Repression of the prolactin promoter: a functional consequence of the heterodimerization between Pit-1 and Pit-1 β

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Abstract

The POU-homeodomain transcription factor Pit-1 is required for the differentiation of the anterior pituitary cells and the expression of their hormone products. Pit-1 β , an alternate splicing isoform, has diametrically different outcomes when it is expressed in different cell types. Pit-1 β acts as a transcriptional repressor of prolactin (PRL) and growth hormone genes in pituitary cells, and as a transcriptional activator in non-pituitary cells. In order to explore these differences, we: (1) identified the transcriptional cofactors necessary for reconstitution of repression in non-pituitary cells; (2) tested the effect of the β -domain on heterodimerization with Pit-1 and physical interaction with the co-activator CREB binding protein (CBP); and (3) determined the β -domain sidechain chemistry requirements for repression. Co-expression of both Pit-1 isoforms reconstituted the repression of the PRL promoter in non-pituitary cells. The β -domain allowed heterodimerization with Pit-1 but blocked physical interaction with CBP, and specific chemical properties of the β -domain beyond hydrophobicity were dispensable. These data strongly suggest that Pit-1 β represses hormone gene expression by heterodimerizing with Pit-1 and interfering with the assembly of the Pit-1–CBP complex required for PRL promoter activity in pituitary cells.

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Introduction

POU domain factors regulate transcription by interacting with themselves and with other proteins. The 33 kDa POU-homeodomain transcription factor Pit-1 helps to establish anterior pituitary lactotroph, somatotroph, and thyrotroph lineages and proper expression of their respective hormone products: prolactin (PRL), growth hormone (GH), and thyroid stimulating hormone subunit β (TSH β). Pit-1's amino- (N-) amino acid (AA) terminal transactivation domain (TAD) activates transcription (Theill *et al.* 1989, Ingraham *et al.* 1990) and mediates dopamine repression (Lew & Elsholtz 1995). Together, the POU-specific (AA 128–198) and POU-homeodomain (AA 214–273) form the carboxyl- (C-) terminal DNA binding and dimerization domain (DBD) (Bodner & Karin 1987, Herr *et al.* 1988, Ingraham *et al.* 1988) (Fig. 1), which binds DNA with high affinity and specificity (Ingraham *et al.* 1990) and interacts with transcription cofactors (Xu *et al.* 1998).

Pit-1 transduces activation by the cyclic AMP (cAMP)-protein kinase A (PKA) pathway (Day *et al.* 1989, Keech & Gutierrez-Hartmann 1989, Iverson *et al.* 1990, Conrad & Gutierrez-Hartmann 1992, Keech

et al. 1992) and by liganded estrogen receptor (Iverson *et al.* 1990). It also transduces the inhibition by dopamine and activin (Maurer 1981, Elsholtz *et al.* 1991, Sanyal & Van 1997, Lacerte *et al.* 2004). In addition, Pit-1 can increase the acetylation state of the PRL promoter (Diamond & Gutierrez-Hartmann 2000) and alter its chromatin structure (Kievit & Maurer 2005). Pit-1 has been shown to interact with other DNA-binding proteins and coregulators that are incapable of independent DNA binding. For instance, recruitment of CREB binding protein (CBP) by Pit-1 is required for PKA activation of the PRL promoter. Competition between the corepressor N-CoR and the coactivator CBP modulates the transcriptional activity of Pit-1 (Xu *et al.* 1998, Cohen *et al.* 1999, Zanger *et al.* 1999, Hashimoto *et al.* 2000, Scully *et al.* 2000, Zanger *et al.* 2001).

The β -isoform of Pit-1, Pit-1 β , differs only by the splice-insertion of the 26 amino acid β -domain at the beginning of exon 2 in the TAD (Fig. 1), yet it has diametrically opposite effects with regard to hormone gene expression in anterior pituitary cells. While Pit-1 enhances PRL and GH gene expression, Pit-1 β blocks their gene expression and decreases the histone acetylation of their promoters in a manner dependent

Figure 1 The structure of Pit-1 and Pit-1 β . Upper, Pit-1, with its TAD, POU-specific, and POU-homeodomains. P_B and HD_B represent POU-specific and POU-homeodomain basic domains respectively; their α -helices are represented graphically. Lower, Pit-1 β , with the location of the 26 amino acid β -domain and its sequence.

upon five hydrophobic β -domain residues (Fig. 1) (Diamond & Gutierrez-Hartmann 1996, 2000, Ferry *et al.* 2005). Yet, in HeLa non-pituitary cells, Pit-1 β activates hormone gene transcription. The reason for this cell-type specificity has remained unknown to date, but it seems that a difference between the pituitary and non-pituitary cell environments modulates Pit-1 β transcriptional activity.

An important feature of POU factors is their ability to form cooperative homodimers and even heterodimers with other POU factors, both in the absence of DNA and even more so when bound to an appropriate DNA site. Consequently, diverse configurations of homodimeric and heterodimeric interactions emerge when different members of the POU domain gene family are coexpressed in the same cells or tissues. On some sites, Pit-1 can bind as a heterodimer with the POU domain factor Oct-1, which results in synergistic transactivation of the PRL promoter. However, on most sites, Pit-1 appears to bind as a homodimer (Elsholtz *et al.* 1990, Ingraham *et al.* 1990, Voss *et al.* 1991, 1993). The specific domain of the POU domain (POU-S) is essential for Pit-1–Pit-1 interactions and the result is efficient transactivation. The importance of this homodimerization is underscored by the fact that dominant-negative

Pit-1 mutants, such as those that occur naturally in combined pituitary hormone deficiency (Arg271 Trp, Pro14 Leu and Pro24 Leu), impede the function of the wild-type Pit-1 transcribed from the other, wild type, allele. This phenomenon occurs despite their ability to bind DNA (Cohen *et al.* 1999, Parks *et al.* 1999, Pfaffle *et al.* 1999).

Similarly, Pit-1 β blocks hormone gene expression even though it effectively binds DNA (Konzak & Moore 1992, Morris *et al.* 1992, Theill *et al.* 1992). Some studies showed that the Pit-1 β might induce repression in pituitary cells by altering the balance between competing coactivator and corepressor interaction with Pit-1 (Xu *et al.* 1998). We recently showed that Pit-1 β reduces CBP recruitment to the PRL and GH promoters (Ferry *et al.* 2005). However, it is not clear whether the Pit-1 β directly inhibits coactivator recruitment, or whether the repression is simply an effect of decreased Pit-1 or Pit-1 β availability due to heterodimerization. In order to distinguish between these possibilities, we tested the constraints for: (1) reconstitution of repression in non-pituitary cells; (2) the further constraints on repression added by CBP coactivator and PKA; (3) the effect of the β -domain on physical interaction with CBP; and (4) β -domain sidechain chemistry. We demonstrate that the

presence of Pit-1, CBP and PKA allow reconstitution of repression by Pit-1 β in non-pituitary cells, and that the β -domain blocks physical interaction between Pit-1 and a Pit-1-binding domain of CBP. In addition, we showed that specific chemical properties beyond hydrophobicity are dispensable. We propose a model in which Pit-1 β heterodimerizes with Pit-1 and thus decreasing their efficiency as transcriptional activators. Furthermore, this heterodimerization may account for the limited access of Pit-1 to the CBP zinc fingers and therefore prevent the formation of the Pit-1–CBP complex required for PRL and GH promoter activity in pituitary cells.

Materials and methods

Cell culture

We maintained monolayer cultures of HeLa non-pituitary and GH3 rat pituitary tumor cells (Tashjian *et al.* 1968) in Dulbecco's Modified Eagle Medium (DMEM), 20% fetal bovine serum (FBS) and 50 μ g/ml penicillin and streptomycin, at 37 °C in 5% CO₂ and changed the medium 16–18 h before each transfection. We harvested cells for transfection at approximately 80% confluence, using 0.05% trypsin and 0.5 mM EDTA.

Plasmids

Plasmid pA3 PRL luc, firefly luciferase driven by the proximal (–425) rat (r) PRL promoter, was described previously (Conrad & Gutierrez-Hartmann 1992, Diamond & Gutierrez-Hartmann 1996, 2000, Diamond *et al.* 1999). Plasmids pRSV HA Pit-1, pRSV HA Pit-1 β , pRSV PKA β and pRSV β -globin, respectively driven by the RSV promoter, were described previously (Diamond & Gutierrez-Hartmann 1996, 2000, Diamond *et al.* 1999). Plasmid RSV CBP was the generous gift of Dr Chee-Gun Lee. Plasmid pCMX NCoR was the generous gift of Dr Kate Horwitz. Plasmid pGEX-Pit-1 was the generous gift of Dr Gutierrez-Hartmann.

Mutant Pit-1 β constructs in mammalian expression vectors were constructed by nested PCR mutagenesis of the Pit-1 transactivation domain as described previously (Diamond & Gutierrez-Hartmann 1996, 2000). The pRSV HA Pit-1 plasmid was used as a substrate for PCR mutagenesis, in which the 26 amino acid β -domain was substituted with altered sequences, and a HA epitope tag was retained at the amino terminus of all of the Pit-1 constructs. Amplified DNA was initially subcloned into pCR 2.1 (Invitrogen, Carlsbad, CA, USA), and the presence of each introduced mutation and the integrity of its TAD region verified by dideoxy sequencing. HA-tagged Pit-1 TAD sequences were then excised from pCR2.1 by digestion with Hind III and PpuM I and were ligated to the unique Hind III and PpuM I sites of pRSV-Pit-1.

Expression vectors for labeled Pit-1 and Pit-1 β , T7 Pit-1 and T7 Pit-1 β , were constructed by PCR amplification of full length rat Pit-1 and Pit-1 β , followed by ligation to pCR4 Blunt TOPO (Invitrogen). The GST-fusion expression vector CBP AA 312–440 was constructed by PCR amplification of mouse CBP nucleotides 936–1320 with primers encoding BamH I and Sma I sites, followed by ligation to pCR4 Blunt TOPO. The CBP insert was then subcloned to the unique BamH I and Sma I sites of pGEX 4T-1.

Transfection

We introduced DNA into GH3 pituitary or HeLa non-pituitary cells by electroporation as follows. Approximately $2\text{--}3 \times 10^6$ enzymatically dispersed cells were mixed with plasmid DNA in a sterile gene-pulse chamber and exposed to a controlled electrical field of 525 μ Farads at 220 V, as described previously (Diamond & Gutierrez-Hartmann 1996, Ferry *et al.* 2005). We performed transient transfections in triplicate in at least three separate experiments. Cells from individual transfections then were maintained in DMEM, 20% FBS, 50 μ g/ml penicillin and streptomycin, at 37 °C in 5% CO₂.

A 1:3 Pit-1:Pit-1 β plasmid DNA ratio that results in equal levels of protein expression was used (Diamond & Gutierrez-Hartmann 1996, 2000, Diamond *et al.* 1999). We controlled the nonspecific effect of the RSV promoters upon transcription factor availability by including amounts of pRSV β -globin plasmid DNA in all assays to render the total pRSV DNA concentration constant. Plasmid pRLC Renilla (15 ng) was included as an internal control for all transfections.

Luciferase assays

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 were normalized for Renilla luciferase light units. We expressed results as mean fold activation of the PRL promoter $\pm$ S.E.M. and used Fisher's protected test of least significant differences to calculate the significance of differences between multiple conditions. In determining the ratios of two independent variables, the relative errors were added in quadrature to determine the S.E.M. of the ratio.

Western Blot analysis of HA-tagged Pit-1 proteins

Transient transfections were performed as above. Three aliquots of cells were pooled and harvested with PBS containing 3 mM EDTA, pelleted and resuspended in

2X Laemmli sample 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 membrane (Millipore, Bedford, MA, USA). The HA-tagged Pit-1 proteins were detected with a mouse monoclonal anti-HA HRP-conjugated antibody (Roche Diagnostics, Indianapolis, IN, USA) and ECL Advance media (Amersham Biosciences, Piscataway, NJ, USA). Dilutions of 1:1000 of the anti-HA monoclonal antibody were used.

GST fusion protein synthesis

Recombinant fusion proteins GST only, GST-Pit-1, and GST-CBP AA 312–440 were prepared from bacterial extracts (Bradford *et al.* 1995, 2000). Overnight cultures of *Escherichia Coli* BL-21 (DE3) pLysS (Stratagene, La Jolla, CA, USA), transformed with plasmids pGEX 4T-1, pGEX Pit-1, or pGEX CBP 312–440 were diluted 1:10 in fresh Luria broth supplemented with ampicillin (50 μ g/ml) and grown at 30 °C to an optical density at 600 nm of 0.4. Cultures were induced by addition of 1 mM isopropyl- β -D-thiogalactopyranoside for 2 h at 30 °C. Bacterial cells were harvested by centrifugation and resuspended and lysed in 1/10 volume of B-Per (Promega, Madison, WI, USA), with the recommended concentration of complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). Cellular debris was removed by centrifugation. Supernatants were bound to glutathione–sepharose (Amersham-Pharmacia, Piscataway, NJ, USA) for 1 h at room temperature and washed extensively in PBS supplemented with protease inhibitors. Bound protein was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and western blot with antibodies against GST. Protein concentration was measured by the Bradford assay (Bradford 1976).

In vitro protein interaction assays

Fluorotect-Green (Promega, Madison, WI, USA) lysine-labeled Pit-1 and Pit-1 β proteins were synthesized *in vitro* with the TNT coupled transcription-translation reticulocyte lysate system (Promega), according to the manufacturer's protocol. Equal amounts (2 μ g) of GST fusion proteins were bound to glutathione–agarose beads and suspended in binding buffer (40 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM PMSF, 0.05% Nonidet P-40, 1 mM dithiothreitol, pH 7.5) supplemented with protease inhibitors. *In vitro*-translated and labeled protein was incubated with immobilized GST, GST-Pit-1, or GST-CBP AA 312–440 in a final volume of 0.5 ml binding buffer containing 50 μ g/ml ethidium bromide and mixed by rocking for 1 h at room temperature. Beads were collected by a rapid, 30 s centrifugation at 1000g, and then washed five times for

5 min each in 0.5 ml binding buffer containing 0.1% Triton X-100. Bound labeled proteins were eluted by heating to 80 °C in SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Bradford *et al.* 1995, 2000). Bands were quantitated using a Molecular Dynamics Typhoon laser-scanning densitometer with Imagequant software (Amersham Biosciences, NJ, USA).

Statistics

The data were analyzed using GraphPad Prism software (San Diego, CA, USA). Differences between groups were considered significant for *P* value <0.05 as analyzed by Fishers least significant differences multiple comparison procedure.

Results

Reconstitution of pituitary-specific repression in non-pituitary cells

Pit-1 and Pit-1 β have a very limited expression pattern and are mainly found in the anterior pituitary gland. Our previous studies showed that Pit-1 β functioned as a repressor of the PRL promoter only in pituitary cells (Diamond & Gutierrez-Hartmann 1996, 2000, Diamond *et al.* 1999, Ferry *et al.* 2005). We speculated that this pituitary-specific repression might be the result of the heterodimerization between Pit-1 and Pit-1 β . To test this hypothesis, we measured the effect of increasing doses of Pit-1 β on activation of the proximal PRL promoter in HeLa non-pituitary cells in the presence of Pit-1. When transfected alone, both Pit-1 and Pit-1 β significantly increased PRL promoter activity by 12-fold over the vector only control (Fig. 2) Combination of equal amounts (10 μ g) of each isoform resulted in a significant drop in the PRL promoter activity compared with each isoform alone. However, increasing the amount of Pit-1 β to 15, 20, 25 and 30 μ g increased PRL promoter activity by 20-fold, 27-fold, 80-fold and 100-fold respectively. These results suggest that repression by the β -domain requires the presence of both isoforms and that the interaction or heterodimerization between Pit-1 and Pit-1 β leads to decreased intracellular availability and consequently to reduced efficiency as transcriptional activators in HeLa cells.

We previously found that repression by Pit-1 β in pituitary cells is limited to promoters that rely on Pit-1 to recruit CBP (Ferry *et al.* 2005). Pituitary cells express an abundance of CBP relative to many non-pituitary cells (Xu *et al.* 1998), providing a possible mechanism for repression. We therefore investigated the effect of the putative heterodimerization between Pit-1 and Pit-1 β on the activation of PRL promoter in the presence of CBP in HeLa non-pituitary cells. As shown in Fig. 3, there

Figure 2 Effects of Pit-1 β on activation of the rPRL promoter in HeLa non-pituitary cells in the presence of Pit-1. Plasmid pA3 PRL luc-425 (3 μ g) and pRSV HA Pit-1 (10 μ g), together with 0–30 μ g RSV HAPit-1 β were introduced into 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 (See Materials and methods).

was no effect on PRL promoter activity when HeLa cells were transfected with CBP alone. Addition of 10 μ g of either Pit-1 β or Pit-1 significantly increased PRL promoter activity 9-fold compared with vector only. Again, the further addition of increasing amounts of 15,

20, 25, and 30 μ g Pit-1 β resulted in augmented PRL promoter activity by 12, 19, 25, 33-fold respectively. Interestingly, addition of equal inputs of Pit-1 and Pit-1 β did not repress PRL promoter activity in the presence of CBP. It is possible that CBP binds Pit-1 before Pit-1 β

Figure 3 Effects of Pit-1 β on activation of the rPRL promoter in HeLa non-pituitary cells in the presence of Pit-1 and CBP. Plasmid pA3 PRL luc-425 (3 μ g), pRSV HA Pit-1 (10 μ g) and pRSV CBP (10 μ g), together with 0–30 μ g RSV HAPit-1 β were introduced into 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 (See Materials and methods).

Figure 4 Effects of Pit-1 β on activation of the rPRL promoter in HeLa non-pituitary cells in the presence of Pit-1, PKA β and CBP. Plasmid pA3 PRL luc-425, (3 μ g), pRSV HA Pit-1 (10 μ g), pRSV CBP (10 μ g) and pRSV PKA β (10 μ g) together with 0–30 μ g RSV HAPit-1 β , were introduced into 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 (See Materials and methods).

does and thus prevents the heterodimerization between Pit-1 and Pit-1 β . As well, interactions between all three players may take place simultaneously and produce a sufficient amount of Pit-1–CBP complex to activate the PRL promoter.

It has been shown that POU factors can be modified by phosphorylation (Kapiloff *et al.* 1991, Segil *et al.* 1991). Anterior pituitary PRL gene expression is high in lactotrophs, due to the tonic activity of the cAMP-signaling cascade (Pickett & Gutierrez-Hartmann 1997). This signal is transduced to the PRL promoter through CBP and Pit-1 (Xu *et al.* 1998, Zanger *et al.* 1999). This left the possibility that heterodimerization between Pit-1 and Pit-1 β blocks PKA-activation of the PRL promoter through Pit-1 and CBP. To test this hypothesis, we introduced Pit-1 β into HeLa non-pituitary cells with Pit-1, CBP, and PKA's catalytic subunit, PKA β (Fig. 4). Either Pit-1 or Pit-1 β in combination with CBP and PKA β activated the PRL promoter approximately 7-fold compared with the vector only control. Addition of both isoforms in combination with CBP and PKA β resulted in a significant drop in the PRL promoter activity. However, addition of 15, 20, 25, and 30 μ g Pit-1 β increased PRL promoter activity in a dose-dependent manner between 7 and 10-fold. Pit-1 β repression was more pronounced in the absence of CBP. Interestingly, activation of the PKC signaling pathway also allows reconstitution of repression by Pit-1 β in the presence of Pit-1 and CBP, while expression of N-CoR does not allow reconstitution of repression in the presence of any

combination of Pit-1 β , Pit-1 and CBP (data not shown). These experiments reconstitute, for the first time, repression by Pit-1 β in non-pituitary cells, and they indicate that the β -domain blocks the ability of Pit-1 and CBP to transduce cAMP/PKA activation of the PRL promoter.

The β -domain blocks physical interaction between Pit-1 and CBP

We next sought to address the question whether Pit-1 β might repress PRL promoter activity by blocking Pit-1 dimerization itself, or by blocking a downstream action of Pit-1 dimers such as recruitment of phosphorylated CBP. In order to test this possibility, we examined the ability of Pit-1 vs Pit-1 β to physically interact with Pit-1 and CBP *in vitro* through a GST pull-down assay. We chose the region of CBP containing AA 312–440 because it contains a CBP zinc-finger domain sufficient to bind Pit-1 *in vitro*, and functionally interact with Pit-1 *in vivo* (Tolon *et al.* 1998, Zanger *et al.* 1999).

Fusion proteins containing GST-linked Pit-1 and CBP AA 312–440 were constructed, immobilized on glutathione agarose, and used in pull-down assays with fluorescently labeled Pit-1 or Pit-1 β (see Materials and methods). Incubations were performed with equivalent amounts of beads, bound GST protein, bound GST-Pit-1 fusion protein, and bound GST-CBP AA 312–440 fusion protein in the presence of ethidium bromide to block nonspecific protein–DNA interactions (Lai & Herr 1992, Bradford *et al.* 2000). As shown in a representative

Figure 5 Effects of The β -domain on physical interaction between Pit-1 and select CBP domains *in vitro*. Aliquots (50 μ l packed volume) of glutathione-Sepharose beads, bound to 2 μ g of GST (lane 1), 1% of labeled Pit-1 protein input (lane 2), GST-CBP AA 312–440 (lane 3), or GST-Pit-1 (lane 5) were incubated with equal amounts of *in vitro*-transcribed and translated labeled Pit-1 (upper panel) or Pit-1 β (lower panel). A representative SDS PAGE gel is shown (A), and the results of seven pull-down experiments are quantified (B).

experiment in Figure 5A, and quantified from seven such experiments in Figure 5B, Pit-1 bound both GST-Pit-1 and GST-CBP AA 312–440 at levels 4-fold above background, while Pit-1 β bound GST-Pit-1 at levels nearly 3-fold above background but failed to bind GST-CBP AA 312–440 at levels significantly above background. These results provide physical evidence that Pit-1 β interacts with Pit-1 but not with the AA 312–440 binding region of CBP, and that the β -domain presence may render the heterodimer incapable of interacting

The sidechain length of hydrophobic residues is not necessary for repression

In order to address how the β -domain insertion might affect Pit-1–CBP interaction, we analyzed the β -domain

sequence constraints on repression. Changes in AA sidechain length can eliminate binding between polypeptides by steric interference or by removing interacting sidechain elements from the same vicinity (Vu *et al.* 2001). In order to test the constraints on β -domain sidechain length, we substituted the five residues previously shown to be required for repression (Leucine 7, Isoleucine 8, Tyrosine 17, Phenylalanine 18 and Methionine 20) with AAs of similar chemistry but different sidechain length (Fig. 6). For Leucine 7 and Isoleucine 8, we substituted Valine and Methionine; for Tyrosine 17, we substituted Tryptophan and Phenylalanine; for Phenylalanine 18, we substituted Tryptophan and Tyrosine; for Methionine 20, we substituted Valine and Isoleucine.

To ensure that mutagenesis did not eliminate transcription function, we tested the mutant Pit-1 β s in isoform-insensitive HeLa non-pituitary cells (Diamond & Gutierrez-Hartmann 1996) in the presence of the rPRL promoter-driven luciferase reporter pA3–425 PRL luc, which contains sequences sufficient to confer cell-type specific gene expression in transient transfection experiments (Fig. 6A) (Camper *et al.* 1985, Gutierrez-Hartmann *et al.* 1987, Elsholtz *et al.* 1991) (See Materials and methods). Pit-1 β increased transcription of the PRL promoter 17-fold compared with the vector-only control, as seen previously (Diamond & Gutierrez-Hartmann 1996, Ferry *et al.* 2005). All of the sidechain length substitution mutants increased PRL promoter activity (from 3- to 21-fold). The variation in transcriptional potency in these experiments was similar to that among single amino acid substitutions in our previous substitution mutagenesis of the β -domain (Diamond & Gutierrez-Hartmann 2000), and most of the mutants had similar transcription potency to wild-type Pit-1 β .

In order to determine the tolerance of repression for these alterations in sidechain-length, we introduced wild type and mutant Pit-1 β s into GH3 pituitary cells by electroporation with pA3 –425 PRL luc (See Materials and methods) (Fig. 6B). Pit-1 β reduced PRL promoter activity by 60% compared with the vector only control, and all of the sidechain-length mutants repressed PRL promoter activity by 41 – 85%. These data demonstrate that the sidechain lengths of these hydrophobic β -domain residues are not necessary for repression.

The aliphatic/aromatic character of hydrophobic residues is not necessary for repression

Hydrophobic amino acids fall into two broad categories with different structures and chemical properties: aliphatic vs aromatic. Aliphatic sidechains are unsubstituted hydrocarbon chains subject to nucleophilic substitution, while aromatic sidechains incorporate closed planar rings and are subject to electrophilic

Figure 6 Sidechain length mutations. Effects of Pit-1 β constructs on activation of the rPRL promoter in non-pituitary HeLa cells (A) and GH3 pituitary cells (B). Plasmid pA3 PRL luc-425 (3 μ g), together with wild type or mutant pRSV HA Pit-1 β (30 μ g), were introduced into GH3 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 (See Materials and methods).

Figure 7 Aliphatic vs aromatic mutations. Effects of Pit-1 β constructs on activation of the rPRL promoter in non-pituitary HeLa cells (A) and GH3 pituitary cells (B). Plasmid pA3 PRL luc-425 (3 μ g), together with wild type or mutant pRSV HA Pit-1 β (30 μ g), were introduced into GH3 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 (See Materials and methods).

substitution. These differences affect protein conformation and intermolecular interactions with ligands and other proteins (Heyl *et al.* 1994, Deardorff & Sachs 1997, Murray *et al.* 1998, Nakagawa *et al.* 2000).

For the aliphatic residues Leucine 7, Isoleucine 8, and Methionine 20, we substituted the aromatic residues Tyrosine and Phenylalanine. For the aromatic residues Tyrosine 17 and Phenylalanine 18, we substituted the aliphatic residues Leucine and Isoleucine. In addition, we constructed two multiple substitution constructs, where multiple hydrophobic residues were similarly substituted: 4 substitutions (Leu 7 Tyr, Ile 8 Phe, Tyr 17 Leu, Phe 18 Ile) and 5 substitutions (Leu 7 Tyr, Ile 8 Phe, Tyr 17 Leu, Phe 18 Ile, Met 20 Phe).

When expressed alone in HeLa non-pituitary cells (Fig. 7A), the sidechain-length substitution mutants increased rPRL promoter activity compared with the vector-only control from 9- to 104-fold versus 54-fold for Pit-1 β . The differences in transcriptional potency in these experiments were smaller than those in our previous substitution mutagenesis of the β -domain (Diamond & Gutierrez-Hartmann 2000), but most of the mutants had a somewhat reduced transcription potency compared with wild-type Pit-1 β . Of note, the four- and five-substitution mutants were among the least transcriptionally active of the constructs. This is reminiscent of the more severe effects on transcription potency among larger epitope substitutions (Diamond & Gutierrez-Hartmann 2000).

Table 1 Random β-domains

	β-domain sequence
Wild type	VPSILSLIQTPKCLHTYFSMTTMGNT
RAN 1	YVLCTPSQFIMTGTLPSTSMTNHL
RAN 2	MKVSCTSSMYPLGTPNTIITFTLQLH
RAN 3	PGFTLQTMLSVSTISPINTCHLMTYK

These data demonstrate that the β-domain mutants retain transcriptional potency.

In GH3 pituitary cells, the sidechain-length mutants repressed rPRL promoter activity by 46% to 75% (Fig. 7B) compared with the vector-only control. Surprisingly, the β-domain tolerated substitution of four or five hydrophobic residues. These data demonstrate that the aliphatic vs aromatic chemistry of Leucine 7, Isoleucine 8, Tyrosine 17, Phenylalanine 18, and Methionine 20 is not required for repression.

β-domain amino acid order is not required for repression

The lack of constraint on sidechain chemistry for these five hydrophobic amino acids suggested that the β-domain might function solely through its hydrophobicity. A prediction of such a model would be that sequence order would not be necessary for repression, so long as hydrophobicity was preserved. We used a random number generator to scramble the β-domain sequence in three different ways. We then substituted a scrambled domain for the wild-type β-domain in mutant constructs (Table 1).

All of the scrambled Pit-1β proteins retained basal transcriptional potency in the isoform-insensitive HeLa reconstitution assay (Fig. 8A). They increased PRL promoter activity 7- to 15-fold compared with the vector-only control, vs 18-fold for wild-type Pit-1β. In GH3 pituitary cells, the scrambled β-domain-containing proteins reduced PRL promoter activity (Fig. 8B) between 69 and 77% compared with the vector only control. These data demonstrate that β-domain amino acid order is not required for repression.

Hydrophobic sidechains are required for repression

If the β-domain functions solely through its hydrophobicity, other hydrophobic β-domain sidechains should be required for repression. We sequentially substituted alanines for five untested hydrophobic β-domain residues – valine 1, isoleucine 4, leucine 5, leucine 14, and methionine 23. Alanine substitution has been used in several systems to probe structure and function by eliminating most sidechain chemistry (Wertman *et al.* 1992, Diamond & Gutierrez-Hartmann 2000) reviewed

Figure 8 Random β-domains. Effects of Pit-1β constructs on activation of the rPRL promoter in non-pituitary HeLa cells (A) and GH3 pituitary cells (B). Plasmid pA3 PRL luc-425 (3 μg), together with wild type or mutant pRSV HA Pit-1β (30 μg), were introduced into GH3 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 (See Materials and methods).

earlier by Diamond & Kirkegaard 1994. Alanine is common (Klapper 1977), uncharged, and has minimal effects on protein secondary structure. While routinely categorized as hydrophobic because of its aliphatic methyl sidechain, it is actually no more hydrophobic

Figure 9 Hydrophobic residue to alanine mutations. Effects of Pit-1 β constructs on activation of the rPRL promoter in non-pituitary HeLa cells (A) and GH3 pituitary cells (B). Plasmid pA3 PRL luc-425 (3 μ g), together with wild type or mutant pRSV HA Pit-1 β (30 μ g), were introduced into GH3 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 (See Materials and methods). Expression levels of Pit-1 and Pit-1 β (C). Plasmids pRSV HA Pit-1 (10 μ g), pRSV HA Pit-1 β (30 μ g), and mutant pRSV HA Pit-1 β (30 μ g) were introduced into GH3 pituitary cells by electroporation. After 24 h cells were harvested and analyzed by SDS PAGE and western blot (See Materials and methods).

than Threonine (Karplus 1997), and is found as often at solvent-exposed as at internal positions (Chothia 1976, Rose *et al.* 1985). Moreover, since there are no alanines in the β -domain (Fig. 1), each substitution is distinct from the endogenous sequence.

All of the alanine-substituted Pit-1 β proteins retained basal transcriptional potency in isoform-insensitive HeLa non-pituitary cells, and activated the PRL promoter when compared with the vector only control (Fig. 9A). Pit-1 β activated the PRL promoter 18-fold, and the five alanine-scanning mutants activated the promoter between 21- and 98-fold. In contrast to the sidechain-length and aliphatic vs aromatic mutations, the five alanine-substitution mutants activated the PRL promoter when compared with the vector only control even more than did wild type Pit-1 β .

In GH3 cells, the wild-type Pit-1 β isoform reduced PRL promoter activity by 41% (Fig. 9B). Two of the

alanine-substitution proteins, Val 1 Ala and Met 23 Ala, failed to reduce PRL promoter activity, and three of the alanine-substitution proteins actually increased PRL promoter activity between 2- and 3-fold. In our previous alanine-substitution mutagenesis studies, we also observed varying degrees of reversal of repression, from loss of repression to activation (Diamond & Gutierrez-Hartmann 2000).

The lack of effect of the Val 1 Ala and Met 23 Ala constructs on rPRL promoter activity in GH3 pituitary cells raised the concern that the mutant proteins may not be expressed, though their retention of transcription potency in HeLa non-pituitary cells argues against considerably altered protein expression. We performed western blot analysis of protein expression for these constructs in GH3 cells (Fig. 9C). The protein expression levels of the mutant constructs were roughly equivalent to that of wild-type Pit-1 β . We detected small differences

in apparent mobility among the Pit-1 mutant constructs, likely due to sequence-specific effects on gel mobility of the mutant proteins, similar to those that we have seen previously with Pit-1 (Diamond & Gutierrez-Hartmann 1996, 2000) and the poliovirus RNA polymerase (Diamond & Kirkegaard 1994).

Nonhydrophobic residues are not required for repression

A further prediction of β -domain function solely through hydrophobicity would be that nonhydrophobic side-chains, unlike the hydrophobic sidechains, would be dispensable for repression. To test this hypothesis, we replaced nonhydrophobic β -domain amino acids with alanine. We chose five nonhydrophobic AAs (serine 6, glutamine 9, lysine 12, cysteine 13, and threonine 21) whose physical properties (Kawashima *et al.* 1999, Kawashima & Kanehisa 2000) had been changed in previous mutageneses only by multiple AA substitution mutations that eliminated repression (Diamond & Gutierrez-Hartmann 1996, 2000).

All of the alanine substitutions retained transcriptional potency in HeLa non-pituitary cells (Fig. 10A). Pit-1 β activated the PRL promoter 7-fold compared with the vector-only control, and the five alanine-scanning mutants activated 2- to 10-fold. For three of the mutants (Ser 6 Ala, Cys 13 Ala and Thr 21 Ala) transcriptional potency was less than that of the wild-type Pit-1 β , and for two of the mutants (Gln 9 Ala and Lys 12 Ala) transcriptional potency was greater than for that of the wild type.

In GH3 pituitary cells, the proteins with alanine-substitutions for non-hydrophobic residues blocked PRL promoter activity between 60 and 83% compared with the vector only control, similar to the 65% repression by Pit-1 β (Fig. 10B). These data demonstrate that the non-hydrophobic β -domain amino acids serine 6, glutamine 9, lysine 12, cysteine 13, and threonine 21 are not required for repression.

Discussion

Small repression domains can block transcription through diverse mechanisms, such as interference with host protein conformation (Necela & Pollenz 1999), DNA binding (Katz *et al.* 1995, Roulet *et al.* 1995), recruitment of corepressors or other proteins (Zhang *et al.* 2002, Xia *et al.* 2003, Townson *et al.* 2004), or interference with positive interactions (Moffett *et al.* 1997). The ability of Pit-1 β to bind DNA as well as Pit-1 (Konzak & Moore 1992, Sanchez-Pacheco *et al.* 1998) argues against a mechanism solely involving interference with DNA binding.

A. Activation in Pituitary (GH3) Cells Effect of non-Hydrophobic Residues

B. Activation in non-Pituitary (HeLa) Cells Effect of non-Hydrophobic Residues

Figure 10 Nonhydrophobic residue to Alanine mutations. Effects of Pit-1 β constructs on activation of the rPRL promoter in non-pituitary HeLa cells (A) and GH3 pituitary cells (B). Plasmid pA3 PRL luc-425 (3 μ g), together with wild type or mutant pRSV HA Pit-1 β (30 μ g), were introduced into GH3 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 (See Materials and methods).

The results of the reconstitution experiments (Figs 2–4) indicate that the decrease in CBP recruitment to the PRL promoter seen previously (Ferry *et al.* 2005) are due to interference by Pit-1 β with the function of the Pit-1–CBP complex to transduce cAMP/PKA signaling. It has been shown that the POU domain of Pit-1 may interact with CBP through two cysteine–histidine rich domains (Xu *et al.* 1998, Zanger *et al.* 1999) and that POU factors can be modified by phosphorylation. For example, Pit-1 is phosphorylated in the POU domain

(Thr-220). This phosphorylation results in decreased DNA binding to GH sites and increased DNA binding to TSH β and PRL promoter sites. The failure of Pit-1 alone, CBP or even PKA alone seen previously (Diamond & Gutierrez-Hartmann 1996, Diamond *et al.* 1999) to allow Pit-1 β to reconstitute repression suggest that this repression is an emergent property of Pit-1 β 's interaction with the Pit-1–CBP complex.

The correlation between the dosage of Pit-1 β and the degree of activation of the PRL promoter by Pit-1, PKA and CBP (Figs 2–4) is typical of the dosage effects seen with dominant-negative poison products, which typically interact with one partner of a complex but fail to interact with others (Mains *et al.* 1990, Mounkes & Fuller 1999). It has been shown that Pit-1 and non-liganded nuclear receptors (NR) can repress each other's signaling (Gonzalez & Carlberg 2002). In this study, we propose a mechanism by which Pit-1 interacts with Pit-1 β resulting in cross-repression. This phenomenon may take place whenever Pit-1 and Pit-1 β are co-expressed. Our finding that the β -domain blocks physical interaction between Pit-1 and CBP AA 312–440 (Fig. 5) provides a physical basis for this model. Thus, the cell-type specificity of repression by Pit-1 β would be due to the unique environment of anterior pituitary lactotroph and somatotrophs, which display tonic activation of the cAMP/PKA signaling cascade, and high levels of both Pit-1 and CBP.

The negative effect of the β -domain on both Ets-1 and Ras signaling transduction (Diamond & Gutierrez-Hartmann 1996) as well as the differential interaction of Pit-1 and Pit-1 β with Ets-1 (Bradford *et al.* 2000) may also be a product of the insertion of the hydrophobic β -domain into the Pit-1 TAD. The β -domain insertion-point lies at the boundary between the two TAD exons, R1 and R2, which regulate Pit-1–ETS-1 synergy and Ras response respectively (Duval *et al.* 2003). The lack of requirement for physical interaction between Ets-1 and Pit-1 for either Ras responsiveness or Pit-1–ETS-1 synergy suggests that the β -domain may interfere with coactivator recruitment by the Pit-1 and Ets-1 complex. Indeed, both Pit-1 and Ets-1 can interact with the AA 312–440 region of CBP.

The AA sequence requirements for β -domain repression are most consistent with a model of β -domain function solely through hydrophobicity. Current models of the function of transcriptional activator require specific charge and hydrophobic interactions to strengthen binding between conformationally flexible TADs and partner proteins (Hermann *et al.* 2001, De Guzman *et al.* 2004, Razeto *et al.* 2004, Zor *et al.* 2004). The lack of constraint for sidechain length (Fig. 6), sidechain chemistry (Fig. 7) or even AA order (Fig. 8) argues against the β -domain forming one side of a specific interface that involves surface complementarity. The requirement for only the β -domain's hydrophobic

(Figs 9 and 10) residues argues against a requirement for charge interactions, and suggests a threshold effect: the β -domain may be hydrophobic enough to block interaction between Pit-1 β and CBP, but losses of hydrophilic properties do not increase the repression.

The effect of the β -domain mutations on transcription potency when expressed alone in HeLa cells correlates with their effect on repression in pituitary cells. This suggests that changes in β -domain sidechain structure have similar effects on both basal transcription potency in nonpituitary cells and the ability of Pit-1 β to interact with CBP. The mechanism by which either Pit-1 or Pit-1 β transduce PKA signaling in HeLa cells in the absence of CBP is clearly different from the mechanism in pituitary cells or in our current reconstitution experiments with CBP, and underlines the importance of characterizing mutant transcription factors in their native environment.

How might the hydrophobic β -domain actually block interaction with and recruitment of CBP? The hydrophobicity of the β -domain is reminiscent of that of the hydrophobic rtARNTa repression domain (Necela & Pollenz 1999), which can repress promoter activation by either ARNT or the yeast transcription factor Gal 4 through protein misfolding, possibly due to hydrophobic sidechain burial in the protein's interior. Similarly, the β -domain might alter the conformation of the Pit-1, perhaps directly affecting the POU-homeodomain, which has been shown to directly interact with CBP (Xu *et al.* 1998, Zanger *et al.* 1999). Alternatively, the β -domain might interfere with the conformational flexibility of the TAD, and prevent necessary interactions between Pit-1 and CBP. Two of CBP's zinc-finger domains in the AA 312–440 region form stable, well-ordered complexes with transcription factor TADs, which appear to involve the induced fit of an otherwise disordered TAD with a CBP zinc finger (Dial *et al.* 2003, Freedman *et al.* 2003).

Support for a model in which the β -domain alters the Pit-1 TAD to interfere with recruitment of CBP comes from the analysis of a mutation in Pit-1 that lead to familial combined pituitary hormone deficiency (CPHD), a genetic disorder of anterior pituitary function in which multiple anterior pituitary hormone genes are under-expressed. Twenty mutations in the Pit-1 locus have been identified that lead to CPHD (Li *et al.* 1990, Ohta *et al.* 1992, Pfaffle *et al.* 1992, Radovick *et al.* 1992, Tatsumi *et al.* 1992, Cohen *et al.* 1995, 1999, Irie *et al.* 1995, Pellegrini-Bouiller *et al.* 1996, Brown *et al.* 1998, Fofanova *et al.* 1998, Pernasetti *et al.* 1998, 1999, Blankenstein *et al.* 2001, Hendriks-Stegeman *et al.* 2001, Vallette-Kasic *et al.* 2001). Of particular interest is a mutation that converts the conserved Proline at position 24 of the TAD to Leucine, and interferes with interaction between Pit-1 and CBP (Ohta *et al.* 1992, Cohen *et al.* 1996). Interestingly, the dominant-negative

Pro 24 Leu Pit-1, like Pit-1 β , fails to block either PRL or GH promoter activity in non-pituitary cells and like Pit-1 β , actually activates those promoters (Kishimoto *et al.* 2002).

Future experiments will investigate the mechanism(s) that underlie the ability of the β -domain to block recruitment of CBP by Pit-1. Owing to its dominant-negative function with regard to the functional recruitment of CBP to the PRL promoter, Pit-1 β also may provide a probe to identify additional downstream targets of Pit-1 and CBP.

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