Transcriptional repressor of vasoactive intestinal peptide receptor mediates repression through interactions with TFIIB and TFIIE β

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The transcriptional repressor for rat vasoactive-intestinal-polypeptide receptor 1 (VIPR-RP) is a recently characterized transcription factor that belongs to a family of proteins, which include components of the DNA replication factor C complex. In this study, I investigated the mechanisms by which VIPR-RP represses transcription. I show here that transcriptional repression by VIPR-RP is mediated by a histone deacetylase-independent mechanism. I provide evidence that VIPR-RP makes direct physical contacts with two proteins of the basal transcription apparatus, the transcription factors TFIIB and TFIIE β . The interaction with TFIIB is mediated by the N-terminal 180

amino acids, whereas the interactive domain with TFIIE β is located between residues 367 and 527 of VIPR-RP. Using gel mobility-shift assays I demonstrated that interaction between VIPR-RP and TFIIB prevents the recruitment of TFIIB into a DNA-TATA-box-binding protein complex. My results indicate that VIPR-RP mediates transcriptional repression through direct interactions with the general transcription machinery.

Key words: general transcription factor, histone deacetylase, pre-initiation complex, VIPR-RP.

INTRODUCTION

Transcriptional repression is an essential mechanism in the control of differential gene expression [1]. Repressor proteins can affect transcription by multiple mechanisms. Repression may occur by directly targeting components of the RNA polymerase II (RNAPII) core transcription machinery to block the formation of the pre-initiation complex (PIC) [2–4]. The RNAPII PIC is composed of RNAPII and a set of general transcription factors (GTFs), which includes the TATA-box-binding protein (TBP), TFIIA, TFIIB, TFIIE, TFIIF and TFIH [3]. RNAPII is recruited to the promoter as a holoenzyme that includes a subset of GTFs and the co-activator complex that mediates the response to transcriptional activators as well as repressors [5–7]. Eukaryotic repressors are typically modular with functionally distinct domains that can target different components of the transcription machinery to affect distinct steps in initiation [8]. For example, the human Dr1/Drap1 complex represses transcription by blocking TFIIB association with TBP and inducing a conformational change in TBP or DNA that alters TFIIA binding [9].

Another mechanism of transcriptional repression involves modification of chromatin structure by histone deacetylation [10]. It is known that hyperacetylated regions of chromatin frequently contain active transcription units, whereas hypoacetylated chromatin is transcriptionally silent [11]. The relative levels of histone acetylation are determined by the enzymic activities of both acetyl transferases and histone deacetylases (HDACs) [12,13]. Several transcriptional repressors such as YY-1, RB and CBF-1 interact directly with HDAC, whereas nuclear hormone receptors and Mad are linked indirectly to

HDAC through formation of co-repressor complexes containing Sin3, SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) or NCoR (nuclear receptor co-repressor) [14–18]. Recruitment of HDAC by these factors results in deacetylation of histone tails and transcriptional repression.

Recently I characterized a transcriptional repressor for rat vasoactive-intestinal-polypeptide receptor 1 (VIPR-RP) [19]. VIPR-RP belongs to a family of nuclear proteins that all contain a region of about 80 amino acids highly homologous with bacterial DNA ligases [20,21]. In my previous studies I have mapped VIPR-RP DNA-binding and transcriptional-repression domains and provided evidence that the ability of VIPR-RP to repress transcription is modulated by phosphorylation [22]. I showed that VIPR-RP contains two separate transcriptional-repression domains located between amino acids 50 and 100 and 469 and 527 [22]. In this study, I sought to determine the molecular mechanisms involved in VIPR-RP transcriptional repression. I show here that VIPR-RP represses transcription via an HDAC-independent mechanism. I provide evidence that VIPR-RP physically interacts with components of the basal transcription machinery and represses transcription by inhibition of recruitment of TFIIB into the PIC.

MATERIALS AND METHODS

Plasmids

The coding region of TBP was amplified by PCR and cloned into the BlueScript vector (Stratagene). Expression plasmids for TFIIB and TFIIA were provided by Dr M. Carey (UCLA, Los

Abbreviations used: RNAPII, RNA polymerase II; PIC, pre-initiation complex; GTF, general transcription factor; TBP, TATA-box-binding protein; HDAC, histone deacetylase; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; NCoR, nuclear receptor co-repressor; VIPR-RP, transcriptional repressor for vasoactive-intestinal-polypeptide receptor 1; TK, thymidine kinase; TKLUC, minimal TK promoter linked to luciferase; GST, glutathione S-transferase; DTT, dithiothreitol; TSA, trichostatin A; ECL, enhanced chemiluminescence.

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Angeles, CA, U.S.A.) and for TFIIE α and TFIIE β by Dr R. Tjian (UC Berkeley, CA, U.S.A.). For eukaryotic expression, the coding region of TFIIB was excised from the pET11d vector and cloned into the *NheI* and *BamHI* sites of the CEP4 vector (Invitrogen). The coding region for TFIIE β was removed from pM10 by *NdeI*/*SmaI* digestion. After filling in the 5' overhang of *NdeI*, the blunt-ended insert was cloned into the *SmaI* site of the pBKCMV vector (Stratagene). The coding region of Mad was amplified by PCR and cloned into the pBKCMV vector. An oligonucleotide containing four reiterations of Mad-Max consensus binding sites (CACGTG) was synthesized with *BamHI* restriction sites at both ends and cloned upstream of the minimal thymidine kinase (TK) promoter linked to luciferase (TKLUC).

***In vitro* transcription/translation and glutathione S-transferase (GST) pull-down assays**

TBP, TFIIB and the subunits of TFIIA and TFIIE were transcribed from the T7 promoter and translated in reticulocyte lysate using the TNT-coupled reticulocyte lysate system (Promega) in the presence of Transcent[™] biotinylated lysyl-tRNA (Promega) following the manufacturer's protocol. Construction of various GST-VIPR-RP fusion plasmids has been described previously [22]. Expression of GST fusion proteins was induced with 0.5 mM isopropyl β -D-thiogalactoside at 37 °C for 90 min. Cells were centrifuged and the resulting pellet resuspended in a sonication buffer containing 150 mM KCl, 40 mM Hepes, pH 7.9, 0.5 mM EDTA, 5 mM MgCl₂, 1.0 mM dithiothreitol (DTT), 0.05 % Nonidet P-40, 1 mM PMSF and 1 μ g/ml aprotinin. Cells were lysed by sonication. Cell debris was removed by centrifugation, and the supernatant was added to Sepharose 4B beads (Pharmacia).

The *in vitro* binding assay was performed as follows. 10 μ l of the *in vitro*-translated protein was incubated with beads containing 200 ng of GST or various GST-VIPR-RP fusion proteins in the sonication buffer for 90 min at 4 °C. Complexes were washed extensively with the sonication buffer, boiled in loading buffer and separated by SDS/PAGE (10 % gels). Gels were transferred to nylon membranes and blocked by incubation with Tris-buffered saline (TBS) containing 0.5 % Tween 20 (TBST). The membranes were incubated with streptavidin-horseradish peroxidase conjugate in TBST for 45 min, washed four times with TBST and three times with TBS. The membranes were then incubated with the chemiluminescent substrate mixture for 1 min and exposed to Kodak X-ray film for 20 min.

Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10 % fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. Trichostatin A (TSA) was purchased from Calbiochem. Transfections were performed using calcium phosphate precipitation as described previously [19]. All transfections were performed in triplicate, and each DNA construct was tested in at least three independent experiments. Post transfection (48 h) cells were lysed in 0.25 M Tris/HCl, pH 7.8, with three freeze-thaw cycles. Cell lysates (50 μ g/assay) were assayed for luciferase activity as described previously [19].

Immunoprecipitation and Western-blot analysis

Various parts of VIPR-RP, TFIIB and TFIIE β were transcribed by T7 polymerase *in vitro* and translated in rabbit reticulocytes as the described above. For *in vitro* binding assays, equal volumes

of translation products (10 μ l each) were mixed gently in association buffer (20 mM Hepes, pH 7.9/50 mM KCl/5 mM MgCl₂/1 mM DTT/10 % glycerol), incubated at 30 °C for 30 min, and then clarified by centrifugation at 1200 *g* for 15 min at 4 °C. The cleared supernatants were diluted into 100 μ l of Nonidet P-40 buffer (10 mM Tris/HCl, pH 7.5/150 mM NaCl/1 mM EDTA/0.2 % Nonidet P-40) containing 2 μ l of either anti-TFIIB (Babco) or anti-TFIIE β (Santa Cruz Biotechnology) antibodies and rotated for 2 h at 4 °C. Immune complexes were then incubated for 1 h at 4 °C with 10 μ l of Protein A/G-agarose, precipitated and washed three times with Nonidet P-40 buffer. Samples were resolved by SDS/PAGE (10 % gels), transferred to nylon membrane, incubated with anti-streptavidin-horseradish peroxidase antibody and detected by enhanced chemiluminescence (ECL).

Gel mobility-shift assays

The probe for gel mobility-shift assays contained the TATA box and initiator region of adenovirus major late promoter. The binding was performed in 10 μ l reactions containing 0.5–100 ng of recombinant proteins [23], 1 fmol of ³²P-labelled probe, 20 ng of poly(dI:dC), 1.25 μ g of BSA, 1 mM DTT, 7.5 mM MgCl₂, 0.1 M KCl, 20 mM Hepes (pH 7.9), 10 % glycerol and 0.2 mM EDTA at 30 °C for 30 min. The samples were analysed on 4.5 % native polyacrylamide gels, dried and exposed to X-ray films.

RESULTS

VIPR-RP represses transcription through an HDAC-independent mechanism

Recent studies [10–18] have provided molecular evidence that modification of chromatin structure by HDAC is an important mechanism in the control of gene transcription. Transcriptional repression by a sequence-specific DNA-binding factor can be mediated by the recruitment of a deacetylase to the promoter region [10]. To determine whether VIPR-RP-mediated transcriptional repression requires HDAC activity, the effect of TSA, a specific HDAC inhibitor [24], was tested in co-transfection experiments. COS-7 cells were co-transfected with either –859LUC (859 bp of the VIPR1 5' flanking sequence linked to luciferase) or 4FTKLUC (four copies of VIPR-RP binding sites cloned in front of TKLUC) and the VIPR-RP expression plasmid. As a positive control, the transcriptional repressor Mad, which is known to repress transcription by an HDAC-dependent mechanism, was co-transfected with M4-TKLUC, a reporter gene. M4-TKLUC consists of four reiterations of Mad-Max consensus binding sites (CACGTG) cloned upstream of TKLUC. As shown in Figure 1, Mad expression resulted in transcriptional repression of M4-TKLUC, and treatment of cells with 50 nM TSA led to complete de-repression of the reporter gene (Figure 1A). Expression of VIPR-RP strongly repressed both VIPR1 (Figure 1B) and TK (Figure 1C) promoter activities. However, treatment of transfected cells with 50 nM TSA for 8 or 24 h had no effect on VIPR-RP repression of either the VIPR1 (Figure 1B) or the TK (Figure 1C) promoter, indicating that an HDAC-independent pathway is required for VIPR-RP-mediated transcriptional repression.

VIPR-RP interacts with TFIIB and the 34 kDa subunit of TFIIE (TFIIE β)

Because VIPR-RP represses transcription through an HDAC-independent mechanism, I sought to determine whether repression occurs through direct interaction of VIPR-RP with the

Figure 1 Effect of TSA on VIPR-RP-mediated transcriptional repression

COS-7 cells were transiently co-transfected with the indicated expression plasmids and the reporter plasmids M4-TKLUC (A), -859LUC (B) and 4FTKLUC (C); see text for details. Cells were treated with 50 nM TSA for 24 h. Cell extracts were then assayed for luciferase activity, represented as fold-activation over the promoterless reporter alone. Values represent means $\pm$ S.E.M. from three independent experiments.

general transcription machinery. To this end, I initially investigated what component of the general transcription machinery VIPR-RP interacted with. Various domains of VIPR-RP were expressed in *Escherichia coli* as GST fusion proteins and used for

Figure 2 Interaction of VIPR-RP with TFIIB and TFIIEβ *in vitro*

(A) and (B) GST pull-down assays. TFIIB and TFIIEβ were transcribed and translated *in vitro* and labelled with Transcent[™] biotinylated lysyl-tRNA. The proteins were allowed to bind to either GST alone or GST fusion proteins containing various regions of VIPR-RP (RP) immobilized on Sepharose 4B beads. The bound proteins were analysed on SDS/polyacrylamide gels in the lanes indicated, blotted and visualized by ECL detection. (C) and (D) Co-immunoprecipitation. VIPR-RP (1-180) and VIPR-RP (178-656) (C) or full-length VIPR-RP (D) were transcribed and translated *in vitro* as described above in the presence of biotinylated lysyl-tRNA. TFIIB and TFIIEβ were transcribed and translated *in vitro* without label. The proteins were mixed and immunoprecipitated with the indicated antibodies. The immune complexes were analysed by SDS/PAGE in the lanes indicated, blotted and visualized by ECL detection.

Figure 3 Effect of TFIIB and TFIIE β on VIPR-RP-mediated repression

COS-7 cells were transiently co-transfected with the indicated expression plasmids and the —859LUC reporter plasmid (see text for details). Post transfection (48 h) cell extracts were assayed for luciferase activity, represented as fold-activation over the promoterless reporter alone. Values represent means $\pm$ S.E.M. from three independent experiments.

Figure 4 Effect of VIPR-RP on TBP-TFIIB-DNA complex formation: gel mobility-shift assay

Lane 1, free probe; lanes 2–7, with 20 ng each of recombinant TBP and TFIIB; lanes 3 and 4, with 1 and 10 ng of wild-type VIPR-RP, respectively; lanes 5 and 6, with 1 and 10 ng of VIPR-RP (178–656), respectively; lane 7, with anti-TFIIB antibody. The arrow and arrowhead indicate the TBP-TFIIB-DNA complex and the supershifted band, respectively.

interactions with *in vitro*-translated TFIIB and TFIIE β . As shown in Figure 2, VIPR-RP (1–180) and VIPR-RP (1–377) interacted with TFIIB whereas VIPR-RP (178–656) did not bind to TFIIB (Figure 2A). On the other hand, VIPR-RP (178–656) and VIPR-RP (367–527) bound to TFIIE β , but VIPR-RP (1–180) and VIPR-RP (1–377) did not (Figure 2B). Using similar approaches, I also tested VIPR-RP binding to TBP, TFIIA, TFIIF and the larger subunit of TFIIE, but no interactions between VIPR-RP and these proteins were detected (results not shown). These results indicate that VIPR-RP interacts with at least two components of the general transcriptional machinery *in vitro*, and that the interaction with TFIIB and TFIIE β is mediated through different regions of VIPR-RP. Whereas interaction with TFIIB requires the N-terminal 180 amino acids of VIPR-RP (containing the N-terminal repressor domain [22]), a region between amino acids 376 and 527 (containing the

second repressor domain [22]) mediates binding to TFIIE β (Figure 2B).

To determine whether the interactions observed with immobilized proteins also occurred in solution, various parts of VIPR-RP were transcribed and translated *in vitro* in the presence of biotylated tRNA and incubated with either TFIIB or TFIIE β synthesized *in vitro*. Antibodies specific for either TFIIB or TFIIE β were added to protein mixtures, and the co-immunoprecipitation products were resolved by SDS/PAGE. Figure 2(C) shows that although VIPR-RP (1–180) co-immunoprecipitated with TFIIB (lane 3) it did not interact with TFIIE β (lane 1). VIPR-RP (178–656) on the other hand was able to interact with TFIIE β (lane 2) but not with TFIIB (lane 4). When the full-length VIPR-RP was used in co-immunoprecipitation, both TFIIB and TFIIE β antibodies were able to detect the repressor protein (Figure 2D), suggesting that VIPR-RP is able to interact with both basal transcription factors simultaneously. These results were consistent with the observations made using immobilized proteins and suggest that VIPR-RP interacts with TFIIB and TFIIE β through different binding domains.

Interaction with the general transcription machinery is required for VIPR-RP-mediated repression

To determine the functional significance of VIPR-RP interaction with the basal transcription machinery, I tested the effect of co-transfection of TFIIB or TFIIE β expression vectors on VIPR-RP-mediated transcriptional repression. As shown in Figure 3, expression vectors for either TFIIB or TFIIE β on their own had little effect on VIPR1 transcription, but their addition along with a VIPR-RP expression plasmid strongly alleviated VIPR-RP-mediated repression of the VIPR1 promoter. When both TFIIB and TFIIE β were transfected into cells, inhibition of transcription by VIPR-RP was almost completely reversed, suggesting that interactions with both of these basal transcription factors are required for VIPR-RP transcriptional repression. Because transfection of either the TFIIB or TFIIE β expression vector alone did not affect VIPR1 promoter activity, the reversal of VIPR-RP inhibition by co-transfection of GTFs is likely to be a specific functional and physiologically relevant interaction.

VIPR-RP prevents TFIID-TFIIB complex formation

Transcription initiation by RNAPII in eukaryotes requires an assembly of GTFs on the promoter to form a PIC [2–4]. The initial complex is formed by TBP/TFIID binding to the TATA element of a promoter [25]. Subsequent interaction with TFIIB connects TFIID, bound to the TATA element, to RNAPII, TFIIF, TFIIE and TFIIH [26]. To determine whether VIPR-RP represses transcription by interference of TFIIB assembly into the PIC, I performed gel mobility-shift assays. Recombinant human TBP and TFIIB interacted with a DNA template containing the adenovirus major late promoter TATA box and initiator region to form a specific TBP-TFIIB-DNA complex (Figure 4, lane 2). Addition of the wild-type VIPR-RP resulted in disappearance of the TBP-TFIIB-DNA complex (Figure 4, lanes 3 and 4). However, inclusion of the N-terminal deletion mutant [RP (178–656)] had no effect on formation of the TBP-TFIIB-DNA complex (Figure 4, lanes 5 and 6). Addition of anti-TFIIB antibody resulted in a supershifted band (Figure 4, lane 7). These results suggest that VIPR-RP inhibits TFIIB assembly into the PIC and that the VIPR-RP-TFIIB interactive domain is required for this function.

DISCUSSION

The data presented here, derived from a combination of cell-transfection and biochemical studies, demonstrate that VIPR-RP represses transcription through an HDAC-independent mechanism and involves direct interaction with the basal transcription machinery.

Chromatin structure is an important component of gene expression, and recent studies have shown that transcriptional repression by a sequence-specific DNA-binding factor can be mediated by the recruitment of a deacetylase to the promoter region [10]. Some transcriptional repressors, such as Mad-Max [27,28], unliganded nuclear receptors [29] and Ume6 [30], are linked to the deacetylases by interactions with Sin3 and NCoR/SMRT, which are related proteins that were originally identified as co-repressors of the unliganded nuclear receptors [31–33]. However, transcriptional repression by VIPR-RP did not require deacetylase activity because TSA, a specific inhibitor of HDAC, was not able to release repression that was mediated by VIPR-RP.

My results showed that VIPR-RP represses transcription by making direct contacts with two components of the basal transcription machinery. One of these factors was TFIIB [34]. TFIIB interacts with a promoter complex containing the TBP to facilitate subsequent interaction with RNAPII through association with TFIIF [35]. TFIIB contains two functionally distinct domains that correlate with these two interactions. An N-terminal zinc-ribbon domain is essential for the RNAPII–TFIIF recruiting activity [36,37], and a proteolytically resistant C-terminal domain is necessary and sufficient for the interaction with the TBP–DNA complex [36–40]. A recent study showed that TFIIB plays a role in transcription-start-site selection, perhaps mediating a conformational change in the polymerase or DNA during the search for initiation sites [41]. My results show that VIPR-RP interacts with TFIIB through the N-terminal repression domain and that this interaction inhibits TFIIB interaction with the TBP–DNA complex. Therefore VIPR-RP represses transcription by interference of an early step during PIC assembly.

VIPR-RP also interacts with TFIIE β through a region containing the DNA-binding domain as well as the second repression domain (amino acids 367–527). TFIIE exists as a heterotetramer composed of TFIIE α and TFIIE β [42]. TFIIE α is known to associate tightly with TFIIF and to recruit it to the PIC [43]. Okamoto et al. [44] showed that TFIIE β interacts with several GTFs including TFIIB and TFIIF β . TFIIE plays essential roles in the regulation of TFIIF activities. The kinase activity that phosphorylates the C-terminal domain of RNAPII and the DNA-dependent ATPase activity of TFIIF are positively regulated by TFIIE, whereas the DNA helicase activity is negatively regulated [45–48]. Thus TFIIE exerts multiple effects on TFIIF and it represents an important control point for the actions of transcriptional regulators. The interaction between VIPR-RP and TFIIE β may inhibit the assembly of the TFIIE α –TFIIF complex with the promoter and/or the efficiency of transcript elongation.

The *Drosophila* zinc-finger protein Kruppel (Kr) has also been shown to interact with both TFIIB and TFIIE β [49]. Monomeric Kr interacts with TFIIB to activate transcription, whereas an interaction of the Kr dimer with TFIIE β results in transcriptional repression [49]. VIPR-RP differs from Kr in that interaction with either TFIIB or TFIIE β resulted in transcriptional repression. Taken together the results from this study indicate that VIPR-RP regulates distinct stages of transcription initiation through interaction with different components of the basal transcription machinery.

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