Pit-1β reduces transcription and CREB-binding protein recruitment in a DNA context-dependent manner

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Abstract

Many transcription factors are expressed as multiple isoforms with distinct effects on the regulation of gene expression, and the functional consequences of structural differences between transcription factor isoforms may allow for precise control of gene expression. The pituitary transcription factor isoforms Pit-1 and Pit-1β differentially regulate anterior pituitary hormone gene expression. Pit-1 is required for the development of and appropriate hormone expression by anterior pituitary somatotrophs and lactotrophs. Pit-1β differs structurally from Pit-1 by the splice-insertion of the 26-residue β-domain in the transactivation domain, and it differs functionally from Pit-1 in that it represses expression of the prolactin promoter in a cell-type specific manner. In order to identify signal and promoter context requirements for repression by Pit-1β, we examined its function in the presence of physiological regulatory signals as well as wild-type and mutant Pit-1-dependent target promoters. Here, we demonstrate that Pit-1β impairs recruitment of cAMP response element-binding protein (CREB)-binding protein to the promoters that it represses. In addition, we show that repression of target promoter activity, reduction in promoter histone acetylation, and decrease of CREB-binding protein recruitment all depend on promoter context. These findings provide a mechanism for promoter-specific repression by Pit-1β.

Journal of Endocrinology (2005) **185,** 173–185

Introduction

Most transcription factors are members of extended families defined by conserved structural motifs, typically in the DNA- and ligand-binding domains, yet they differ in other domains, especially the transactivation domain (TAD). These proteins can have distinct effects on the regulation of target gene expression, tumorigenesis, or the effectiveness of pharmacological approaches to cancer treatment (e.g. c-Jun vs v-Jun (Jurdic *et al.* 1995, Huguier *et al.* 1998); estrogen receptor-α vs -β (Chu *et al.* 2000); glucocorticoid receptor-α vs -β (Longui *et al.* 2000); and progesterone receptor A vs B (Richer *et al.* 1998, McGowan & Clarke 1999)). The pituitary-specific transcription factor isoforms Pit-1 (recently renamed POU1F1) and Pit-1β differentially regulate anterior pituitary hormone gene expression. Pit-1 and Pit-1β differ only by the insertion of the 26-amino acid (AA) β-domain in the Pit-1 TAD; yet they have diametrically opposed actions with regard to prolactin (PRL) expression in pituitary cells.

Pit-1, a pituitary-specific member of the POU homeodomain family of transcription factors, is required for the normal growth and development of three anterior pituitary cell types: somatotrophs, lactotrophs and thyrotrophs. Pit-1 is necessary for the proper temporal and spatial expression of their respective hormones – PRL, growth hormone (GH) and thyroid stimulating hormone β-subunit (TSHβ) – as well as for the expression of the thyroid hormone receptor, GH-releasing hormone receptor and Pit-1 itself (Dolle *et al.* 1990, Simmons *et al.* 1990, Treier & Rosenfeld 1996, Wood *et al.* 1996, Pickett & Gutierrez-Hartmann 1997, Ryan & Rosenfeld 1997).

The transcriptional activity of Pit-1 is determined partly by a balance of interactions with the coactivator cAMP response element-binding protein (CREB)-binding protein (CBP) and an N-CoR/mSin3-containing corepressor (Xu *et al.* 1998). Indeed, Pit-1 can increase histone acetylation and alter the chromatin structure of the PRL promoter (Diamond & Gutierrez-Hartmann 2000, Kievit & Maurer 2005). Pit-1 transduces Ras, cAMP/protein

DOI: 10.1677/joe.1.05738
0022–0795/05/0185–173
Online version via http://www.endocrinology-journals.org

kinase A (PKA), and growth factor/protein kinase C, activating signals to the rat PRL promoter, but phosphorylation of Pit-1 is not required for signal mediation of any of these (Keech *et al.* 1992, Fischberg *et al.* 1994, Howard & Maurer 1994, Okimura *et al.* 1994).

Pit-1 transduces growth factor signals to the rat PRL promoter through the three most proximal DNA binding sites for Pit-1, FP I–III, and transduces Ras signaling through the more distal FP IV (Keech *et al.* 1992, Liang *et al.* 1992, Bradford *et al.* 1995, Rajnarayan *et al.* 1995). Pit-1 functionally interacts with Oct-1 (Diamond *et al.* 1999), as well as pituitary CREB (Fliss *et al.* 1999), to reconstitute PKA-enhanced PRL promoter expression. Pit-1 interacts with Ets-1 to transduce Ras signaling, and Pit-1 and Ets-1 synergistically increase PRL promoter activity in HeLa non-pituitary cells (Bradford *et al.* 1995, 1997).

The Pit-1–Ets-1 interaction is quite specific, and neither Ets-2 nor Pit-1β is able to take part in this interaction (Bradford *et al.* 1997). The synergy domain on Ets-1 maps to the region III TAD (Augustijn *et al.* 2002), and each of the two exons of the Pit-1 TAD has recently has been shown to contribute differing activities to the functional synergy between Pit-1 and Ets-1 and to transduce the Ras response (Duval *et al.* 2003). Specifically, the first TAD exon modulates Pit-1′s ability to synergize with Ets-1 in reconstitution systems, while the second TAD exon acts as a tissue-specific transducer of Ras signaling.

Pit-1β arises from use of an alternate 3′ splice acceptor at the end of the first intron (Konzak & Moore 1992, Morris *et al.* 1992, Theill *et al.* 1992), which inserts the 26 AA β-domain at position 48 in the TAD. The β-domain dictates a range of isoform-specific transcriptional properties, such that Pit-1β acts as a repressor of transcription from the PRL promoter in pituitary somatolactotrophs, which express both PRL and GH, and α-TSH thyrotrophs (Konzak & Moore 1992, Morris *et al.* 1992, Theill *et al.* 1992, Haugen *et al.* 1994), and blocks Ras signaling to the PRL promoter in somatolactotroph (Conrad *et al.* 1994, Bradford *et al.* 1995). The β-domain acts as a transcription switch motif (Diamond *et al.* 1999) that dictates active repression of the PRL promoter by modifying the acetylation state of the PRL promoter (Diamond & Gutierrez-Hartmann 2000).

The AA sequence of the β-domain is conserved across the vertebrates (Konzak & Moore 1992, Morris *et al.* 1992, Ono & Takayama 1992, Theill *et al.* 1992, Wong *et al.* 1992, Delhase *et al.* 1995) and dictates the dominant-negative properties of Pit-1β (Diamond *et al.* 1999). Analysis of the β-domain itself has revealed that two regions consisting of five hydrophobic AAs (leucine 7, isoleucine 8, tyrosine 17, phenylalanine 18 and methionine 20) are required for repression of the PRL promoter (Diamond & Gutierrez-Hartmann 2000).

The transcriptional activity of Pit-1 is dictated by the DNA-binding-induced conformation of Pit-1 on different promoters (Scully *et al.* 2000). These results suggest that the β-domain might function by modulating the conformational changes induced in Pit-1 by promoter context or by different signals impinging on Pit-1 target promoters.

In this manuscript, we describe the promoter-, sequence- and signal-specificity of transcriptional repression by Pit-1β. We demonstrate that Pit-1β impairs recruitment of CBP to promoters that it represses, and that repression of target promoter activity, and reduction in promoter histone acetylation, depend on promoter context. Specifically, we demonstrate that repression by Pit-1β is independent of the mechanism that determines Ras signaling to the PRL promoter. We show that Pit-1β represses the PRL and GH promoters, which rely on Pit-1 to recruit CBP, but it does not repress its own promoter, which relies on CREB to recruit CBP. Our demonstration that Pit-1β reduces CBP recruitment to repressed promoters provides a mechanism for promoter-specific repression by Pit-1β.

Materials and Methods

Cell culture

Monolayer cultures of GH_3 and GH_4 rat pituitary tumor cells (Tashjian *et al.* 1968) were maintained in Dulbecco's modified Eagle's medium, 20% fetal bovine serum and 50μg/ml penicillin and streptomycin at 37 °C in 5% CO_2. The medium was changed 16–18 h before each transfection. Cells used for transfection were harvested at approximately 60–80% confluence using 0·05% trypsin and 0·5 mM EDTA. Forskolin (FSK) was used at 9 μM in DMSO.

Plasmids

Plasmids pA_3PRLluc:firefly luciferase driven by the proximal (−425) rat PRL promoter and pA_3GHluc:firefly luciferase driven by the proximal (−593) rat GH promoter have been described previously (Conrad & Gutierrez-Hartmann 1992). Plasmid pA_3Pit-1 luc:firefly luciferase driven by the proximal (−200) rat Pit-1 promoter was constructed as follows: the −200 to +1of the Pit-1 promoter was amplified from p-200 GHF1-CAT plasmid (the generous gift of M Karin, UCSD, San Diego, CA, USA) using the following primers, CGGTACCgtccc acttattgacg and CCAAGCTTggcggctctgaggc, and the resulting fragment cloned into pCR2·1 (Invitrogen).

The fragments were confirmed by sequencing, then were isolated and cloned into the HindIII–KpnI sites upstream of the pA3-luc plasmid (Maxwell *et al.* 1989) – plasmid pSV Ras: H-Ras valine 12 mutant, driven by the SV40 early promoter; and plasmids pRSV influenza hemaglutinin (HA) epitope Pit-1, HA Pit-1β and β-globin (Tokitou *et al.* 1999), driven by the RSV promoter (Diamond & Gutierrez-Hartmann 2000).

Five mutant $pA_3 - 425$ PRL plasmids were used: (i) plasmid pA_3 mFP IV, a mutation of -200 to -195 from ATTAAT to GTCGAC; (ii) plasmid pA_3EBS, which contains a mutation from -214 to -209 – the sequence was changed from AAGGAA to an Xho1 site, CTCGAC; (iii) plasmid pA_3PRLLuc(-425)mFP III, a mutation of -160 to -155, from GAATAT to CTCGAG; (iv) plasmid pA_3PRLLuc(-425)mFP II, a mutation of -128 to -123, from GTTTAA to CTCGAG; and (v) plasmid pA_3PRLLuc(-425)mFP I, a mutation of -52 to -47, from ATTCAT to CTCGAG (Jackson *et al.* 1992, Keech *et al.* 1992, Bradford *et al.* 1996).

Transfection

DNA was introduced into HeLa, GH_3 or GH_4 cells by electroporation as follows. Approximately $2–3 \times 10^6$ enzymatically dispersed cells were mixed with plasmid DNA in a sterile gene-pulse chamber and exposed to a controlled electrical field of 500 μF at 220 V, as described previously (Diamond *et al.* 1999, Diamond & Gutierrez-Hartmann 2000). Cells from individual transfections then were maintained in Dulbecco's modified Eagle's medium, 15% horse serum, 2·5% fetal bovine serum and 50 μg/ml of penicillin and streptomycin, at 37 °C.

A 1:3 Pit-1:Pit-1β plasmid DNA ratio that results in equal levels of protein expression was used (Diamond *et al.* 1999, Diamond & Gutierrez-Hartmann 2000). The non-specific effect of the RSV promoters upon transcription factor availability was controlled by including amounts of pRSV β-globin plasmid DNA in all assays to render the total pRSV DNA concentration constant. Twenty-five nanograms of plasmid pRLC Renilla were included as an internal control for all transfections. Cells from individual transfections were maintained for 24 h.

Luciferase assays

Transient transfections were performed in triplicate, in at least three separate experiments. After incubation for 24 h, cells were harvested and assayed, using the Dual Luciferase Reporter Assay System (Promega) and a Monolight 3010 Luminometer (Analytical Luminescence Laboratories, San Diego, CA, USA). Firefly luciferase light units for each transfection are normalized for Renilla luciferase light units. Results are expressed as mean fold activation of the PRL promoter ± S.E.M. for at least three experiments, in triplicate. Fishers protected least significant differences (one-way ANOVA between groups, followed by two-tailed Student's *t*-tests) was used to calculate significance of differences between multiple conditions.

Western blot analysis of HA-tagged Pit-1 proteins

Transient transfections using pRSV HA Pit-1 and Pit-1β were performed as above. Three aliquots of cells were pooled and harvested with PBS containing 3 mM EDTA, pelleted and resuspended in 5 × Lowry loading buffer and passed through a 25 G needle seven times. Equal volumes of each extract were separated on 15% SDS polyacrylamide gels and transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA, USA). The HA-tagged Pit-1 proteins were detected with a mouse monoclonal anti-HA horseradish peroxidase-conjugated antibody (Roche Diagnostics, Indianapolis, IN, USA) and ECL Advance media (Amersham Biosciences). Dilutions of 1:1000 of the anti-HA monoclonal antibody were used.

Chromatin immunoprecipitation studies

Chromatin immunoprecipitation (ChIP) assays were performed according to the protocol for the Acetyl-Histone H4 ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA), as described previously (Diamond & Gutierrez-Hartmann 2000, Lambert & Nordeen 2003). Transient transfections were performed as above. Twenty-four hours after transfection, $1·6 \times 10^7$ GH_3 cells were cross-linked by addition of formaldehyde into the medium at a final concentration of 1% and incubated for 15 min at room temperature.

Cells were washed with ice-cold PBS and resuspended in a 500 μl ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8·0, with protease inhibitors). The lysates were sonicated with a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT, USA) at power setting 2, with three, 10 s pulses at duty cycle 90, and diluted to 3 ml with a ChIP dilution buffer (0·01% SDS, 1·1% Triton X-100, 1·2 mM EDTA, 16·7 mM Tris–HCl, pH 8·0, 167 mM NaCl). One milliliter of each sample was precleared by incubating with 80 μl Protein A beads agarose for 30 min at 4 °C with rotation. Five microliters of anti-acetyl histone H4 antibody (Upstate Biotechnology) or anti-CBP antibody (Santa Cruz Biotechnology, Santa Cruz, CA. USA) were added, and immunoprecipitation was done overnight at 4 °C, with rotation.

Immune complexes were collected with 60 μl Protein A agarose and washed once with 1 ml each of the following buffers in sequence: low-salt wash buffer (0·1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8·0, 150 mM NaCl), high-salt wash buffer (0·1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8·0, 1500 mM NaCl), LiCl wash buffer (250 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8·0). Then they were washed twice with 10 mM Tris–HCl, pH 8·0, 1 mM EDTA.

Immune complexes were eluted, cross-links were reversed by heating at 65 °C, and extracts were subjected to proteinase K treatment to eliminate protein. DNA was recovered by phenol–chloroform extraction, followed by ethanol precipitation, and used as a template for PCR (30 cycles), using pA3 − 425 PRL Luc promoter-specific, commercially synthesized deoxyoligonucleotides (Gibco/BRL) that contain a PRL promoter-specific sequence,

GACTCAAGATGTCAGTCAGC, and a luciferase-specific sequence, GCCTTTCTTTATGTTTTTGGC.

In addition, internal control PCR reactions were performed with pSV 40 plasmid-specific, commercially synthesized deoxyoligonucleotides (Gibco/BRL) that contain SV40 promoter-specific sequences, GCATCTCAAT TAGTCAGC and GGAATAGCTCAGAGGCCGAG. Control reactions were performed to ensure that all PCR assays took place in the linear range of response to input DNA. PCR products were separated by agarose gel electrophoresis, and bands were imaged and quantified (relative to a 50 ng control band) on a Foto/Eclipse-CCD (Fotodyne Corp., Hartland, WI, USA) and Gel Documentation System with NIH image (Bethesda, MD, USA). Densitometric units were normalized to those of the SV40 control in the presence of wild-type (WT) PRL promoter.

Results

Pit-1β blocks multiple signaling pathways to the PRL promoter

We have previously demonstrated that the β-domain insertion converts Pit-1 from an activator to a repressor of the PRL promoter (Conrad *et al.* 1994, Bradford *et al.* 1995). Moreover, we have shown that the AA sequence of the β-domain dictates this repression and that the activity of a histone deacetylase is required (Diamond *et al.* 1999, Diamond & Gutierrez-Hartmann 2000). If the β-domain simply alters the balance between Pit-1-associated histone acetylation vs deacetylation in a simple manner, one would expect that Pit-1β would block the PRL promoter, regardless of signals impinging upon it, and repress all Pit-1 target promoters. Signal- or promoter-specificity of repression would suggest that repression requires the array of transcription cofactors involved in the particular signal or promoter context, and thus it may involve an alteration in the mechanism of signaling.

In order to test whether Pit-1β can repress the PRL promoter in the presence of signals other than oncogenic Ras, we examined the effects of Pit-1β in the presence of an activated cAMP/PKA pathway. This signaling pathway is independent of, and actually antagonistic toward, that of Ras (Conrad & Gutierrez-Hartmann 1992). Pit-1 and Pit-1β were introduced into GH_3 pituitary somatolactotroph cells by electroporation in the presence of a PRL promoter-driven luciferase reporter and either pSV Ras or FSK, an activator of the PKA signaling pathway (Conrad & Gutierrez-Hartmann 1992) (Fig. 1A). We used a 3:1 ratio of Pit-1β:Pit-1 plasmid DNA that we previously have shown to result in comparable levels of protein expression (Diamond *et al.* 1999, Diamond & Gutierrez-Hartmann 2000) (Fig. 1B).

Ras and FSK increased PRL promoter activity 12- and 9-fold respectively (data not shown). Co-transfection with Pit-1 enhanced the activity of the PRL promoter by more than 74% in the presence of no signal, by 64% in the presence of oncogenic Ras and by 37% in the presence of FSK. Co-transfection of the Pit-1β isoform not only failed to enhance the activity of the PRL promoter but actually reduced it by 40–54%. Thus, Pit-1β can repress PRL promoter activity in the presence of two independent signaling pathways (Ras and FSK) of the PRL promoter.

Pit-1β shows promoter selectivity for repression

In order to test whether Pit-1β would repress multiple Pit-1 target promoters, Pit-1 and Pit-1β were introduced into GH_4 pituitary somatolactotroph cells by electroporation in the presence of luciferase reporter constructs regulated by the proximal region of either the rat PRL promoter, GH promoter or Pit-1 promoter (Fig. 2). For each promoter, the firefly luciferase gene was placed under the control of a region that previously has been shown sufficient to confer cell-type specific gene expression in transient transfection experiments: the −425 to +65 bp of the 5′ flanking region of the PRL promoter (Camper *et al.* 1985, Gutierrez-Hartmann *et al.* 1987, Elsholtz 1992, Bradford *et al.* 1997), the −593 to +65 bp of the 5′ flanking region of the GH promoter (Flug *et al.* 1987, Ye *et al.* 1988, Schaufele 1996, Palomino *et al.* 1998), and the −200 to +1bp of the 5′ flanking region of the Pit-1 promoter (Chen *et al.* 1990, McCormick *et al.* 1990, 1991) respectively.

Co-transfection of Pit-1 enhanced the PRL promoter activity by 26%, and co-transfection of the Pit-1β isoform reduced activity by approximately 67% (Fig. 3A). Co-transfection with Pit-1 had no significant effect on the GH promoter activity, and co-transfection of the Pit-1β isoform reduced activity by 61%. Co-transfection with either Pit-1 or Pit-1β had no significant effect on the Pit-1 promoter activity. As documented previously, co-transfection with Pit-1 did little to enhance target promoter activation in pituitary cells, but Pit-1β showed great differences in action on the different target promoters. Similar results were seen in GH_3 pituitary cells (data not shown). These data show that the β-domain confers repression to a subset of Pit-1 target promoters.

A trivial explanation for lack of repression of the Pit-1 promoter by Pit-1β would be that Pit-1β could not interact with the proximal Pit-1 promoter and thus could not repress its activity in somatolactotrophs. We previously have demonstrated that Pit-1β displays cell-type specificity in its ability to repress the PRL promoter in pituitary cells, and it actually activates the PRL promoter in HeLa non-pituitary cells (Diamond *et al.* 1999). Thus, we used HeLa non-pituitary cells, which lack endogenous Pit-1 expression, in order to demonstrate that Pit-1 and Pit-1β could indeed interact with and activate all three target promoters in non-pituitary cells.

HA-tagged Pit-1 and HA-tagged Pit-1β were introduced into HeLa non-pituitary cells by electroporation in

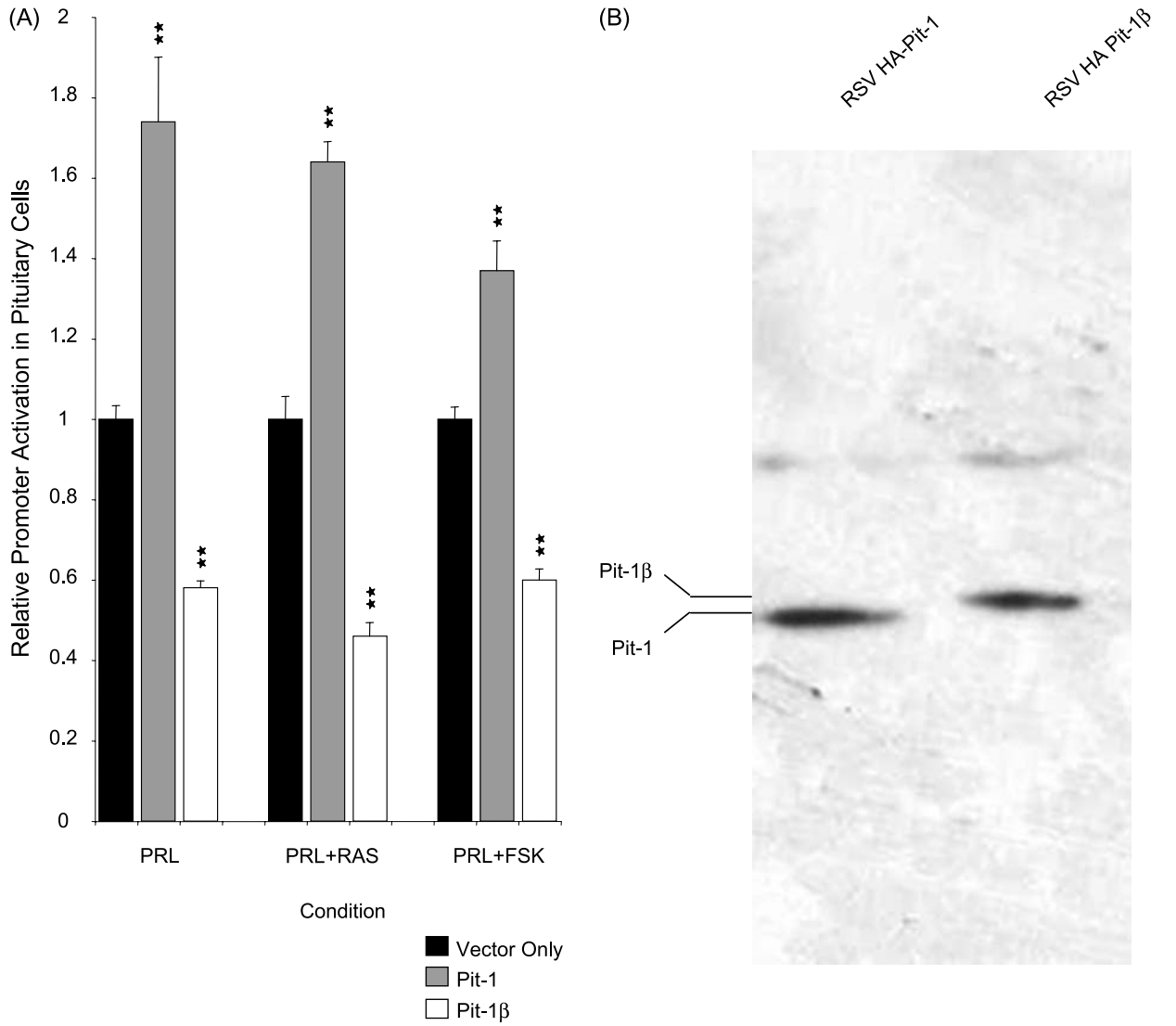

Figure 1 Pit-1β blocks multiple signaling pathways to the PRL promoter. (A) Effects of Pit-1 and Pit-1β on activation of the PRL promoter by the Ras and PKA signaling pathways in GH_3 cells. Plasmid pA3 PRL luc − 425 (3 μg) and combinations of pSV Ras (2 μg), pRSV HA Pit-1 (10 μg) and pRSV HA Pit-1β (30 μg) were introduced into 4×10^6 GH_3 pituitary cells by electroporation. Vehicle (DMSO) or 9 μM FSK in DMSO was added for the last 3 h of incubation. Total RSV and SV40 promoter amounts were maintained constant with pRSV β-globin and pSV40 DNA. After 24 h, cells were harvested and total light units were measured. Starred bars are significantly different from vector only: $^{**}P<0{\cdot}01$. (B) Expression levels of Pit-1 and Pit-1β. Plasmids pRSV HA Pit-1 (10 μg) and pRSV HA Pit-1β (30 μg) were introduced into 4×10^6 GH_3 pituitary cells by electroporation. After 24 h, cells were harvested and analyzed by SDS-PAGE and Western blot (See Materials and Methods).

the presence of luciferase reporter constructs regulated by either the proximal regions of the PRL promoter, the GH promoter, or the Pit-1 promoter (Fig. 3B). Pit-1 and Pit-1β activated the PRL promoter by 66- and 100-fold respectively. Pit-1 and Pit-1β activated the GH promoter by 3- and 10-fold respectively, and the Pit-1 promoter by 3- and 21-fold respectively. Of note, the GH and Pit-1 promoters were activated to similar extents by both Pit-1 and Pit-1β. These data demonstrate that the failure of Pit-1β to repress the Pit-1 promoter was not due to an inability to interact with or activate that promoter, but rather to a specific loss of ability to repress the promoter.

Pit-1β shows promoter context requirements for repression

Target promoter-specific repression by Pit-1β suggests that specific promoter elements, and thus, specific transcription cofactors brought to these elements, might be

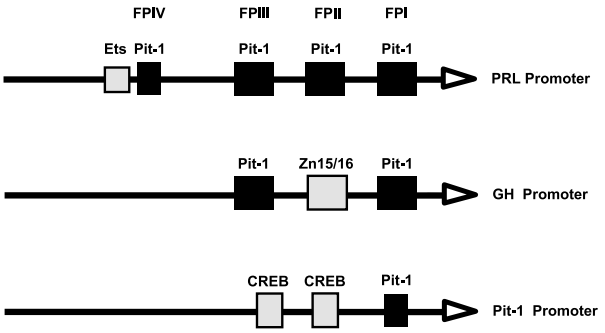

Figure 2 Three Pit-1 target promoters. The proximal PRL, GH and Pit-1 promoters are shown (not to scale). Pit-1 binding sites are shown as black squares, and other transcription factor binding sites appear as grey squares. Mutations of FP I II, III and IV, as well as the EBS of the RRE were tested (See Materials and Methods).

required for repression. In order to test this hypothesis, Pit-1 and Pit-1β were introduced into GH_3 pituitary somatolactotroph cells by electroporation in the presence of a series of mutant PRL promoter–reporter constructs with previously characterized substitutions for four Pit-1 binding sites, FP I, FP II, FP III and FP IV in the proximal PRL promoter (Fig. 2) (Jackson *et al.* 1992, Keech *et al.* 1992, Bradford *et al.* 1996, Kievit & Maurer 2005).

The FP IV Pit-1 binding site forms part of a composite Ras response element (RRE), together with a binding site for Ets-1 (Bradford *et al.* 1996). The failure of Pit-1β to interact functionally with Ets-1 in non-pituitary cells had led to a model of Pit-1β repression in which repression of PRL promoter activity takes place in the context of this composite FP IV/Ets-binding site (EBS), required for optimal Ras signaling to the PRL promoter (Bradford *et al.* 2000).

Mutation of the FP I, FP II, FP III and FP IV elements decreased PRL promoter activity by 14-, 6-, 5- and 3-fold respectively (data not shown). As documented previously, co-transfection with the Pit-1β isoform reduced the response of the WT PRL promoter in the presence of oncogenic Ras (Fig. 4A) by 37%. Co-transfection with the Pit-1β isoform did not reduce, but rather enhanced, the response of the mutant (m) FP I, II and III PRL promoters in the presence of oncogenic Ras by 67, 42 and 55% respectively. However, Pit-1β did repress the response of the mFP IV reporter in the presence of oncogenic Ras by 46%. These data demonstrate that FP I, FP II and FP III, but not FP IV, are necessary for repression. Thus, specific sequence requirements exist for repression by the Pit-1β isoform.

The demonstration that the Pit-1 binding site in the RRE is unnecessary for repression suggests that previous models of Pit-1β repression that involve interactions with Ets-1 and require the composite FP IV RRE in the PRL promoter (Bradford *et al.* 1995, 1996, 1997) are incorrect. To further test this model, we tested the ability of a mutation of the FP IV-proximal EBS that prevents Ets-1 binding (Bradford *et al.* 1996) to block repression by the Pit-1β isoform (Fig. 4B).

Mutation of the EBS element, like that of the FP IV element, reduced PRL promoter activity 3-fold (data not shown). In the presence of oncogenic Ras, co-transfection with the Pit-1 isoform activated the WT PRL promoter by 2·2-fold, and co-transfection with the Pit-1β isoform reduced the response of the WT PRL promoter by 45%. Co-transfection with the Pit-1 isoform shows no significant effect on the mFP IV promoter, and co-transfection with the Pit-1β isoform reduced the response of the mFP IV promoter by 55%, as Fig. 4A also shows. Co-transfection with the Pit-1 isoform activated the mEBS PRL promoter by 2·3-fold, and co-transfection with the Pit-1β isoform reduced the response of the mEBS promoter by 58%. These data demonstrate that neither element of the FP IV composite Pit-1/EBS is required for repression by the Pit-1β isoform.

In order to test whether the DNA-context dependence of repression by Pit-1β shows signal specificity, we introduced Pit-1 and Pit-1β into GH3 pituitary somatolactotroph cells by electroporation in the presence of the FP I, FP II, FP III and FP IV mutant PRL promoter–reporter constructs in the presence of FSK (Fig. 5). Mutation of the FP I, FP II, FP III and FP IV elements decreased PRL promoter activity by 10-, 7-, 3- and 3-fold respectively (data not shown).

Co-transfection with the Pit-1β isoform reduced the response of the WT PRL promoter in the presence of FSK by 64%. Co-transfection with the Pit-1β isoform did not reduce, but rather enhanced, the response of the mFP I, II and III PRL promoters in the presence of FSK 7, 21 and 26% respectively, similar to the 37% increase in the presence of Pit-1 (Fig. 1A). However, Pit-1β did repress the response of the mFP IV reporter in the presence of FSK by 77%. Similar results were seen in GH_4 pituitary cells (data not shown). These data demonstrate that the DNA-context requirements for repression are the same in the presence of FSK or Ras.

Pit-1β shows promoter-context requirements for alterations in target promoter histone acetylation

We previously have shown that Pit-1β represses PRL promoter activity by altering the histone acetylation state of its target promoter (Diamond & Gutierrez-Hartmann 2000). In order to test whether the DNA sequence requirements for promoter repression were reflected in the sequence requirements for reduction of histone acetylation, HA-tagged Pit-1 and HA-tagged Pit-1β were introduced into GH_3 pituitary cells by electroporation in the presence of the mFP I, mFP II, mFP III or mFP IV PRL promoters (Fig. 6). In addition, we used the SV40 promoter of the pSV plasmid as a control promoter, to demonstrate that Pit-1β-dependent changes in histone

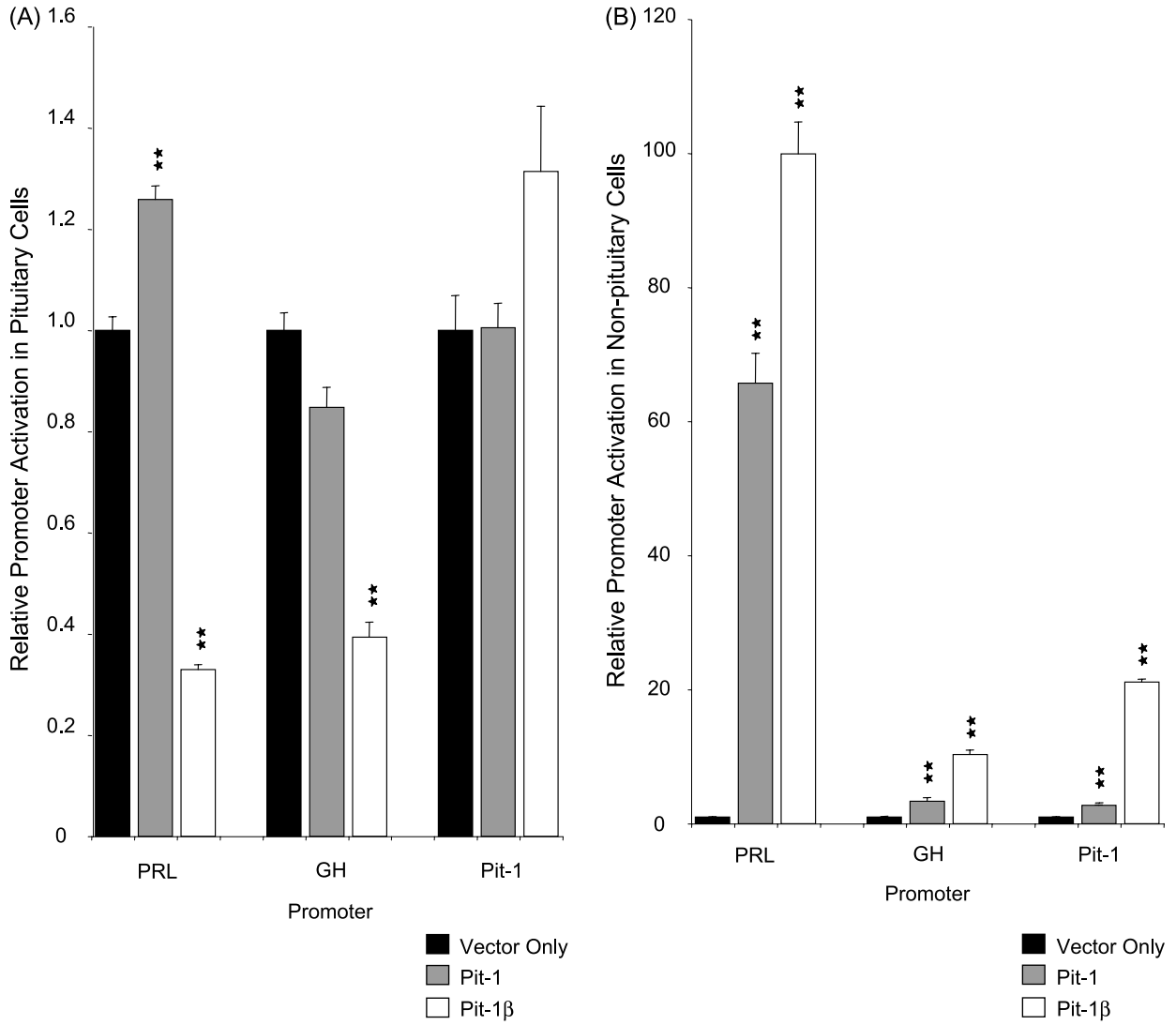

Figure 3 Pit-1β shows promoter selectivity for repression. (A) Effects of Pit-1 and Pit-1β constructs on activation of the target promoters PRL, GH and Pit-1 promoters in GH_4 cells. Combinations (5 μg) of plasmids pA3 PRL luc − 425, pA3 GH luc − 593 or pA_3 Pit-1 luc − 200, together with pRSV HA Pit-1 (4 μg) and pRSV HA Pit-1β (15 μg), were introduced into 4×10^6 GH_4 pituitary cells by electroporation. Total RSV promoter amounts were maintained constant with pRSV β-globin DNA. After 24 h, cells were harvested and total light units were measured. Starred bars are significantly different from vector only: $^{**}P<0{\cdot}01$. (B) Effects of Pit-1 and Pit-1β constructs on activation of the target promoters PRL, GH and Pit-1 promoters in HeLa cells. Combinations (5 μg) of plasmids pA3 PRL luc − 425, pA3 GH luc and pA_3 GH luc − 593, together with pRSV HA Pit-1 (4 μg) and pRSV HA Pit-1β (14 μg), were introduced into 4×10^6 HeLa non-pituitary cells by electroporation. Total RSV promoter amounts were maintained constant with pRSV β-globin DNA. After 24 h, cells were harvested and total light units were measured. Starred bars are significantly different from vector only: $^{**}P<0{\cdot}01$.

deacetylation were specific for the proximal PRL promoter and not global changes in histone deacetylation. The SV40 promoter is activated by CBP (Tabakin-Fix *et al.* 2004) and is not affected by Pit-1 or Pit-1β (Eckel *et al.* 2003).

As documented previously, co-transfection with Pit-1 increased the acetylation level of the WT, mFP II and mFP III PRL promoters and had no effect on the acetylation level of the mFP I and m FP IV promoters in the presence of oncogenic Ras. Co-transfection with Pit-1β reduced the acetylation level of the WT and mFP IV PRL promoters but not of the mFP I, mFP II and mFP III PRL promoters. Indeed, Pit-1β significantly increased (67%) the acetylation level of the mFP III promoter. These data suggest that the same sequence requirements that dictate repression by the Pit-1β isoform also dictate its ability to alter histone acetylation of the PRL promoter.

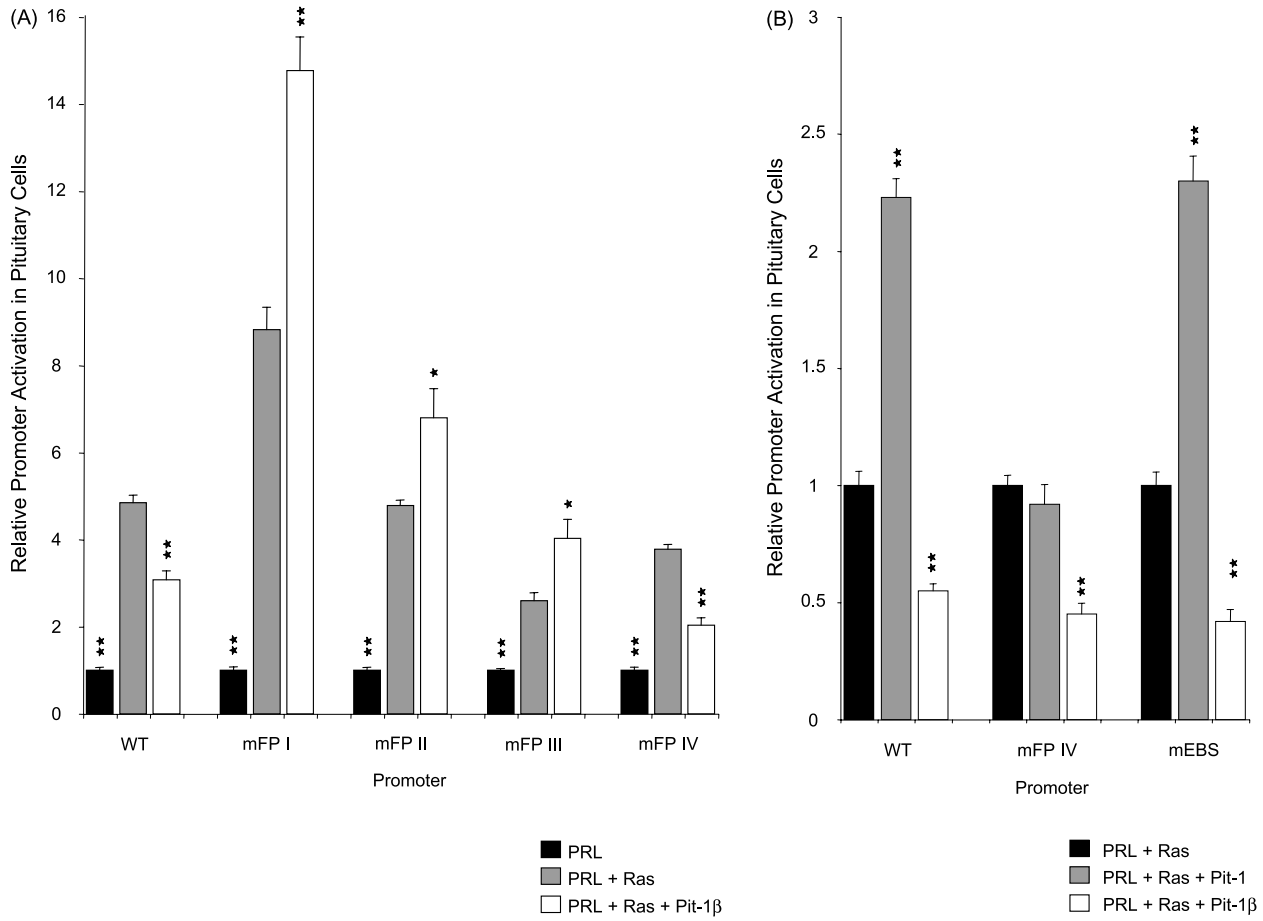

Figure 4 Pit-1β shows promoter-context requirements for repression. (A) Effects of Pit-1β on activation of mutant PRL promoter in the Ras signaling pathway in GH_3 cells. Combinations (3 μg) of plasmids pA_3-pA_3PRLLuc(− 425), pA_3PRLLuc(− 425)mFP IV, pA_3PRLLuc(− 425)mFP III, pA_3PRLLuc(− 425)mFP II, and pA_3PRLLuc(− 425)mFP I together with combinations of pSV Ras (2 μg) and pRSV HA Pit-1β (30 μg), were introduced into 4×10^6 GH_3 pituitary cells by electroporation. Total RSV and SV40 promoter amounts were maintained constant with pRSV β-globin and pSV40 DNA. After 24 h, cells were harvested and total light units were measured. Starred bars are significantly different from vector only+Ras: $^{**}P<0{\cdot}01$; $^{*}P<0{\cdot}05$. (B) Effects of Pit-1 and Pit-1β on activation of PRL promoters with mutant RREs by the Ras signaling pathway in GH_3 cells. Combinations (3 μg) of plasmids pA_3-pA_3PRLLuc(− 425), pA_3PRLLuc(− 425)mFP IV and pA_3EBS together with combinations of pSV Ras (2 μg), pRSV HA Pit-1 (10 μg) and pRSV HA Pit-1β (30 μg), were introduced into 4×10^6 GH_3 pituitary cells by electroporation. Total RSV and SV40 promoter amounts were maintained constant with pRSV β-globin and pSV40 DNA. After 24 h, cells were harvested and total light units were measured. Starred bars are significantly different from vector only+Ras: $^{**}P<0{\cdot}01$.

In order to test a model in which Pit-1β can reduce recruitment of CBP to the PRL promoter, we examined the level of CBP recruitment to the PRL, GH and Pit-1 promoters in GH_3 pituitary cells. HA-tagged Pit-1 and HA-tagged Pit-1β were introduced into GH_3 pituitary cells by electroporation in the presence of the mFP I, mFP II, mFP III or mFP IV PRL promoters (Fig. 7). In addition, we used the SV40 promoter, which CBP can activate (Tabakin-Fix *et al.* 2004), as a control to demonstrate that the Pit-1β-dependent decrease in CBP recruitment is not global, but rather specific for the proximal PRL promoter. Co-transfection with Pit-1 had no significant effect on the recruitment of CBP to the PRL, GH or Pit-1 promoters in GH_3 pituitary cells. However, co-transfection with Pit-1β significantly reduced recruitment of CBP to the PRL and GH promoters but not to the Pit-1 promoter.

Discussion

The influence of promoter context on the transcriptional activity of Pit-1 itself (Scully *et al.* 2000) suggests that repression by Pit-1β also might be modulated by promoter context. This hypothesis would predict that the activity of the β-domain itself might be affected by either promoter

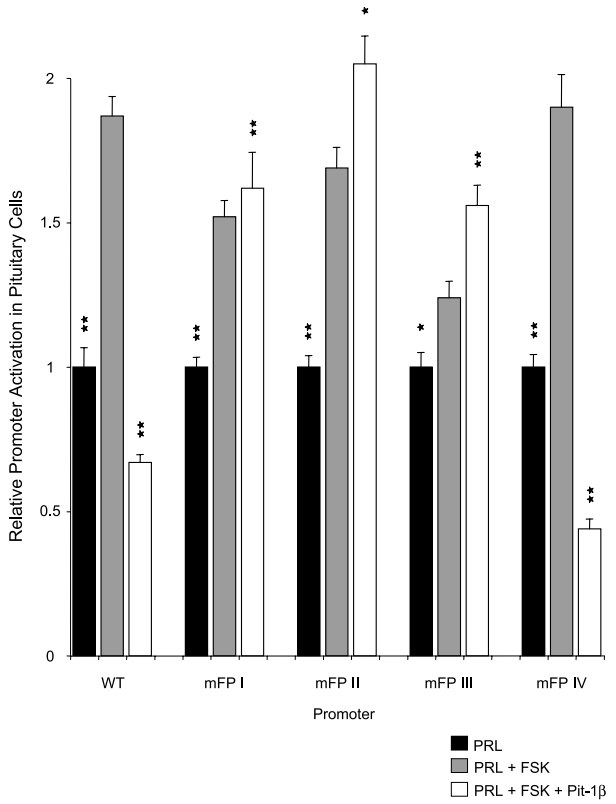

Figure 5 Pit-1β shows no signal specificity in its promoter-context requirements for repression. Effects of Pit-1β on activation of mutant PRL promoter by the cAMP signaling pathway in GH_3 cells. Combinations (3 μg) of plasmids pA_3-pA_3PRLLuc(− 425), pA_3PRLLuc(− 425)mFP IV, pA_3PRLLuc(− 425)mFP III, pA_3PRLLuc(− 425)mFP II and pA_3PRLLuc(− 425)mFP I, together with combinations of pRSV HA Pit-1β (30 μg), were introduced into 4×10^6 GH_3 pituitary cells by electroporation. Total RSV promoter amounts were maintained constant with pRSV β-globin. After 24 h, cells were harvested and total light units were measured. Starred bars are significantly different from vector only+FSK: $^{**}P<0{\cdot}01$; $^{*}P<0{\cdot}05$.

context or by differing signaling pathways. Promoter context has been shown to modulate the transcriptional activity of many transcription factors, either through direct effects of DNA sequence elements on protein conformation (reviewed in Lefstin & Yamamoto 1998) or through interaction with neighboring cofactors. Here, we demonstrate that Pit-1β impairs CBP recruitment to promoters that it represses. In addition, we show that repression of target promoter activity, reduction in promoter histone acetylation, and decrease of CBP recruitment all depend on promoter context.

Our experiments demonstrate that Pit-1β represses target promoters in a promoter-context-dependent manner. The ability of Pit-1β to repress PRL promoter activity in the presence or absence of Ras or FSK (Fig. 1A) is consistent with our previous findings that the mechanism for isoform-specific regulation involves the alteration of histone acetylation, which should inhibit promoter activity broadly. The promoter selectivity of repression by Pit-1β in our experiments (Fig. 3A) suggests that repression depends on promoter elements present in the proximal GH and PRL promoters but absent in the proximal Pit-1 promoter.

The ability of Pit-1β to activate the Pit-1 promoter in parallel experiments in HeLa non-pituitary cells (Fig. 3B) allows us to rule out the possibility that the lack of repression by Pit-1β of the Pit-1 promoter could be due to an inability of Pit-1β to bind to and modulate the Pit-1 promoter. We have previously have demonstrated that Pit-1β acts as a repressor in a pituitary-specific manner and actually activates PRL promoter activity in HeLa non-pituitary cells (Diamond & Gutierrez-Hartmann 1996, 2000).

What are the promoter-specific attributes that provide the contextual information to Pit-1β? We investigated this question with a series of mutant PRL promoter constructs, each of which is defective in one of the principal transcription factor binding motifs present in the PRL promoter (Figs 4 and 5). Previous models of Pit-1β repression have assumed that the repression of Ras signaling to the PRL promoter took place in the context of the composite FP IV monomer Pit-1/EBS site, required for optimal Ras signaling to the PRL promoter (Bradford *et al.* 1995, 1996, 1997, 2000). However, our results show that repression requires neither the FP IV nor EBS elements even in the presence of Ras (Fig. 4A and B). This decoupling of the promoter requirements for Ras signaling and β-domain implies that repression is not due to alterations of interaction between Pit-1 and Ets-1.

A pattern emerges when we examine our results more closely. Both the promoter-specificity of repression and the DNA sequence requirements for repression of PRL promoter activity display a pattern of requirement for Pit-1 binding sites that transduce cAMP/PKA and growth factor signaling to the target promoter. The proximal GH promoter, which is repressed, contains a pair of elements that bind Pit-1 and allow Pit-1 tethering of activated CBP, while the proximal Pit-1 promoter, which is not repressed, binds Pit-1 as a monomer and contains two CREB-binding sites that themselves recruit CBP (Holloway *et al.* 1995, Gaiddon *et al.* 1996, Cohen *et al.* 1999) (Fig. 2).

The PRL promoter contains three elements that bind Pit-1 and transduce cAMP/PKA and growth factor signaling (FP I, II and III). The promoter also contains the FP IV Pit-1 monomer site that transduces Ras signaling (Nelson *et al.* 1988, Mangalam *et al.* 1989, Elsholtz *et al.* 1990, Voss *et al.* 1993, Kievit & Maurer 2005). Our data show that mutant PRL promoters that lack FP I, FP II or FP III are not repressed. However, a mutant PRL promoter that lacks the FP IV site but retains the other sites is repressed. The results of the ChIP assays in Fig. 6

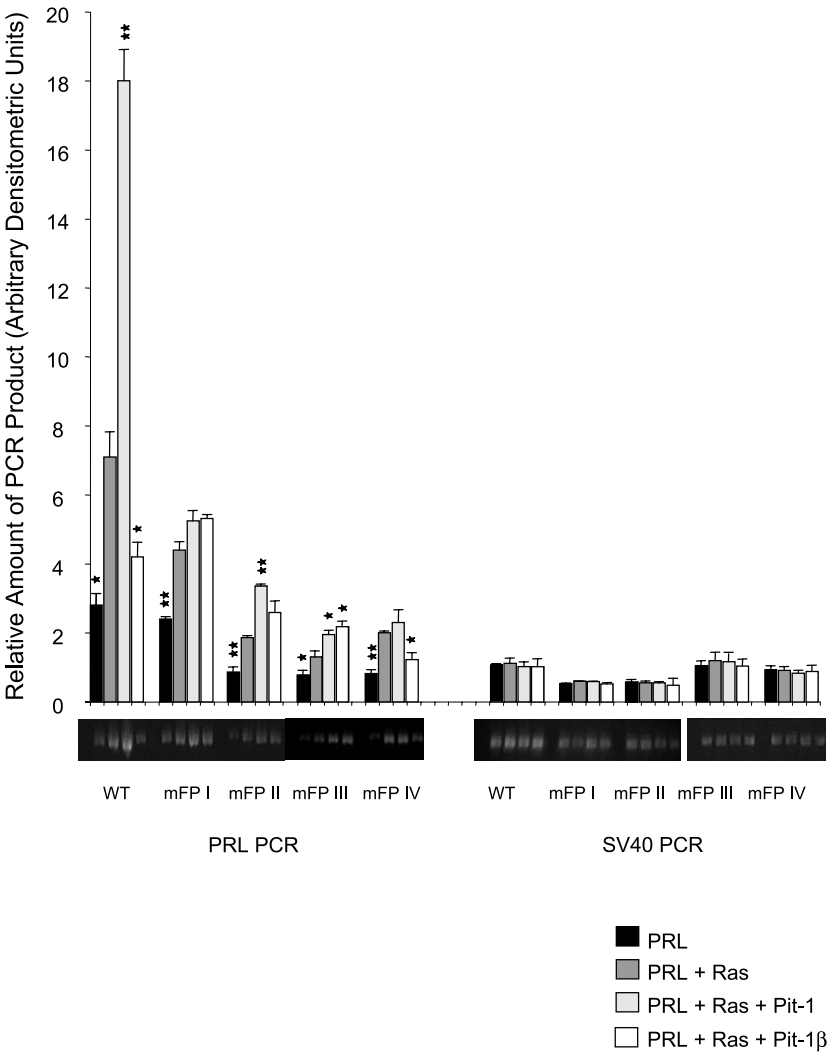

Figure 6 Pit-1β shows promoter-context requirements for alteration of the promoter acetylation state. Above, the effects of Pit-1β on histone acetylation of mutant PRL promoter by the Ras signaling pathway in GH_3 cells. Three micrograms of plasmids pA_3PRLLuc(− 425), pA_3PRLLuc(− 425)mFP I, pA_3PRLLuc(− 425)mFP II, pA_3PRLLuc(− 425)mFP III, pA_3PRLLuc(− 425)mFP IV, or pA_3EBS, together with combinations of pSV Ras (2 μg), pRSV HA Pit-1 (10 μg), and pRSV HA Pit-1β (30 μg), were introduced into 4×10^6 GH_3 pituitary cells by electroporation, ten times for a total of 4×10^7 cells. After 24 h, cells were harvested for ChIP assays (See Materials and Methods). Below, a representative ChIP assay is shown below the results of three ChIP experiments in triplicate, expressed as relative amounts of target and SV40 control promoter DNA associated with acetylated histone H4 (in arbitrary densitometric units) (See Materials and Methods). Starred bars are significantly different from vector+Ras: $^{**}P<0{\cdot}01$; $^{*}P<0{\cdot}05$.

demonstrate that the DNA requirements for repression are the same as those for deacetylation of target promoters. The results of the ChIP assay for CBP recruitment (Fig. 7) demonstrate directly that Pit-1β reduces recruitment of CBP to the PRL and GH promoters, which recruit CBP through Pit-1, but has no negative effect on the Pit-1 promoter, which recruits CBP through CREB.

The requirement for multiple PRL Pit-1 binding sites for maximal gene activation in the pituitary, and for maximal repression by Pit-1β, may reflect a requirement for multiple interactions to recruit CBP. Such a requirement has been shown for other genes such as the P31 element of the interferon-beta gene (Yang *et al.* 2002, 2003). The GH promoter also contains multiple

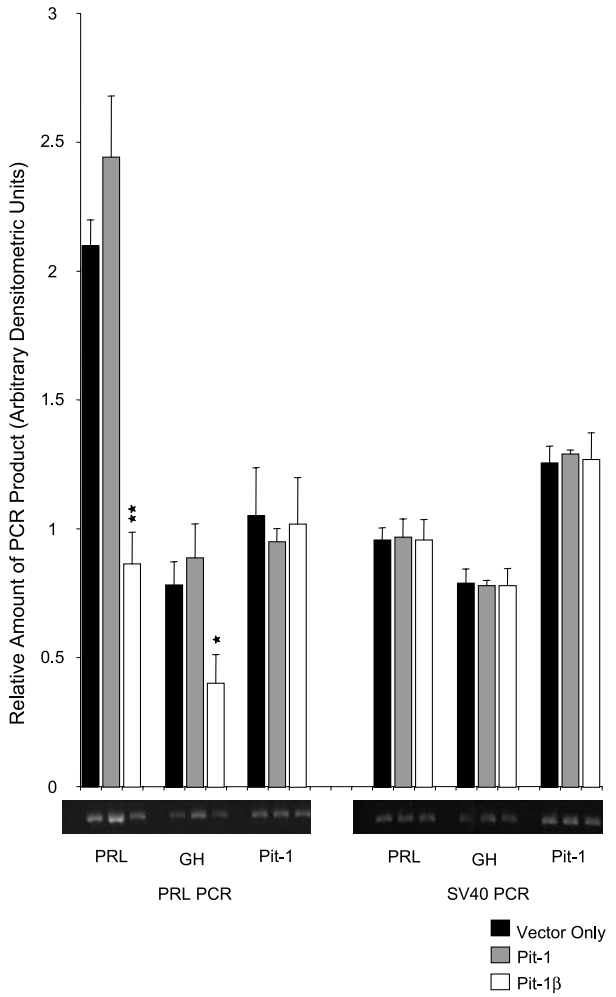

Figure 7 Pit-1β shows promoter-context requirements for reduction of CBP recruitment. Above, the effects of Pit-1β on CBP recruitment to PRL, GH and Pit-1 promoters by the Ras signaling pathway in GH_3 cells. Three micrograms of plasmids pA3 PRL luc − 425, pA3 GH luc − 593 or pA_3 Pit-1 luc − 200, together with pSV40 and combinations of pRSV HA Pit-1 (10 μg) and pRSV HA Pit-1β (30 μg), were introduced into 4×10^6 GH_3 pituitary cells by electroporation, ten times, for a total of 4×10^7 cells. After 24 h, cells were harvested for ChIP assays (See Materials and Methods). Below, a representative ChIP assay is shown below the results of three ChIP experiments in triplicate, expressed as relative amounts of target and SV40 control promoter DNA associated with CBP (in arbitrary densitometric units) (See Materials and Methods). Starred bars are significantly different from vector only: $^{**}P<0{\cdot}01$; $^{*}P<0{\cdot}05$.

transcription factor binding sites. One, between the two Pit-1 sites, binds Zn-15/16, a Cys/His zinc-finger transcription factor that acts synergistically with Pit-1 to activate GH expression (Lipkin *et al.* 1993, Tuggle & Trenkle 1996, Petersenn *et al.* 1997, Shewchuk *et al.* 1999, VanderHeyden *et al.* 2000).

We found that when FP I, FP II or FP III are altered, Pit-1β does not simply fail to repress the promoter, but instead it acts as an activator. We have shown previously that Pit-1β acts as an activator in non-pituitary cells (Diamond & Gutierrez-Hartmann 1996, 2000, Diamond *et al.* 1999). It seems likely that Pit-1 can activate target promoters through two separate mechanisms, a pituitary-specific mechanism dependent upon recruitment of CBP by Pit-1, which Pit-1β disrupts, and a second non-specific mechanism, independent of recruitment of CBP by Pit-1, for which Pit-1β is quite competent. Indeed, a mutant Pit-1 lacking the TAD, the location of the β-domain insertion, can alter PRL promoter nucleosome assembly but cannot activate transcription in a *Xenopus laevis* oocyte cell system (Kievit & Maurer 2005). This suggests that the second, isoform-insensitive, mechanism of promoter activation seen in non-pituitary cells, may be due to chromatin alteration by Pit-1 and Pit-1β that allows ubiquitous factors to bind and activate target promoters.

Thus, our findings support a model in which Pit-1β inhibits hormone gene expression by inhibiting Pit-1 recruitment of CBP to target promoters. Further experiments will investigate the physical mechanisms that underlie this inhibition.

Acknowledgements

The authors thank Ms Ashley Buckingham and Ms Cortney Stringer for technical assistance, Dr Dawn Duval for critical reading of this manuscript, other members of the Diamond and Gutierrez-Hartmann laboratories for their helpful suggestions and comments, and Dr Maria Cadwallader of The Editor Inc.com and Ms Dana Manning for careful proofreading.

Funding

S E D was supported by the American Cancer Society (RSG TBE-105036), the National Institutes of Health (K01 DK02752 and P20 RR15592) and the Kentucky Research Challenge Trust Fund. A G H was supported by the National Institutes of Health (R01 DK037667 and R01 DK046868). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 11 October 2004
Accepted 6 January 2005