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Transcriptional activation of the human rod cGMP-phosphodiesterase β-subunit gene is mediated by an upstream AP-1 element

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ABSTRACT

During photoactivation retinal cGMP-phosphodiesterase (PDE) mediates signal transduction in the photoreceptor outer segments. Mutations in the β-subunit gene of rod-specific PDE (β-PDE) have been associated with inherited retinal degeneration in a number of species, including human. Here we have investigated the proximal upstream sequences that participate in transcriptional activation of this gene. Transient transfections demonstrated that the sequence from –72 to +53 bp contained sufficient information to direct high levels of gene expression in cells of retinal origin. Deletion or mutagenesis of an AP-1 motif present in this region caused 90–95% reduction in reporter gene expression. By gel mobility shift assay we demonstrated specific interactions between putative nuclear transcription factors and this AP-1 element. These findings indicate that the proximal AP-1 site in the human β-PDE promoter is functionally relevant and necessary for transcriptional activation of this gene.

INTRODUCTION

The rod cGMP-phosphodiesterase (PDE), a key enzyme in phototransduction, is a heterotetramer composed of α and β catalytic subunits and two inhibitory γ subunits (1,2). This enzyme serves as an important biochemical link between the rhodopsin-activated transducin and the cGMP-gated cationic channels present in the photoreceptor plasma membrane. Activation of PDE results in fast hydrolysis of cGMP, closure of the cationic channels and subsequent plasma membrane hyperpolarization that leads to cell signaling (reviewed in 3).

The human β-PDE gene has been characterized and comprises 22 exons spanning approximately 43 kb of genomic DNA (4). Retinal degeneration affecting human (5–10), mouse (11,13) and Irish setter dog (13) has been associated with molecular defects in this gene, thus it is important to understand how retina-specific expression of the β-PDE gene is regulated.

In this study we examined the functional role of upstream sequences as well as the putative nuclear factors that may participate in transcriptional activation of the β-PDE gene. Human retinoblastoma cells in culture, which retain many photoreceptor-specific markers and express the β-PDE transcript endogenously (14), were used in transient transfection experiments to test the ability of several β-PDE upstream sequences to direct gene expression. A construct carrying 72 bp upstream of the first transcription start site of the β-PDE gene contained sufficient information to direct high levels of expression of the reporter gene which was specific to retinoblastoma cells.

Deletion and point mutational analysis of the region from –72 to +53 bp indicated that an AP-1 element (5′-TGAGTCA-3′), the DNA recognition site for members of the Jun/Fos family of transcription factors, is functionally relevant for β-PDE transcription. In addition, binding of nuclear factors present in retinoblastoma cells to this AP-1 element was demonstrated using gel shift assays.

MATERIALS AND METHODS

Plasmids

Various lengths of the 5′-flanking region of the human β-PDE gene were generated by PCR using sequence-specific primers. The 3′ primer, complementary to positions +34 to +53 of the human β-PDE 5′-sequence, was common to all the fragments and contained a *Bgl*II linker. The 5′ primers varied for each of the constructs to encompass different lengths and contained an *Nhe*I linker. PCR products were directionally subcloned into the pGL2-Basic vector (Promega, Madison, WI) upstream of the firefly luciferase cDNA. Inserts were fully sequenced in both directions by the dideoxy chain termination method (15) to assure 100% identity with the original template. Plasmid nomenclature (p–1356 to p–34) refers to the 5′-most nucleotide in the subcloned fragment relative to the transcription start site of the gene (16). Plasmid p+4 was engineered by annealing two complementary

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oligonucleotides spanning +4 to +53 bp with *Nhe*I and *Bgl*II linkers for directional subcloning. Mutant constructs were generated by introducing site-specific A→C and G→T transversions in the primers used for PCR and subcloned as described above. The primers used to generate mutant fragments were: p–93M, 5′-GGGCTAGCAGCGCAGGCCCCCATGGTTAG-3′, and p–72M, 5′-GGGCTAGCGAGGTCTGAAGCTGACCC-3′.

Cell cultures and transient transfections

Human Y-79 retinoblastoma cells (ATCC HTB 18) were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 15% (v/v) fetal bovine serum. HeLa cells and human 293 kidney cells were maintained in D-MEM medium (Gibco BRL) supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml). Cells were kept at 37°C in a humidified incubator (5% CO_2). Retinoblastoma cells were maintained in suspension and grown in monolayers for transfection experiments as previously described (14). Transient transfections were performed when cells were 60–70% confluent by the calcium phosphate precipitation method (17). For normalization of transfection efficiency, cells were routinely co-transfected with 5 µg internal control plasmid containing the bacterial *lacZ* gene driven by the SV40 promoter. Forty eight hours after transfection, cells were harvested and lysed in 250 µl lysis buffer in the presence of 5% Triton X-100. Luciferase and β-galactosidase assays were performed as described previously (14).

Nuclear protein extracts

Crude nuclear protein extracts were prepared from Y-79 and Weri-Rb1 human retinoblastoma cells as previously described with minor modifications (18). Retinoblastoma cells were grown in suspension and the packed cell volume (PCV) determined after centrifugation of the cells for 6 min at 1000 *g* at 4°C. Cells were washed with 30× PCV cold phosphate-buffered saline, gently resuspended in 5× PCV buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.75 mM spermidine, 0.15 mM spermine, 100 µM PMSF, 1 µg/ml aprotenin, 1 µg/ml leupeptin and 1 µg/ml pepstatin) and allowed to swell on ice for 10 min. Cells were centrifuged for 3 min at 1000 *g* at 4°C. The pellet was resuspended in 2× initial PCV hypotonic buffer, transferred to a Dounce homogenizer and cells were broken with five strokes of the tight pestle. Immediately, 0.3× PCV buffer B (50 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.75 mM spermidine, 0.15 mM spermine, 100 µM PMSF, 1 µg/ml aprotenin, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 67% sucrose) was added and mixed with five strokes of the loose pestle. Nuclei were separated from lysed cells by centrifugation for 30 s at 16 000 *g* at 4°C. The supernatant was removed and the nuclear pellet resuspended in 0.66× PCV buffer C (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 100 µM PMSF, 1 µg/ml aprotenin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 20% glycerol) and rapidly frozen and thawed three times to lyse the nuclei. Lysed nuclei were centrifuged for 10 min at 16 000 *g* at 4°C. The supernatant was collected, aliquoted and stored at –80°C. Protein concentration was determined using the Bradford assay (19).

Gel shift assays

The following oligonucleotide DNA probes containing AP-1 sequences were synthesized using an Applied Biosystems 392 DNA/RNA synthesizer for use in gel shift assays: βAP-1, a probe identical to the fragment of the human β-PDE upstream sequence from –72 to –60, 5′-GAGTGAGTCAGCTGA-3′ (16); cAP-1, corresponding to the sequence 5′-GCATGAGTCAGACAC-3′ of the human collagenase promoter (20). A probe identical to βAP-1 containing a mutated AP-1 sequence (5′- GAGGTCTTCAGCTGA-3′) was designated βAP-1M (nucleotide transversions underlined). The βAP-1 and βAP-1M probes were radiolabeled, whereas the cAP-1 probe was mainly used as an unlabeled competitor. To generate radiolabeled probes, each of the oligonucleotides was end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP and annealed to its complementary counterpart. The labeled double-stranded probes (~3×10^5 c.p.m.) were incubated with 5–20 µg nuclear extract from Y-79 or Weri-Rb1 cells in the presence of 2 µg poly(dI·dC) as non-specific DNA competitor. The binding reactions were performed at room temperature for 20 min in a total volume of 20 µl containing 10 mM Tris, pH 7.5, 50 mM KCl, 0.2 mM EDTA, 1 mM $MgCl_2$, 4% (v/v) glycerol, 1 mM dithiothreitol, 0.05% (v/v) Nonidet P-40 and 1 mM PMSF. Reaction mixtures were resolved on 8% non-denaturing polyacrylamide gels and electrophoresed using a Tris–borate buffer system at 45 V for 5 h at room temperature. Gels were dried and autoradiographed.

RESULTS

The human β-PDE proximal 5′-region contains at least one strong positive regulator

Several constructs containing different lengths of the human β-PDE 5′-flanking region were transiently transfected into Y-79 retinoblastoma cells to delineate the 5′-sequences involved in transcriptional activation. Transfection of serially truncated segments of the 5′-flanking region linked to the luciferase gene resulted in strong reporter gene expression that peaked at –197 bases upstream of the first transcription start site. Deletion of the sequence between –167 and –34 bp caused a drastic reduction in the luciferase activity to levels comparable with those observed when a vector lacking a promoter was transfected, suggesting that one or more strong regulatory elements are present in the proximal promoter region of the β-PDE gene (Fig. 1). We have previously identified several areas of DNA–protein interaction in the human β-PDE sequence from –167 to +53 by DNase I footprinting using nuclear extracts of both Y-79 retinoblastoma cells and pig retinas (16), which are summarized in Figure 2. Briefly, several potential regulatory elements were identified: (i) an E box (consensus 5′-CANNTG-3′), the binding site of the helix–loop–helix (HLH) family of transcriptional activators (reviewed in 21); (ii) an AP-1 motif (consensus 5′-TGAg/cTCA-3′), which is the DNA recognition site for members of the Jun and Fos family of transcription factors (reviewed in 22); (iii) a GC box, which normally binds the ubiquitous factor Sp1 (23); (iv) an element spanning the sequence 5′-CCTAATC-3′, which bears similarity to Ret1, a binding site for a retina-specific transcription factor (24); (v) a degenerate TATA box located at –31 to –25 bases upstream of the first transcription start site (16).

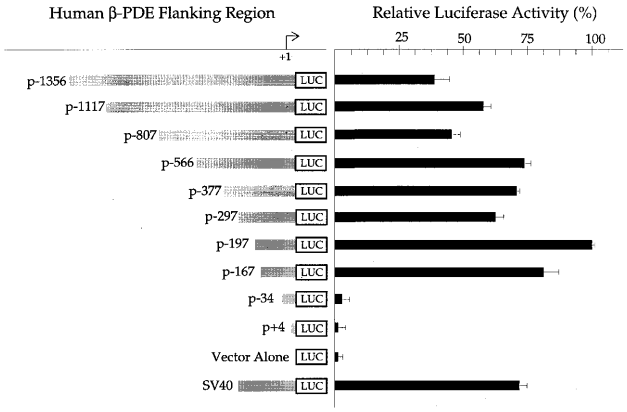

Figure 1. Relative luciferase activity of constructs containing different lengths of the human β-PDE 5′-flanking region from –1356 to +4 upon transient transfection into Y-79 human retinoblastoma cells. Plasmids (15 µg) were co-transfected with the control *lacZ* gene driven by the SV40 promoter (5 µg). Luciferase activity was normalized to the corresponding β-galactosidase activity for each sample and expressed as percentage activity of the construct p–197. Values represent the average of at least three transfections and standard deviation bars are shown.

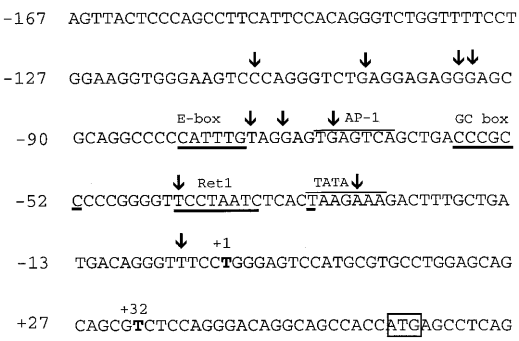

Figure 2. Sequence of the human β-PDE proximal promoter region showing the potential regulatory elements previously identified by DNase I footprinting analysis (16). Protected areas are underlined and hypersensitivity bands are shown as arrows. The putative regulatory elements, E box, AP-1, GC box, Ret1 and TATA box are labeled. The AP-1 site is overlined.

Mutational analysis of the functional human β-PDE proximal 5′-region

To determine more precisely the sequences in the proximal upstream region important for β-PDE transcription, several constructs were engineered carrying small deletions or point mutations in elements previously defined by protein–DNA interaction. The results of transient transfections using these constructs are shown in Figure 3. Deletion of the segment between –72 and –61 bp, which includes the putative AP-1 site, caused a strong inhibition (~90–95%) of gene expression, therefore raising the possibility that this sequence is implicated in regulation of β-PDE gene transcription. Transfection of the construct p–72M, which contained point mutations in the AP-1 element, resulted in a similar reduction of reporter gene expression. Deletion of the sequence from –167 to –93 bp as well as point mutations in the putative E box had no significant effect

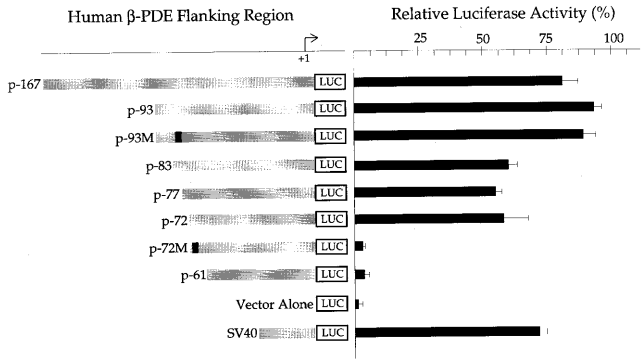

Figure 3. Relative luciferase activity of serial deletion constructs carrying the human β-PDE 5′-flanking region from –167 to –72 bp upon transient transfection into Y-79 human retinoblastoma cells. Plasmids p–93M and p–72M carry point mutations in the E box and AP-1 element, respectively. Luciferase activity was normalized to the corresponding β-galactosidase activity for each sample and expressed as percentage activity of the construct p–197. Values represent the average of at least three transfections and standard deviation bars are shown.

on gene expression. Further 5′-end deletions from –61 bp or point mutations introduced into the putative Ret1 and TATA elements did not cause significant changes in gene transcription levels (not shown).

In order to investigate what sequence was sufficient to support high levels of retina-specific transcriptional activation of the reporter gene, we transfected constructs p–197, p–93, p–83, p–72 and p–61 into 293 kidney and HeLa cells in addition to Y-79 retinoblastoma cells (Fig. 4). The relative luciferase activity of constructs p–197, p–93, p–83 and p–72 detected in the 293 and HeLa cell lines was several orders of magnitude lower than that observed in Y-79 retinoblastoma cells. Transfection of the p–61 construct, which lacked the AP-1 site, showed no significant difference in luciferase activity among all three cell lines tested, which was comparable with activity of the vector alone. The high reporter gene expression obtained in all three cell lines using the SV40 promoter validated the efficiency of the assay. These results suggest that the sequence between –72 and +53 of the human β-PDE 5′-region is sufficient to drive high levels of luciferase activity in retinoblastoma cells and that the AP-1 element may be functionally relevant for transcriptional activation of this gene.

Analysis of nuclear protein interactions with the human β-PDE AP-1 site

To initiate characterization of nuclear proteins that bind to the AP-1 motif in the β-PDE promoter, gel shift assays were performed with several probes in the presence of either Y-79 or Weri-Rb1 retinoblastoma cell nuclear extracts. Two shifted complexes were detected with the βAP-1 probe, one with slower and less prominent (I) and another faster and more abundant (II) (Fig. 5). No shifted bands were detected when Y-79 or Weri-Rb1 nuclear extracts were incubated with labeled AP-1M probe, which contained four mutations in the core AP-1 sequence.

To further test whether formation of complexes I and II reflected specific interactions between nuclear proteins and the AP-1 sequence, we estimated the relative affinity of the protein–DNA interaction using a competition assay in which binding of the Y-79 nuclear extract to the βAP-1 probe was

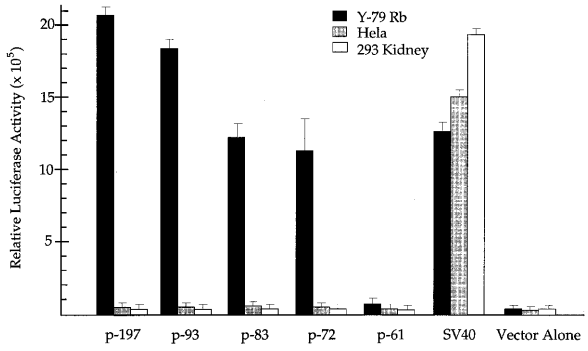

Figure 4. Reporter gene expression driven by serial deletion constructs of the human β-PDE promoter from –197 to –61 upon transient transfection into retinoblastoma, 293 kidney and HeLa cells. The activities of SV40-promoted vector and vector alone controls are shown. Values represent the average of at least three transfections and standard deviation bars are shown.

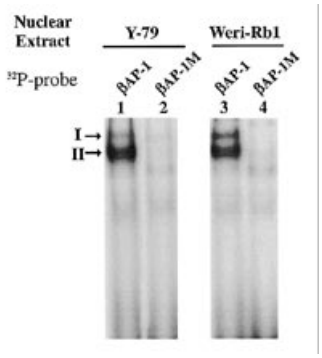

Figure 5. Two major shifted complexes (I and II) were detected when probe βAP-1 (3×10^5 c.p.m.) was incubated with 5–20 µg either Y-79 or Weri-Rb1 cell nuclear extracts. The absence of complexes I and II was confirmed when a labeled probe containing mutations on the AP-1 site (βAP-1M) was used.

performed in the presence of excess amounts of several unlabeled oligonucleotides (Fig. 6). The intensity of both complexes I and II decreased progressively upon addition of increasing amounts of unlabeled βAP-1 competitor oligonucleotide (lanes 1–3). However, no significant inhibition of complex formation was observed with up to 100-fold molar excess of the βAP-1 oligomer containing four nucleotide transversions in the AP-1 core sequence (lanes 4–6). Unlabeled cAP-1 (lanes 7–9) competed efficiently with the βAP-1 probe for formation of shifted complexes upon incubation with nuclear extracts.

DISCUSSION

Several independent mutations in the β-PDE gene have been directly associated with different forms of human retinal degeneration that invariably lead to blindness (5–10). The upstream sequences of the human β-PDE gene that might be necessary for transcriptional activation and potentially retina-specific expression were investigated in this study. Transient transfection assays using a retinoblastoma cell line demonstrated that deletion of the sequence –167 to –34 upstream of the first transcribed nucleotide reduced reporter gene expression by 90% (Fig. 1), indicating the presence of important regulatory elements in this region. This

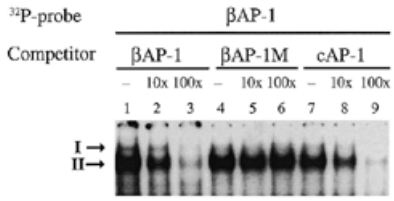

Figure 6. Competition gel shift experiments using the βAP-1 probe and Y-79 nuclear extract in the presence of excess amounts of various unlabeled oligonucleotides. The intensity of complexes I and II decreased progressively upon addition of excess amounts of unlabeled βAP-1 (lanes 1–3) or cAP-1 (lanes 7–9) competitors. No significant inhibition of complex formation was observed with excess unlabeled βAP-1M (lanes 4–6).

sequence contained several potential sites for DNA–protein interactions, including an AP-1 consensus motif located at –69 to –63 bp (Fig. 2). This putative AP-1 element is highly conserved among the human, bovine and mouse β-PDE genes (16). Transfection experiments demonstrated that the human β-PDE gene sequence from –72 to +53 bp, which contained the AP-1 motif, was sufficient to support high levels of reporter gene expression in a retinoblastoma-specific fashion. The transcriptional activity of this construct was three orders of magnitude higher in retinoblastoma cells than in HeLa or 293 cells. Therefore, the sequence from –72 to +53 is a good candidate to comprise the minimal promoter of the human β-PDE gene.

Deletion or site-directed mutagenesis of the proximal AP-1 element caused a 10- to 12-fold reduction in the levels of luciferase gene expression in transfection assays using retinoblastoma cells. However, this effect was not observed when other cell lines, such as HeLa and 293, were used. These findings suggest that the AP-1 motif present in the β-PDE gene proximal upstream region is functionally relevant in transcriptional activation of this gene. In addition, participation of this element seemed to be restricted to cells of retinal origin. The potential participation of this sequence in retina-specific β-PDE gene expression is supported by our recent finding that the sequence from –297 to +53 was sufficient to direct photoreceptor-specific expression of a reporter gene in transgenic mice (manuscript in preparation).

Mobility shift assays using a probe corresponding to the human β-PDE proximal AP-1 element (βAP-1) demonstrated that this sequence interacted with potential *trans*-acting factors present in Y-79 and Weri-Rb1 retinoblastoma cell nuclear extracts. Within members of the AP-1 family of transcription factors leucine zipper dimerization juxtaposes basic regions to form a DNA binding surface (25). DNA–protein interactions may occur due to electrostatic forces between basic amino acid residues and the DNA phosphodiester backbone. Such interactions might allow non-specific binding between AP-1 proteins and a variety of DNA sequences. We established that the βAP-1–protein complex was sequence specific by testing complex formation in the presence of increasing amounts of wild-type βAP-1, mutant βAP-1 (βAP-1M) and a related AP-1 sequence found in the collagenase promoter (cAP-1). The results of these competition experiments demonstrated that the AP-1 motif present in the β-PDE promoter mediates formation of sequence-specific complexes with putative nuclear factors present in retinoblastoma cell nuclear extracts.

The finding that the proximal AP-1 element is functionally implicated in β-PDE gene activation in cells of retinal origin is novel. Although Fos, Jun and related gene products are present in most cell types, there is substantial evidence that this family of transcriptional activators may support expression of cell-specific genes. For example, expression of the lymphoid-specific interleukin-2 gene was shown to be regulated by AP-1 proteins (26). The functional versatility observed in members of the AP-1 family seems to be due to their distinct tissue distribution, level of expression and interaction with other factors giving rise to dimers with different DNA binding and transcriptional activities (27–30). The mechanism by which the proximal AP-1 element mediates β-PDE gene activation remains to be elucidated, but might involve direct interaction with AP-1 proteins or interactions with dimers between AP-1 proteins and members of other families of *trans*-activators (31,32). It is also possible that other transcription factors or cofactors are required for retina-specific β-PDE activation. Recently a neural retina leucine zipper factor (Nrl) was shown to activate transcription of the rhodopsin gene in a retina-specific fashion (33). The possibility that factors such as Nrl could be involved in regulating β-PDE gene transcription is currently under investigation in our laboratory.

Future studies will allow further characterization of the nuclear proteins that interact with the AP-1 element in the human β-PDE promoter as well as the molecular mechanisms that mediate transcriptional activation. *In vivo* studies using transgenic mouse technology will be instrumental in understanding the role of proximal AP-1 sequence in cell-specific β-PDE expression.

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