA. * p < 0.01 vs SP + CP-99,994, n = 6, ± SD; ** p < 0.01 vs SCF + CP-99,994. The results are shown with optimum concentration of CP-99,994, derived from dose-response studies. The levels of NK-1 mRNA in stroma stimulated with SCF in the presence or absence of CP-99,994 were 49,185 ± 110 and 5,085 ± 140 molecules/μg total RNA, respectively.

Discussion

The 5′ untranslated region of PPT-I has a high content of G-C and this hindered easy cloning. Because the sequence of human PPT-I is important to several biological disciplines, this report removes major limitation in understanding the complex mechanisms by PPT-I in immune and hemopoietic functions (1). The findings in this study provide adequate scientific information that will initiate an understanding of immune and hemopoietic pathophysiology associated with dysregulation of PPT-I (1). With current technology, we can use the sequence shown in Fig. 1 as the basis to determine whether specific mutations are associated with different cancers that overexpress PPT-I (4–6) and refine potential targets for drug design. Of interest is the high CpG content of the noncoding regions of the sequence shown in Fig. 1. Because PPT-I is overexpressed in breast and other cancers, another surrogate of mutation, DNA methylation (29), is being investigated in our laboratory.

In Fig. 6A, NK-1R specific antagonist (CP-99,994) did not completely blunt promoter activity of SCF. There could be several explanations for this, e.g., SP could be interacting with other subtypes of neurokinin receptors (28). Another explanation may be explained by a lag in the expression of the inducible NK-1R (13) so that the antagonist has a window in which it is not binding to a receptor and could become unstable. By the time NK-1R is expressed, the antagonist could be at suboptimum concentration and thus reflect the observed effect.

Studying the role of CRE and CRE-like sequences in PPT-I regulation could be important because the possibility that PPT-I may be induced through the cAMP pathway is likely to be physiologically relevant. For example, PPT-I is overexpressed in breast and other endocrine cancers, and the high levels of PPT-I peptides appear to be involved in autocrine proliferation of the cancer cells (4). In fact, overexpression of the inducible repressor, ICERIIγ, in endocrine and neuroendocrine cancers alters the growth of these tumors (30). Also, PKA, a cAMP-dependent kinase, is implicated in different types of cancer (31–34).

It is intriguing that the two CRE are necessary for the activation of PPT-I promoter by two cytokines (Fig. 5). PPT-I and cytokines form a complexed network in several biological responses. Many cytokines use different pathways to activate cAMP (35, 36). Therefore, the two CRE found in the promoter could be important in the understanding of signal pathways mediated by various cytokines in biological processes such as inflammation and hemopoiesis (1). PPT-I has consensus sequences for other transcription factors associated with cytokines such as NF-κB (Fig. 1). However, the two sequences for NF-κB are not within the promoter regions, and although these sequences may have a role in PPT-I regulation, the CRE sites may be more relevant. Detailed analyses to characterize PPT-I promoter will provide further understanding of its regulation.

In BM stroma, NK-1R and PPT-I are induced by common stimuli (Table II). This could be relevant for unraveling the steps between cytokine stimulation and PPT-I induction. Fig. 6B shows a model of a direct and indirect mechanism by which CRE could be stimulated to regulate PPT-I expression. Direct mechanism could occur through stimulation by a particular cytokine such as SCF, GM-CSF, or IL-3 (23, 35, 36) and indirect mechanism by the induction of PPT-I and NK-1R (Table I). Because BM stroma can express endogenous SCF, an additional indirect mechanism could also occur after PPT-I is released because its peptides could induce the production of this and other cytokines (1). The produced PPT-I peptides could interact with NK-1R, which is a G protein-coupled receptor (28) to activate cAMP pathway. This could lead to auto-regulation of PPT-I (Table III). The second promoter of CREM gene encodes ICER, a repressor of activators of CRE-interacting proteins (15). Despite compelling evidence for PPT-I induction by SCF, it is yet to be determined whether indirect stimulation of SCF, through PPT-I peptide-NK-1R interactions (Fig. 6B), could also lead to the production of ICER and consequently a negative feedback of PPT-I expression. Depending on the interacting transcription factor (CRE vs ICER), CRE could activate or repress PPT-I. Therefore, if SCF could also lead to the induction of ICER, this would be important in the understanding of PPT-I regulation. These questions are currently being studied in our laboratory. The model shown in Fig. 6B might be extended to other factors known to induce PPT-I, such as NGF (3).

The results described for Fig. 7 suggest that in addition to tissue specificity for PPT-I promoter activity, there could be differences based on the type rather than source of the cell. Future studies will examine these observations in detail with the inclusion of other cells such as those of neural origin and deleted fragments of PPT-p1.2 (Fig. 1). Because both exon 1 and intron 1 are included in PPT-I-p1.2, the relevant sequences are yet to be determined. Furthermore, it is uncertain whether these downstream sequences (exon 1 and intron 1) consist of regions that are inhibitory to Upstream/N0 in fibroblasts. As PPT-I is a hemopoietic regulator and is overexpressed in breast cancer (4), this study could provide insights into breast and other cancers that metastasize to the BM.
Further studies are in progress to understand the intricacies of PPT-I regulation in normal cells from different tissues, and immune and nonimmune cells. Defining the regions within PPT-I-p1.2 will be important for future studies to determine the mechanism that leads to overexpression of PPT-I in various clinical and ultimately to manipulate this gene in different organs.

This report is pertinent to the understanding and identification of putative cytosolic factors that enhance β-PPT-I translation (4). Furthermore, this study applies to a new understanding in the network between cytokines and peptides encoded by PPT-I in the regulation of hemopoiesis, modulation of immune responses, and also CNS functions such as inflammation. Although cotransfection with CREM was used as a model to represent multiple cytokines, experiments in the presence of multiple cytokines and/or neurotrophic factors will address this question in a more precise manner. However, these and the other studies presented in this report could provide potential targets for cancer therapy and has relevance to nerve injury, gene therapy, and BM transplantation.

References


